Pathways of bradykinin degradation in blood and plasma of normotensive and hypertensive rats

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Pathways of bradykinin degradation in blood and plasma of normotensive and hypertensive rats. Am J Physiol Heart Circ Physiol 280: H2182–H2188, 2001.—Kinins are vasodilatory peptide hormones that can confer protection against the development of hypertension. Because their efficacy is greatly influenced by the rate of enzymatic degradation, the activities of various kininases in plasma and blood of spontaneously hypertensive rats (SHR) were compared with those in normotensive Wistar-Kyoto rats (WKY) to identify pathogenic alterations. Either plasma or whole blood was incubated with bradykinin (10 μM). Bradykinin and kinin metabolites were measured by high-performance liquid chromatography. Kininase activities were determined by cumulative inhibition of angiotensin I-converting enzyme (ACE), carboxypeptidase N (CPN), and aminopeptidase P (APP), using selective inhibitors. Plasma of WKY rats degraded bradykinin at a rate of 13.3 ± 0.94 μmol·min⁻¹·l⁻¹. The enzymes ACE, APP, and CPN represented 92% of this kininase activity, with relative contributions of 52, 25, and 16%, respectively. Inclusion of blood cells at physiological concentrations did not extend the activities of these plasma kininases further. No differences of kinin degradation were found between WKY and SHR. The identical conditions of kinin degradation in WKY and SHR suggest no pathogenic role of kininases in the SHR model of genetic hypertension.

Kinins; metabolism; angiotensin I-converting enzyme; aminopeptidase P; carboxypeptidase N

Kinins are small peptide hormones that mediate a variety of cardiovascular effects, such as vasodilation, release of endothelial autacoids, natriuresis, myocardial preconditioning, and inhibition of cell proliferation (6). These actions have acquired therapeutic significance due to the development of angiotensin I-converting enzyme (ACE) inhibitors that abolish kinin degradation by this enzyme and consequently increase the availability of kinins. Meanwhile, a multitude of studies have demonstrated that the potentiation of kinin effects contributes decisively to the cardioprotective actions of ACE inhibitors, which include inhibition of vascular and myocardial hypertrophy, inhibition of cardiac remodeling and fibrosis, and reduction of infarct size (23).

Several lines of evidence suggest that endogenous kinins can exert protection against cardiovascular diseases and against hypertension in particular. Enhanced production of bradykinin (BK) after overexpression of kallikrein, as well as long-term infusion of a stable B2-agonist, were effective in preventing the development of hypertension in spontaneously hypertensive rats (SHR) (26, 38). Moreover, an association of hypertension with an impaired state of the kinin-kallikrein system has been described, which even suggests a physiological role for kinins in the stabilization of normotensive blood pressures. One of the first observations (15) regarded the urinary formation of kinins by renal kallikrein, which was found to be decreased in hypertensive patients, and its impairment seemed to predispose the development of hypertension (2). Such conclusions were further supported by experimental studies involving genetic deletion of B2-receptors, which led to a significant salt sensitivity and a slight increase in blood pressure (24).

The rapid breakdown of kinins by various kininases exerts a significant influence on the functional potency of kinins. This has been most convincingly demonstrated by the use of ACE inhibitors, agents that sensitize the dose response for the hypotensive effect of intravenously administered BK in humans by a factor of ~40 (4). Consequently, the pharmacological potentiation of endogenous kinins through kininase inhibition has been shown to mimic the cardioprotective properties of kinins in various ways (23). Kinin potentiation has even been identified as the mechanism by which ACE inhibitors prevent the development of hypertension in later life when SHR are treated during early development (29). A distinction between circulating and tissue-bound enzyme entities has also been made using ACE inhibitors. Therapeutic experimental studies have determined that very low doses of ACE inhibitors selectively inhibit tissue ACE, an effect that leads to a reduction of cell hypertrophy and proliferation in the absence of blood pressure alterations, whereas higher...
MATERIALS AND METHODS

Animals. Male WKY and SHR (300–350 g) were obtained from Charles River (Sulzfeld, Germany). The animals were kept under controlled temperature, humidity, and 12:12-h light-dark conditions and were allowed free access to food and water. Systolic blood pressures, as determined using a tail-cuff method, were 130 ± 9 and 204 ± 12 mmHg in the WKY and SHR groups, respectively (n = 6). The study was carried out in accordance with the Declaration of Helsinki and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals as adopted by the Ministerium für Natur und Umwelt des Landes Schleswig-Holstein, Germany.

Substances. BK, peptide standards, Earle’s balanced salt mixture (EBSS), and HEPES were purchased from Sigma-Aldrich (Deisenhofen, Germany). 2-Mercaptoethanol, acetone-nitrile, methanol, and trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany), and DL-2-mercaptoethyl-3-guanidinoethylthiopropanoic acid (MGTA) was purchased from Calbiochem (Bad Soden, Germany). Ramiprilat was donated by Hoechst-Marion-Roussel (Frankfurt, Germany).

Preparation of blood and plasma. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and blood was drawn from the aorta with the use of a syringe that contained 10 IU/ml heparin sodium (Liquemine N 25,000, Roche; Basel, Switzerland). One portion of the samples was centrifuged (15,000 g, 10 min) to obtain plasma and to determine hematocrit.

Incubation procedure. Incubations were performed at 37°C in EBSS supplemented with 10 mM HEPES (pH 7.4). Either 33 μl of plasma or whole blood containing this amount of plasma were added to 1.1 ml of incubation buffer containing 10 μM BK. Four independent incubations were performed: 1) in the absence of kininase inhibitors; 2) with ACE inhibited by 0.25 μM ramiprilat; 3) with ACE and CPN inhibited by 0.25 μM ramiprilat and 10 μM MGTA; and 4) with ACE, CPN, and APP inhibited by 0.25 μM ramiprilat, 10 μM MGTA, and 1 mM of 2-mercaptoethanol. Aliquots (225 μl) from the incubations were taken at 0, 10, 30, 90, and 240 min, supplemented with TFA (120 mM final concentration), and stored at −80°C until analysis.

HPLC analysis. The spectrum of BK and its degradation products were determined as described previously (8). Peptides were separated by HPLC with the use of a C18 column (model ET200 Nucleosil 5, Machery-Nagel; Düren, Germany). Separation was performed at 50°C and at a flow rate of 0.8 ml/min by linearly increasing the organic component (60% acetonitrile, 1% methanol, and 0.1% TFA) in 12 mM HCl from 2 to 45%. Peptides were detected by absorption at 210 nm.

Calculation and statistics. Complete degradation kinetics were fitted with the monoexponential function \( \text{BK}(t) = \text{BK}_0 e^{-kt} \), where \( k \) is degradation rate, \( t \) is time by nonlinear regression (Prism, GraphPad Software; San Diego, CA), and \( \text{BK}_0 \) is the BK concentration at 0 min. The activities of individual kininases were calculated from the decrease of the BK degradation rate brought about by inclusion of the respective inhibitors (8). All of the data are given as means ± SD determined in five independent experiments per group. Differences between the treatment groups were evaluated by analysis of variance with Tukey’s post hoc test. The treatment effects were considered statistically significant at an error level of \( P < 0.05 \).

RESULTS

Spectrum of kininases. To identify the kininases of blood and plasma and to demonstrate the specificity and efficacy of the applied inhibitors, the BK fragments produced under the different incubation conditions were analyzed by HPLC. Figure 1 shows the original tracings that represent the spectrum of BK metabolites after 30 min of incubation with WKY rat plasma. Elution of peptide standards and an analysis of plasma and substrate before incubation are depicted in Fig. 1, A and B, respectively. Under control conditions, BK was mostly (by 69%) metabolized, yielding the fragments [1–5]-BK, [1–7]-BK, and [1–8]-BK as a result of
carboxyterminal cleavage and [2–9]-BK (Fig. 1C). Inhibition of ACE with ramiprilat increased intact BK (by 84%) and greatly diminished the ACE products [1–5]-BK and [1–7]-BK, whereas the generation of [1–8]-BK and [2–9]-BK was not affected (Fig. 1D). The additional inhibition of CPN with MGTA preserved intact BK further and prevented the formation of [1–8]-BK (Fig. 1E). The amino-terminal cleavage of BK (generation of [2–9]-BK) was largely reduced by addition of 2-mercaptoethanol (Fig. 1F). This indicated a major involvement of APP in BK degradation in rat plasma. No further significant amino-terminal degradation of BK could be shown. A combination of all three enzyme inhibitors conserved 85% of BK over 30 min of incubation. Identical spectra of kinin degradation products were obtained when degradation of BK by SHR plasma was studied, and for this reason the spectra were not shown in this paper.

**Kininase activities.** The participation of the described enzymes in BK degradation was determined by incubating BK with blood or plasma in the presence of various inhibitors. The degradation kinetics in blood and plasma are presented for WKY and SHR in Figs. 2 and 3, respectively. Kinin degradation activities calculated from monoexponential fits are listed in Table 1. The degradation rates corresponded to virtually identical half-lives of 18 and 20 min for BK in diluted plasma of WKY and SHR, respectively. BK degradation was not enhanced by the inclusion of blood cells at physiological concentrations. All of the enzyme inhibitors significantly reduced the degradation rates in plasma and blood ($P < 0.05$ vs. control groups). The rates of kinin degradation determined in the presence of ramiprilat or ramiprilat plus MGTA showed no significant differences among all groups. Only the residual activity, resistant to ramiprilat, MGTA, and 2-mercaptoethanol, was elevated in blood samples of SHR compared with plasma of either SHR or WKY (Table 1).

The reduction of kinin degradation rates caused by the addition of each of the kininase inhibitors was taken as a measure for the kininase activities of the respective enzymes. Accordingly, the average enzyme activities in all (plasma or blood of either WKY or SHR) samples were 6.2 ± 0.9 μmol·min$^{-1}$·l$^{-1}$ for ACE, 2.0 ± 0.3 μmol·min$^{-1}$·l$^{-1}$ for CPN, and 2.8 ± 0.6 μmol·min$^{-1}$·l$^{-1}$ for APP. Hence, ACE, CPN, and APP accounted for 51, 16, and 23% of the total kininase activity.

![Fig. 1. Spectra of bradykinin (BK) metabolites after incubation in Wistar-Kyoto rat (WKY) plasma. Original high-performance liquid chromatography (HPLC) tracings demonstrate retention times of peptide standards (A) and those of BK and its degradation fragments at the start (B) and after 30 min of incubation (C) in WKY plasma. Pretreatment with enzyme inhibitors was used to identify the kininases responsible for BK degradation. Inhibition of angiotensin-I converting enzyme (ACE) by ramiprilat (0.25 μM) greatly reduced the formation of [1–5]-BK and [1–7]-BK (D). Additional inhibition of carboxypeptidase N (CPN) by 10 μM DL-2-mercaptomethyl-3-guanidinioethylthiopropanoic acid (MGTA) abolished the generation of [1–8]-BK (E). Role of aminopeptidase P (APP) in the degradation of BK was revealed by generation of the fragment [2–9]-BK, which was significantly reduced after inhibition of aminopeptidase P with 2-mercaptoethanol (1 mM) (F). Combination of all three enzyme inhibitors effectively blocked BK degradation, so that 85% of the incubated BK was still present after 30 min of incubation. Residual generation of [1–5]-, [1–7]-, and [2–9]-BK was still apparent under these conditions. Phe, phenylalanine (standard); ø, peak has been abolished or greatly reduced by respective enzyme inhibitor.](http://alphahrd.physiology.org/d)
activity, respectively. The distribution of these enzymes showed no significant differences among blood or plasma samples of either WKY or SHR (Fig. 4). However, the unidentified residual kininase activity corresponded to 8.3 or 12% of total kininase activities in all (WKY or SHR) plasma or blood samples, respectively ($P < 0.05$).

**DISCUSSION**

These studies demonstrate that ACE, representing more than half of the total kininase activity, is the most important kininase in plasma of both SHR and normotensive WKY rats. Additional kininase activity involves the enzymes APP and CPN, which also contribute significantly to kinin hydrolysis. The total kinin degradation activity of WKY plasma (13.3 μmol·min$^{-1}·l^{-1}$) corresponds to a half-life of 31 s calculated for 10 μM BK in undiluted plasma. The relative contribution of ACE in this study is consistent with earlier findings that attributed 46.8 and 50% of rat plasma kininase activity to this enzyme (1, 14).

Kinin degradation was determined in the present study at a high (10 μM) substrate concentration, but it must be considered the degree to which those activities reflect the relevance of the individual kininases at physiological (picomolar) kinin levels. All of the rat plasma kininases display relatively high Michaelis-Menten constant ($K_m$) values (16.9, 189, and 19.7 μM for ACE, CPN, and APP, respectively (10, 37)), and therefore the biological activity of each enzyme is reflected by its catalytic efficacy ($k_{cat}/K_m$) value, which corresponds to the relative degradation per time at any given physiological substrate concentration well below these $K_m$ values. At a higher substrate concentration [S], the relative degradation rate underestimates the actual $k_{cat}/K_m$ value by a factor $(1 + [S]/K_m)$. Consequently, the $k_{cat}/K_m$ of ACE, CPN, and APP exceed the relative degradation rates obtained at 10 μM by 59, 5, and 51%, respectively.

Because of this relationship, the half-life of BK at physiological (picomolar) concentrations will be ~30% less than the 31 s determined at 10 μM BK concentration, and the relative contribution of CPN will not reach the 16% of total kininase activity determined under these conditions. On the other hand, the ratio of ACE and APP activities should correspond well to the relative significance of these enzymes under physiolog-
ical conditions. This conclusion is confirmed by measurements on kinin degradation by ACE and APP in the isolated rat heart, where the contribution of ACE measured by using 0.1 nM tritiated BK (54%) was almost as high (46%) when a 3 μM BK concentration was used as substrate (8).

Recent investigations (19) on kininase activities in human plasma have emphasized that the physiological significance of ACE may be greatly underestimated at substrate concentrations in the micromolar range. This is explained by the lower $K_m$ values of human ACE [0.2–1 μM, (9, 16)] and CPN [19 μM, (34)], which cause discrepancies between the $k_{cat}/K_m$ of ACE and the relative degradation rate measured at 10 μM BK by factors of between 10 and 50. Such decisive differences need not be taken into account for the present study because of the higher $K_m$ values of all major kininases in rat plasma.

APP has been found in rat plasma at a low activity, 0.6 μmol·min$^{-1}$·l$^{-1}$, corresponding to 6% of total activity (1), substantially lower than that determined in this study (3.1 μmol·min$^{-1}$·l$^{-1}$, corresponding to 24% of total activity), although an identical substrate concentration was used. The reason for this discrepancy is unclear; however, the higher activity found in this study complies with the 42% residual kininase activity, which could not be attributed to ACE or CPN in an earlier study (14). The fact that unidentified kininases represent only minor activity in our determinations also suggests that no relevant enzymes were neglected. Therefore, this is the first report demonstrating a significant role of APP as a kinin-degrading enzyme in rat plasma. For the reasons stated above, the relative kininase contribution of APP would not have been substantially altered had picomolar, physiological kinin concentrations been investigated. In support of a physiological significance of APP, a marked potentiation of BK-induced hypotension or coronary vasodilation has been observed in anesthetized rats and in isolated perfused rat hearts during inhibition of APP (7, 17). APP has also been detected in human plasma (12); in this case, however, it does not appear to contribute to BK metabolism significantly (19).

The comparison between kininases of plasma and whole blood in the present study revealed that blood cells do not exert a significant influence on overall kinin degradation in blood. This was surprising because APP in particular has previously been identified as a membrane-bound enzyme of platelets and lymphocytes (20, 36), and NEP (EC 3.4.24.11) is present in all kinds of cells do no exert a significant influence on overall kinin degradation in blood. This was surprising because APP in particular has previously been identified as a membrane-bound enzyme of platelets and lymphocytes (20, 36), and NEP (EC 3.4.24.11) is present in all kinds of leukocytes (5, 28) and serves as an established marker enzyme of immature lymphocytes (NEP = CD10, a common acute lymphocytic leukemia antigen) (21).

However, a quantitative determination of the role of cellular kininases in blood has not yet been performed. Apparently, the highly abundant erythrocytes appear to lack any kind of extracellular kininases (13), and the leukocytes and platelets are either inadequately furnished with activity, or the enzymes are not externalized on the cell surface of quiescent cells.

A minor kininase activity in blood and plasma could not be attributed to either ACE, APP, or CPN by the described inhibitor studies. Indeed, all of the known additional kinin-degrading enzymes, such as NEP, prolyl endopeptidase (EC 3.4.21.26), and metalloendopeptidase (EC 3.4.24.15) have already been detected either in plasma (25) or in leukocytes (5). In this study, the spectrum of BK fragments still generated under maximum enzyme inhibition ([1–5], [1–7], and [2–9]-BK) indicates a residual contribution of at least three different enzymes. Because a complete inhibition of ACE by 0.25 μM ramipril can be presumed (8), metalloendopeptidase 24.15 must have led to the genera-

![Fig. 4. Relative contributions of ACE, APP, and CPN to the breakdown of BK in plasma and blood of WKY and SHR. Residual refers to amount of unidentified kininases (means of 5 experiments per group). Data were derived from the inhibitory effects of ramipril (0.25 μM), 2-mercaptoethanol (1 mM), and MGTA (10 μM), respectively, on the degradation of BK (10 μM).](http://ajpheart.physiology.org/)

Table 1. Bradykinin degradation rates in plasma and blood

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ramiprilat</th>
<th>Ramiprilat + MGTA</th>
<th>Ramiprilat + MGTA + Mercaptoethanol</th>
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<tbody>
<tr>
<td>WKY</td>
<td></td>
<td></td>
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<tr>
<td>Plasma</td>
<td>13.32 ± 0.94</td>
<td>6.45 ± 0.62</td>
<td>4.34 ± 0.53</td>
<td>1.01 ± 0.09</td>
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<tr>
<td>Blood</td>
<td>11.96 ± 0.78</td>
<td>6.14 ± 0.46</td>
<td>3.96 ± 0.52</td>
<td>1.29 ± 0.16</td>
</tr>
<tr>
<td>SHR</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>12.22 ± 1.52</td>
<td>6.05 ± 0.38</td>
<td>3.92 ± 0.35</td>
<td>1.12 ± 0.10</td>
</tr>
<tr>
<td>Blood</td>
<td>11.76 ± 0.39</td>
<td>5.65 ± 0.36</td>
<td>4.00 ± 0.59</td>
<td>1.55 ± 0.36</td>
</tr>
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Values are means ± SD; n = 5 rats. MGTA, N-2-mercaptopropyl-3-quanidinothiopropionic acid; WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rats. Influence of kininase inhibitors on the rate of bradykinin degradation in rat plasma or blood (μmol·min$^{-1}$·l$^{-1}$) is shown. Ramiprilat (0.25 μM), MGTA (10 μM), and mercaptoethanol (1 mM) were used to inhibit the kinin-degrading enzymes angiotensin-I converting enzyme, carboxypeptidase N, and aminopeptidase P, respectively. *P < 0.05 vs. control; †P < 0.05 vs. plasma of SHR and WKY.

![Diagram](http://ajpheart.physiology.org/)
tion of [1–5]-BK, whereas the fragment [1–7]-BK indicates the involvement of either NEP or proly endopeptidase. Because APP is the only enzyme known to cleave BK at the amino-terminal side, the generation of [2–9]-BK must have been due to a residual activity of APP. Indeed, 2-mercaptoethanol at 1 mM concentration has been described as a selective, but not fully effective inhibitor of APP (30).

In the blood of SHR, and to a lower extent also in that of WKY, the presence of blood cells augments the contribution of unidentified kininases without significantly altering total kinin degradation. This might reflect the well-known endowment of neutrophils with NEP and the presence of both metalloendopeptidase 24.15 and NEP in T- and B-lymphocytes and in monocytes (5). Because the present investigation was aimed at quantifying kinin degradation pathways that might have a significant impact on the availability of kinins in the circulation, the small contribution that rat leukocytes make to the kinin-degrading abilities of plasma was not characterized further.

The impact of the kallikrein-kinin system on the regulation of blood pressure has been shown previously. Overexpression of kallikrein led to a marked reduction of blood pressure in SHR (38), whereas genetic deletion of B2-receptors sensitizes the blood pressure to salt intake (24). With regard to the kinin-kallikrein system, a variety of differences between SHR and WKY have been described that link kinins as causal or compensatory factors in the development of hypertension. These include an altered sensitivity of the vasculature, renal sensory nerves, and central nervous system to exogenous BK (3, 18, 27) and a renal permeability defect in SHR that is caused by endogenous kinins (31). Although a common basis for those alterations has not yet been identified, changes in kininase activities may be involved. The role of kininases in the physiological regulation of kinin efficacy has been demonstrated with regard to tissue-bound ACE activity, which is upregulated in the heart and vessels under hypertension conditions due to cellular hypertrophy and proliferation (32). Whereas tissue ACE has been emphasized as a target for the antiproliferative actions of ACE inhibitors, the supplementary inhibition of circulating ACE appears to be involved in their antihypertensive actions (22). Thus genetic or environmental alterations in circulating kininase activities should have the potential to influence the manifestation or development of hypertension. However, no differences in the spectrum or potency of plasma or blood kininases have been found between WKY and SHR in the present study. Hence this study shows that there is no evidence that plasma or blood kininase activity contribute to the dysregulation of blood pressure in SHR.

In conclusion, ACE, APP, and CPN are the main BK degrading enzymes in plasma of WKY as well as of SHR. Membrane-bound kininases of blood cells play a minor role in the overall kinin metabolism of blood. There is no evidence for a primary or compensatory alteration of kininase activities in SHR, a well-established model of genetic hypertension.

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