Bradykinin Peptides in Kidney, Blood, and Other Tissues of the Rat

Duncan J. Campbell, Athena Kladis, and Ann-Maree Duncan

The bradykinin peptide system is a tissue-based system with potent cardiovascular and renal effects. To investigate the regulation of this system, we developed a highly sensitive amino terminal–directed radioimmunoassay that, with high performance liquid chromatography, enables the measurement of bradykinin-(1-7), bradykinin-(1-8), and bradykinin-(1-9). Together with a carboxy terminal–directed radioimmunoassay, we characterized bradykinin peptides in rat kidney and blood. The predominant bradykinin peptides in kidney were bradykinin-(1-9) (~100 fmol/g wet weight of tissue) and bradykinin-(1-7) (~70 fmol/g), with low levels of bradykinin-(1-8) (~8 fmol/g) and bradykinin-(4-9) (~12 fmol/g) detectable; bradykinin-(2-9) and bradykinin-(3-9) were below the limits of detection. In blood, the levels of bradykinin-(1-9) were very low (~2 fmol/ml), and other bradykinin peptides were below the limits of detection. Ile,Ser-bradykinin and Met,Ile,Ser-bradykinin were below the limits of detection in both kidney and blood, indicating that T-kininogen makes no detectable contribution to renal or circulating bradykinin peptides. Administration of the angiotensin converting enzyme inhibitor perindopril was associated with an approximate twofold increase in renal levels of bradykinin-(1-8) and bradykinin-(1-9) and a decrease in the bradykinin-(1-7)/bradykinin-(1-9) ratio. The amino terminal–directed radioimmunoassay was also applied to heart, aorta, brown adipose tissue, adrenal, lung, and brain. For these tissues, bradykinin-(1-7) and bradykinin-(1-9) were of similar abundance (16–340 fmol/g), with lower levels of bradykinin-(1-8). These studies demonstrate that tissue levels of bradykinin peptides are much higher than circulating levels, consistent with their formation at a local tissue site. Of peptides derived from K-kininogen, bradykinin-(1-9) is the predominant bioactive peptide in all tissues, and a major pathway of bradykinin-(1-9) metabolism involves the formation of bradykinin-(1-7). In kidney, angiotensin converting enzyme plays an important role in bradykinin-(1-9) metabolism, and increased bradykinin-(1-9) and bradykinin-(1-8) levels may mediate in part the renal effects of converting enzyme inhibition. (Hypertension 1993;21:155–165)

KEY WORDS • radioimmunoassay • chromatography, high performance liquid • peptides • kinins • angiotensin converting enzyme inhibitors

The nonapeptide bradykinin [bradykinin-(1-9), BK-(1-9)] has important actions on blood vessels, the heart, and kidney. By far the most important hemodynamic effect of BK-(1-9) in vivo is the hypotensive vasodilation produced by stimulation of endothelial B2 receptors of arteries and arterioles, with subsequent endothelial release of nitric oxide and prostaglandins. Additional renal actions of BK-(1-9) include the production of a diuresis and natriuresis. Evidence that endogenous bradykinin peptides influence blood pressure and renal function includes the hypertensive effect of bradykinin antagonists in normotensive and hypertensive rats and the decrease in renal blood flow and glomerular filtration rate in response to aprotinin (a non-specific serine protease inhibitor that inhibits renal kallikrein) and bradykinin antagonists. Endogenous bradykinin peptides may also participate in the antihypertensive and renal effects of inhibitors of angiotensin converting enzyme (ACE) (kininase II, dipeptidyl carboxypeptidase, peptidyl dipeptidase hydrolase; EC 3.4.15.1) and neutral metalloendopeptidase-24.11 (EC 3.4.24.11, EP 24.11). Intravenous administration of bradykinin antagonists or kinin antibodies partially reverses the hypertensive effect of ACE inhibition in rats and also reverses the effects of ACE inhibitors on renal papillary blood flow, urine flow, and sodium excretion. Moreover, the kallikrein-kinin system has been implicated in the genesis of hypertension in spontaneously hypertensive rats and of essential hypertension in humans. Investigation of the kallikrein-kinin system presents several concerns. Circulating concentrations of immuno reactive bradykinin are low, and the bradykinin system is considered to operate mainly at a local tissue level. Consequently, a reliable method for the measurement of tissue levels of bradykinin peptides is required. Moreover, the system is complex, in that there are multiple precursors of the kinin peptides, multiple enzymes that can cleave kinins from these precursors, multiple bioactive kinin peptides, and multiple kinin receptors with different specificities. In the rat, the
high and low molecular weight forms of K-kininogen are precursors for BK-(1-9) and, in addition, T-kininogen (major acute phase protein) is a potential precursor for Ile, Ser-bradykinin-(1-9) (Ile, Ser-BK, T-kinin) and Met, Ile, Ser-bradykinin-(1-9) (Met, Ile, Ser-BK). Of the two bioactive bradykinin peptides derived from K-kininogen, BK-(1-9) is the more potent agonist of B, receptors, whereas bradykinin-(1-8) [BK-(1-8)] is the more potent agonist of B, receptors.29 There are no previous estimates of bradykinin peptides in tissues; estimates of bradykinin peptides in blood, plasma, and urine are based on the measurement of immunoreactive bradykinin, and the nature of this immunoreactive material has not been defined.8,21,30-40

To investigate the role of bradykinin peptides in normal renal physiology and in hypertensive states, we developed a highly sensitive amino (N)-terminal-directed radioimmunoassay (RIA) that, with high performance liquid chromatography (HPLC), enables the measurement of bradykinin-(1-7) [BK-(1-7)], BK-(1-8), and BK-(1-9). In addition, a carboxy (C)-terminal-directed RIA was applied to the measurement of BK-(1-9), bradykinin-(2-9) [BK-(2-9)], bradykinin-(3-9) [BK-(3-9)], bradykinin-(4-9) [BK-(4-9)], Ile, Ser-BK, and Met, Ile, Ser-BK. We applied both RIAs to the characterization of all eight peptides in rat kidney and blood and also applied the N-terminal-directed RIA to the measurement of bradykinin peptides in heart, aorta, brown adipose tissue, adrenal, lung, and brain. Neither Ile, Ser-BK nor Met, Ile, Ser-BK was detected in kidney or blood, indicating that T-kininogen makes no detectable contribution to renal or circulating bradykinin peptides. We show that tissue bradykinin peptide levels are higher than circulating levels, consistent with their production in tissue, and we demonstrate that perindopril, an ACE inhibitor, increases renal levels of BK-(1-8) and BK-(1-9).

Methods

Peptides

BK-(1-9), BK-(1-8), bradykinin-(1-4) [BK-(1-4)], BK-(2-9), Ile, Ser-BK, [Lys 8]-bradykinin-(1-9) (Lys-BK), and Met, Lys-bradykinin-(1-9) (Met, Lys-BK) were obtained from Novabiochem AG, Laufelfingen, Switzerland; BK-(1-7), bradykinin-(1-6) [BK-(1-6)], bradykinin-(1-5) [BK-(1-5)], bradykinin-(2-7) [BK-(2-7)], and [Tyr 8]-bradykinin-(1-9) [Tyr 8-BK-(1-9)] were obtained from Bachem Inc., Torrance, Calif.; Met, Ile, Ser-BK, BK-(3-9), BK-(4-9), and bradykinin-(5-9) [BK-(5-9)] were obtained from Auspep Pty. Ltd., Parkville, Australia. Amino-acetyl-[Lys 8]-bradykinin-(1-9) [Acetyl, Lys 8]-BK-(1-9) was synthesized by Ken Mitchelhill and Bruce Kemp at the peptide synthesis facility, St. Vincent's Institute of Medical Research. All peptide concentrations were determined by amino acid analysis using stocks of approximately 1 mg/ml of 20% acetic acid, which were stored at -30°C. Working solutions of RIA standards (1 µM in 1 mg/ml lysozyme, 10 mM acetic acid) were stored at -30°C and were discarded after thawing once.

Animals

Male Sprague-Dawley rats (250-300 g), maintained in a room with a 12-hour light/dark cycle (lights on 6 AM to 6 PM), were fed a diet of GR 2+ pellets (Clarke King & Co., Melbourne, Australia) and received tap water to drink. These studies were performed in accordance with the guidelines of St. Vincent's Hospital Animal Experimentation Ethics Committee.

For the measurement of bradykinin peptides in tissues, rats were killed by decapitation without prior anesthetize (unless specified otherwise), and tissues were rapidly removed, weighed, and immediately homogenized in 4 M guanidine thiocyanate (GTC) and 1% (vol/vol) trifluoroacetic acid (TFA) in water at room temperature, using a Polytron with a 1-cm aggregate (model PT 10-35, Kinematica, Lucerne, Switzerland) operating at maximum speed. For kidney, the time delay between decapitation and homogenization was approximately 30 seconds. Each kidney was homogenized in 20 ml GTC/TFA, and each of the other tissues was homogenized in 10 ml GTC/TFA. Each pair of adrenals was dissected free of connective tissue before homogenization; the whole of each kidney was homogenized after removal of surrounding connective tissue. For the heart, the ventricles were rapidly excised at the atrioventricular junction, then blotted and homogenized. The aorta was excised between the aortic arch and the level of the diaphragm, and the periaortic brown adipose tissue and connective tissue were rapidly stripped from the vessel; then the aorta, free from blood, was homogenized. The periaortic brown adipose tissue, with associated connective tissue, was then homogenized; a portion of peripheral lung was homogenized; a block of brain, including the brain stem, hypothalamus, thalamus, septum, and midbrain, was homogenized. Tissue homogenates were sonicated briefly and then centrifuged at 5,000 rpm for 20 minutes.

For the measurement of bradykinin peptides in blood, rats were first anesthetized with ether, the abdominal aorta was exposed, and 2 ml blood was rapidly collected into a syringe containing 20 ml GTC/TFA. The blood and GTC/TFA were then mixed, transferred to a 50-ml tube, and homogenized briefly, as described above, for tissues.

Extraction of Bradykinin Peptides From Tissues and Blood

Ten milliliters of the supernatant from each homogenate was extracted on a Sep-Pak C 18 cartridge (Waters Chromatography Division, Milford, Mass.), as described previously for angiotensin peptides.41 The eluate was collected into a siliconized 13 x 100 mm borosilicate glass tube and evaporated to dryness in a vacuum centrifuge. Each extract was then dissolved in 1 ml of 1 M hydrochloric acid and was extracted twice with 1 ml diethyl ether. The extracts were then evaporated to dryness again before HPLC. Extracts were subjected to HPLC either 1) without further modification, 2) after acetylation, or 3) after both acetylation and subsequent piperidine treatment. For extracts subjected to HPLC without further modification, the extract was dissolved in 120 µl of 20% glacial acetic acid in water, then transferred to a siliconized microtube and centrifuged at top speed in a microfuge for 5 minutes to remove particulate material, and the supernatant (~100 µl) was injected onto the chromatograph. Extracts were acetylated by sequential addition of 100 µl water, 10 µl triethylamine, and 5 µl acetic anhydride, with mixing...
after each addition. The acetylated extract was then centrifuged as described above and immediately injected onto the chromatograph. Extracts that were both acetylated and piperidine treated were acetylated as described above and then taken to dryness under vacuum before addition of 100 μl water and 10 μl piperidine; after mixing, the extract was left at 20°C for 60 minutes before evaporation to dryness again. The extract was then dissolved in 120 μl acetic acid, centrifuged, and injected onto the chromatograph.

For the determination of the recovery of bradykinin peptides from GTC/TFA homogenates, 20 ml kidney homogenate or 20 ml pooled homogenate of other tissues was divided into two equal portions, to one of which was added 50 μl of a peptide cocktail. For the N-terminal-directed RIA, this cocktail contained 500 fmol/50 μl of each of BK-(1–9), BK-(1–8), and BK-(1–7). Because of the number of kinin peptides measured by the C-terminal–directed RIA and their elution positions on HPLC, two different peptide cocktails were used for recovery determinations: one cocktail contained 500 fmol/50 μl of each of BK-(1–9), BK-(2–9), BK-(3–9), and BK-(4–9), and the second cocktail contained 500 fmol/50 μl of Ile,Ser-BK and Met,Ile,Ser-BK. The homogenates were processed in parallel, recoveries were calculated by subtracting the endogenous peptide levels, and the result was expressed as a percentage of the amount added.

The blank for each assay was assessed by extraction of 10 ml GTC/TFA, and these blank extracts (n = 4 for each assay) were processed as described above and then subjected to HPLC before RIA.

High Performance Liquid Chromatography of Bradykinin Peptides

All separations were performed on a 100×4.6 mm Brownlee RP-18 Spheri-5 column preceded by a 15×3.2 mm RP-18 guard column (Applied Biosystems, Inc., Foster City, Calif.). Solvent A was 0.1% TFA and 0.15 M sodium chloride in water; solvent B was 0.1% TFA and 90% acetonitrile in water. Peptides were eluted by a linearly increasing gradient of 22–42% solvent B over 30 minutes. The flow rate was 1 ml/min, and 0.5-minute fractions were collected into 10×75 mm borosilicate tubes. Extracts that were both acetylated and piperidine treated were acetylated as described above and then taken to dryness under vacuum before addition of 100 μl water and 10 μl piperidine. After mixing, the extract was left at 20°C for 60 minutes before evaporation to dryness again. The extract was then dissolved in 120 μl acetic acid, centrifuged, and injected onto the chromatograph.

For the determination of the recovery of bradykinin peptides subjected to HPLC before RIA. Each assay tubes of the RIA standard curves was 0.5 ml of 5 mg/ml protease-free bovine serum albumin. Fractions and solvent blank fractions were collected into 10×75 mm borosilicate tubes were evaporated to dryness under vacuum and then dissolved in water immediately before RIA.

Radioimmunoassay of Bradykinin Peptides

Two different antisera were used for RIA of bradykinin peptides. The N-terminal–directed antisem R56 was used as a tracer for the N-terminal-directed RIA; Tyr-BK-(1–9) was iodinated with 125I using the chloramine T procedure43 then acetylated as described above, and the moniodinated Tyr-BK-(1–9) was purified by HPLC. The standard for the N-terminal assay was N-acetyl,Lys3-BK-(1–9). The C-terminal–directed antiserum R56 (a generous gift from David Casley, Austin Hospital, Melbourne, Australia) was raised in a rabbit immunized by subcutaneous injection with BK-(1–9) coupled via the N-terminus to bovine thyroglobulin, using glutaraldehyde; this rabbit also received intraperitoneal injections of BK-(1–9) adsorbed onto charcoal. Monoiodinated 125I-Tyr8-BK-(1–9) was purified by HPLC for use as tracer in the C-terminal–directed RIA. The standard for the C-terminal RIA was BK-(1–9). Each RIA was performed with a total volume of 250 μl per assay tube. Tracer (–2,500 cpm), standard, and antiserum were diluted in assay buffer (100 mM sodium phosphate, 10 mM EDTA, 154 mM sodium chloride, 1 g/l sodium azide, 1 g/l casein, pH 7.0). Each assay was incubated at 4°C for 48 hours before separation of free from bound radioactivity with albumin/dextran-coated charcoal. For antiserum B24 at a dilution of 1/267,000, 50% displacement was obtained with 2 fmol per tube N-acetyl,Lys3-BK-(1–9), the detection limit was approximately 0.05 fmol per tube, and the RIA had a within-assay coefficient of variation of 5.7% and a between-assay coefficient of variation of 14.5%. The antiserum R56 at a dilution of 1/50,000, 50% displacement was obtained with 40 fmol per tube BK-(1–9), the detection limit was 1 fmol per tube, and the RIA had a within-assay coefficient of variation of 5.7% and a between-assay coefficient of variation of 9.0%.

The cross-reactivities of each antiserum are shown in Table 1. Antiserum B24 showed a high cross-reactivity with acetylated BK-(1–9), BK-(1–8), and BK-(1–7) but did not recognize nonacetylated peptides or peptides either lacking the N-terminal residue or with an N-terminal extension. Antiserum R56 showed a high cross-reactivity with BK-(1–9), BK-(2–9), BK-(3–9), BK-(4–9), Ile,Ser-BK, and Met,Ile,Ser-BK (Table 1). However, after acetylation, these peptides showed much reduced binding to R56; this may be because of acetylation at sites other than the N-terminus (e.g., Ser*), because binding to R56 was restored by hydrolysis of these extraneous acetyl esters with 10% piperidine while retaining the N-terminal acetyl group and thus binding to antibody B24. Thus, for acetylated/piperidine-treated samples, HPLC fractions could be assayed with both antisera B24 and R56. An advantage of this approach was that the identity of the N-acetyl-BK-(1–9) peak of immunoreactivity was confirmed by its recognition by both antisera. Moreover, N-acetyl-BK-(1–9) was separated from N-acetyl-BK-(3–9) by HPLC, whereas BK-(1–9) and BK-(3–9) coelute when not acetylated (Figure 1).

For routine assay, samples were acetylated before HPLC when bradykinin peptides were measured with the N-terminal–directed RIA, and samples were both acetylated and piperidine treated when bradykinin peptides were measured with the C-terminal–directed RIA.

Validation of Measurement of Bradykinin Peptides

Our identification of individual bradykinin peptides was based on criteria additional to their recognition by bradykinin antisera. The identified bradykinin peptides were shown to coelute with standard peptides on HPLC under at least two different conditions; for peptides recognized by antibody B24, BK-(1–7), BK-(1–8), and BK-(1–9) were shown to coelute with standard peptides recognized by antibody R56, BK-(1–9), BK-(1–8), and BK-(1–7) were shown to coelute with standard peptides.
peptides both for acetylated extracts and for acetylated/piperidine-treated extracts (Figure 1). Similarly, for peptides recognized by antibody R56, BK-(1-9), BK-(2-9), BK-(3-9), BK-(4-9), Ile,Ser-BK, and Met,Ile,Ser-BK, where detected, were shown to coelute with standard peptides both for nonacetylated extracts and for acetylated/piperidine-treated extracts (Figure 1). Moreover, BK-(1-9) was recognized by both antisera B24 and R56 for acetylated/piperidine-treated extracts (Figure 1).

The possible change in bradykinin peptide levels in kidney before homogenization was examined by study of six rats, the left and right kidneys of which were homogenized 30 and 90 seconds after decapitation.
respectively, and then processed in parallel as described above and assayed with both the N-terminal– and C-terminal-directed RIAs.

To determine the stability of bradykinin peptides in the GTC/TFA homogenate, two studies were performed. First, rat kidney homogenates of 20 ml were divided into two equal portions; half of each homogenate was extracted approximately 1 hour after homogenization and the second half extracted approximately 2 hours after homogenization. These extracts were processed in parallel and assayed with the N-terminal–directed RIA. A similar study was performed for blood extracts. Second, rat kidney homogenates of 20 ml were divided into two equal portions, to one of which was added approximately 1,000 fmol BK-(1-9); the homogenates were then processed as described above and assayed with both the N-terminal– and C-terminal–directed RIAs.

To examine whether homogenization of tissue in GTC/TFA causes an immediate arrest of bradykinin peptide degradation and generation and avoids the possible consequences of activation of prekallikrein, we also measured bradykinin peptide levels in kidneys that were snap-frozen. After decapitation of each of six rats, the left kidney was immediately homogenized in GTC/TFA at room temperature, and the right kidney was immediately clamped between two metal plates that had been cooled to the temperature of liquid nitrogen. The powdered frozen kidney was added to GTC/TFA at 0°C and was immediately homogenized by a Polytron. The two renal extracts from each rat were processed in parallel as described above and assayed with the N-terminal–directed RIA.

**Effect of Ether Anesthesia on Bradykinin Peptide Levels in Rat Kidney**

Given that bradykinin peptides were measured in blood of ether-anesthetized rats, we examined whether ether anesthesia might affect bradykinin peptides by studying the effect of ether anesthesia on bradykinin peptide levels of the kidney. Twelve rats were killed by decapitation, ether anesthesia being administered to half of them (ether group) and the other half left untreated (control group). After decapitation of each of the 24 rats, the left kidney was immediately homogenized in GTC/TFA. Twelve milliliters of the homogenate were then processed as described above and assayed with both the N-terminal– and C-terminal–directed RIAs.

**Effect of Perindopril on Bradykinin and Angiotensin Peptides in Rat Kidney**

For the study of the effect of perindopril on bradykinin peptides in the kidney, rats received either tap water to drink or tap water containing either 16 or 48 μg/ml perindopril. Perindopril (a generous gift from Servier Laboratories, Courbevoie, France) was freshly prepared each evening. Water intake was approximately 30 ml per rat per 24 hours, resulting in a perindopril intake of approximately 1.4 and 4.2 mg/kg per 24 hours, respectively. These doses of perindopril produce near-maximal inhibition of plasma ACE activity and reduction of blood pressure in spontaneously hypertensive rats. After 7 days, the rats were killed at 10 AM by decapitation. Kidneys were homogenized, extracted, processed, and acetylated as described above, and the HPLC fractions were assayed for BK-(1-7), BK-(1-8), and BK-(1-9) using antibody B24, and for angiotensin II, angiotensin-(1-9), and angiotensin I using antibody A41.46

**Statistical Analysis**

Comparisons between groups were made by either unpaired t test or paired t test, as appropriate. Differences were termed significant if the t value exceeded the critical value for the 5% level.

**Results**

** Bradykinin Peptides in Kidney, Blood, and Other Tissues**

The HPLC-based RIA used in this study enabled the precise identification of eight bradykinin peptides in kidney. The elution positions of bradykinin peptides from HPLC were highly reproducible, with peptides eluting in no more than three fractions. No detectable endogenous levels were measured for BK-(4-9) and BK-(7-9). The low recoveries of angiotensin I and angiotensin II were attributed to the method of extraction.

**Endogenous Levels of Bradykinin Peptides in Rat Kidney**

A comparison of the recoveries (from extraction, acetylation, and HPLC) of these peptides, as determined by the HPLC-based RIA, is shown in Table 2. The minimum detectable levels apply to a mean wet weight of 0.62 g for kidney. Endogenous levels have been corrected for antibody cross-reactivity and recovery. Recoveries and endogenous levels are mean±SEM. For recoveries, n=5–6; for endogenous levels, n=18–27.

**Table 2. Recoveries, Minimum Detectable Levels, and Endogenous Levels of Bradykinin Peptides in Rat Kidney**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Recovery (%)</th>
<th>Minimum detectable (fmol/g)</th>
<th>Endogenous peptide (fmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino terminal-directed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BK-(1-9)</td>
<td>59±3</td>
<td>3</td>
<td>96±9</td>
</tr>
<tr>
<td>BK-(1-8)</td>
<td>64±4</td>
<td>2.5</td>
<td>8±1</td>
</tr>
<tr>
<td>BK-(1-7)</td>
<td>53±4</td>
<td>3.7</td>
<td>70±10</td>
</tr>
<tr>
<td>Carboxy terminal-directed</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BK-(1-9)</td>
<td>46±3</td>
<td>11</td>
<td>125±21</td>
</tr>
<tr>
<td>BK-(2-9)</td>
<td>59±4</td>
<td>10</td>
<td>&lt;10</td>
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<tr>
<td>BK-(3-9)</td>
<td>46±3</td>
<td>18</td>
<td>&lt;18</td>
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<tr>
<td>BK-(4-9)</td>
<td>68±2</td>
<td>12</td>
<td>12±3</td>
</tr>
<tr>
<td>Ile,Ser-BK</td>
<td>35±1</td>
<td>15</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Met,Ile,Ser-BK</td>
<td>13±2</td>
<td>57</td>
<td>&lt;57</td>
</tr>
</tbody>
</table>

- BK-(1-9), bradykinin-(1-9); BK-(1-8), bradykinin-(1-8); BK-(1-7), bradykinin-(1-7); BK-(2-9), bradykinin-(2-9); BK-(3-9), bradykinin-(3-9); BK-(4-9), bradykinin-(4-9); Ile,Ser-BK, Ile,Ser-bradykinin-(1-9); Met,Ile,Ser-BK, Met,Ile,Ser-bradykinin-(1-9).
- The minimum detectable levels apply to a mean wet weight of 0.62 g for kidney. Endogenous levels have been corrected for antibody cross-reactivity and recovery. Recoveries and endogenous levels are mean±SEM. For recoveries, n=5–6; for endogenous levels, n=18–27.

The use of these RIAs allowed the identification of the levels of these peptides in kidneys from rats that had received either perindopril or vehicle. The predominant bradykinin peptides in kidney were BK-(1-7) and BK-(1-9), with low levels of BK-(1-8) and BK-(4-9) detectable (Table 2). BK-(2-9), BK-(3-9), Ile,Ser-BK, and Met,Ile,Ser-BK were below the minimum detectable. Similarly, for the other tissues examined with the N-terminal–directed RIA, BK-(1-7) and BK-(1-9) were the predominant peptides, with lower levels of BK-(1-8) detected (Table 3, Figure 2). By contrast, for blood the levels of...
TABLE 3. Recoveries, Minimum Detectable Levels, and Endogenous Levels of Bradykinin Peptides in Rat Tissues as Determined With Amino Terminal-Directed (B24) Assay (Acetylated)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Recovery (%)</th>
<th>Minimum detectable (fmol/g)</th>
<th>Endogenous peptide (fmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BK-(1-9)</td>
<td>57±2</td>
<td>0.9</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>BK-(1-8)</td>
<td>54±2</td>
<td>0.9</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>BK-(1-7)</td>
<td>50±3</td>
<td>1.3</td>
<td>&lt;1.3</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BK-(1-9)</td>
<td>50±3</td>
<td>2.4</td>
<td>18±3</td>
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<td>BK-(1-8)</td>
<td>50±3</td>
<td>2.5</td>
<td>2.6±0.8</td>
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<tr>
<td>BK-(1-7)</td>
<td>41±2</td>
<td>4</td>
<td>16±3</td>
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<td>Aorta</td>
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<tr>
<td>BK-(1-9)</td>
<td>49±2</td>
<td>35</td>
<td>342±38</td>
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<td>BK-(1-8)</td>
<td>44±3</td>
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<tr>
<td>BK-(1-7)</td>
<td>38±2</td>
<td>60</td>
<td>191±58</td>
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<td>Brown adipose tissue</td>
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<tr>
<td>BK-(1-9)</td>
<td>53±2</td>
<td>14</td>
<td>239±58</td>
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<td>BK-(1-8)</td>
<td>50±3</td>
<td>15</td>
<td>68±18</td>
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<tr>
<td>BK-(1-7)</td>
<td>42±2</td>
<td>24</td>
<td>181±49</td>
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<td>Adrenal</td>
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<tr>
<td>BK-(1-9)</td>
<td>62±3</td>
<td>33</td>
<td>106±26</td>
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<td>BK-(1-8)</td>
<td>58±3</td>
<td>35</td>
<td>37±13</td>
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<tr>
<td>BK-(1-7)</td>
<td>51±2</td>
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<td>84±14</td>
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<tr>
<td>Lung</td>
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<tr>
<td>BK-(1-9)</td>
<td>57±3</td>
<td>2.2</td>
<td>107±23</td>
</tr>
<tr>
<td>BK-(1-8)</td>
<td>58±2</td>
<td>2.1</td>
<td>15±2</td>
</tr>
<tr>
<td>BK-(1-7)</td>
<td>50±2</td>
<td>3.3</td>
<td>41±7</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BK-(1-9)</td>
<td>56±2</td>
<td>4</td>
<td>28±8</td>
</tr>
<tr>
<td>BK-(1-8)</td>
<td>57±2</td>
<td>4</td>
<td>3±1</td>
</tr>
<tr>
<td>BK-(1-7)</td>
<td>44±2</td>
<td>7</td>
<td>26±7</td>
</tr>
</tbody>
</table>

**Note:** BK-(1-9), bradykinin-(1-9); BK-(1-8), bradykinin-(1-8); BK-(1-7), bradykinin-(1-7). Minimum detectable levels apply to a blood volume of 1 ml and mean wet weight of 0.81 g for heart, 0.06 g for aorta, 0.13 g for brown adipose tissue, 0.05 g for adrenal, 0.82 g for lung, and 0.46 g for brain. Endogenous levels have been corrected for antibody cross-reactivity and recovery. Recoveries and endogenous levels are mean±SEM. For recoveries, n=6; for endogenous levels, n=12 for blood and n=6 for other tissues.

Validation of Measurement of Bradykinin Peptides in Rat Kidney

A 60-second delay in homogenization did not result in any significant change in the levels of bradykinin peptides measured in kidney (Table 4). Moreover, bradykinin peptides were stable in GTC/TFA kidney homogenate in that a 1-hour delay in extraction was not associated with any change in the measured levels of bradykinin peptides (Table 5). We also studied the effect of a 1-hour time delay in extraction on bradykinin peptides were very low, with only BK-(1-9) detected (<2 fmol/ml) (Table 3). We also applied the C-terminal-directed RIA to measurement of bradykinin peptides in blood. These measurements were complicated by the elution of "nonspecific" immunoreactivity in the elution positions of N-acetyl-BK-(1-9) and N-acetyl-BK-(4-9). However, for BK-(2-9) (<4 fmol/ml), BK-(3-9) (<7 fmol/ml), Met,Ile,Ser-BK (<3 fmol/ml), and Met,Ile,Ser-BK (<5 fmol/ml), all levels were below the minimum detectable.

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peptides in blood; the measured levels of BK-(1-9) were 3.00±0.03 fmol/ml (mean±SEM, n=6) at 1 hour after homogenization and 2.3±0.3 fmol/ml (p<0.05) at 2 hours after homogenization; BK-(1-7) and BK-(1-8) were below the minimum detectable at both times. When approximately 1,000 fmol BK-(1-9) was added to rat kidney homogenate, there was no evidence of metabolism of the BK-(1-9) except for a small increase in BK-(1-8) levels, which represented <1% of the added amount of BK-(1-9) (Table 6).

Further evidence for the efficiency by which bradykinin peptide degradation and generation were arrested by homogenization in GTC/TFA is the finding that similar bradykinin peptide levels were measured for kidneys that were either immediately homogenized in GTC/TFA at room temperature or rapidly cooled to the temperature of liquid nitrogen before pulverization and homogenization (Table 7).

### Table 6. Metabolism of Bradykinin-(1-9) During Processing of Rat Kidney Homogenate for Assay

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Control homogenate</th>
<th>Homogenate plus BK-(1-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino terminal-directed (B24)</strong> assay (acyetylated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BK-(1-9)</td>
<td>68±5</td>
<td>1,129±46*</td>
</tr>
<tr>
<td>BK-(1-8)</td>
<td>3.7±0.3</td>
<td>9.8±1.1*</td>
</tr>
<tr>
<td>BK-(1-7)</td>
<td>41±9</td>
<td>38±7</td>
</tr>
<tr>
<td><strong>Carboxy terminal-directed (R56)</strong> assay (acyetylated/piperidine treated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BK-(1-9)</td>
<td>139±45</td>
<td>1,926±288*</td>
</tr>
<tr>
<td>BK-(2-9)/Ile,Ser-BK</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>BK-(3-9)</td>
<td>&lt;18</td>
<td>&lt;18</td>
</tr>
<tr>
<td>BK-(4-9)</td>
<td>7±2</td>
<td>19±5</td>
</tr>
<tr>
<td>Met,Ile,Ser-BK</td>
<td>&lt;57</td>
<td>&lt;57</td>
</tr>
</tbody>
</table>

**BK-(1-9), bradykinin-(1-9); BK-(1-8), bradykinin-(1-8); BK-(1-7), bradykinin-(1-7); BK-(2-9), bradykinin-(2-9); BK-(3-9), bradykinin-(3-9); BK-(4-9), bradykinin-(4-9); Ile,Ser-BK, Ile, Ser-bradykinin-(1-9); Met,Ile,Ser-BK, Met,Ile,Ser-bradykinin-(1-9).** Different homogenates were used for amino terminal-directed and carboxy terminal-directed assays. For amino terminal-directed assays, 950 fmol BK-(1-9) was added to homogenates for which the mean wet weight of kidney was 0.64 g, resulting in an addition of 1.480 fmol/g kidney. For carboxy terminal-directed assays, 1,220 fmol BK-(1-9) was added to homogenates for which the mean wet weight of kidney was 0.58 g, resulting in an addition of 2,100 fmol/g kidney. Data are mean±SEM, n=6.

Effect of Ether Anesthesia on Bradykinin Peptide Levels in Rat Kidney

Ether anesthesia was not associated with any significant change in bradykinin peptide levels in the kidney (Table 8).

Effect of Perindopril on Bradykinin and Angiotensin Peptides in Rat Kidney

Perindopril had different effects on bradykinin and angiotensin peptides in the kidney (Figure 3). The changes in angiotensin peptides were similar to those we have described previously, with marked falls in renal angiotensin II levels for both doses of perindopril and a reduction in the angiotensin II/angiotensin I ratio for the highest dose of perindopril; the levels of angiotensin-(1-9) and angiotensin I showed no change. By contrast, BK-(1-7) showed no change, and there were increases in both BK-(1-8) and BK-(1-9) for the highest dose of perindopril, with a consequent fall in the BK-(1-7)/BK-(1-9) ratio.

### Discussion

The use of HPLC and specific RIA in the present study enabled the precise identification and measurement of individual bradykinin peptides in blood and tissues. The N-terminal RIA we have developed is the most sensitive bradykinin RIA described, and it is of considerable utility given that it enables the measurement of two bioactive peptides BK-(1-9) and BK-(1-8), and also of BK-(1-7), a major metabolite of BK-(1-9). Particular care was taken to validate the measurement of bradykinin peptides in kidney. As we

### Table 7. Comparison of Bradykinin Peptide Levels in Rat Kidneys Immediately Homogenized and Kidneys Snap-Frozen and Pulverized After Homogenization as Determined With Amino Terminal-Directed (B24) Assay (Acetylated)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Left kidney</th>
<th>Right kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK-(1-9)</td>
<td>77±14</td>
<td>72±4</td>
</tr>
<tr>
<td>BK-(1-8)</td>
<td>7±1</td>
<td>6±1</td>
</tr>
<tr>
<td>BK-(1-7)</td>
<td>30±6</td>
<td>37±4</td>
</tr>
</tbody>
</table>

**BK-(1-9), bradykinin-(1-9); BK-(1-8), bradykinin-(1-8); BK-(1-7), bradykinin-(1-7).** Data are mean±SEM, n=6.

### Table 8. Effect of Ether Anesthesia on Bradykinin Peptide Levels in Rat Kidney as Determined With Amino Terminal-Directed (B24) Assay (Acetylated)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>No anesthesia</th>
<th>Ether anesthesia</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK-(1-9)</td>
<td>81±14</td>
<td>61±8</td>
</tr>
<tr>
<td>BK-(1-8)</td>
<td>9±2</td>
<td>7±1</td>
</tr>
<tr>
<td>BK-(1-7)</td>
<td>39±11</td>
<td>30±2</td>
</tr>
</tbody>
</table>

**BK-(1-9), bradykinin-(1-9); BK-(1-8), bradykinin-(1-8); BK-(1-7), bradykinin-(1-7).** Data are mean±SEM, n=5–6.
**Figure 3.** Line graphs show bradykinin-(1–7) (BK(1–7)), bradykinin-(1–8) (BK(1–8)), bradykinin-(1–9) (BK(1–9)), BK(1–7):BK(1–9) ratio, angiotensin II (Ang II), angiotensin-(1–9) (Ang(1–9)), angiotensin I (Ang I), and Ang II:Ang I ratio for kidney of control rats and rats administered either 1.4 or 4.2 mg/kg per day perindopril for 7 days. Each point represents mean±SEM (n=6 for control and 4.2 mg/kg perindopril; n=4 for 1.2 mg/kg perindopril). *p<0.02 compared with control.
have previously shown for angiotensin peptides, a 60-second delay in processing of the kidney did not affect the measured levels of bradykinin peptides. This does not indicate that renal bradykinin peptides are protected from metabolism, which is very rapid in the kidney. Rather, the relatively constant level of bradykinin peptides during the delay before processing indicates that during this time peptide metabolism approximates the production rate of these peptides in the kidney.

For the measurement of bradykinin peptides in blood and tissues, it is necessary to prevent both degradation and generation of these peptides during extraction and processing of the samples. The use of GTC/TFA proved effective in this regard. Measurement of bradykinin peptide levels in blood requires collection of blood directly into an effective inhibitor mixture. If trunk blood from decapitated rats is collected immediately into GTC/TFA, the measured levels of BK-(1-9) are >1,000 fmol/ml (unpublished data from our laboratory). This probably reflects the activation of the blood clotting cascade by contact of blood with tissue factor, although the role of plasma prekallikrein in normal coagulation is debatable. It is evident that for reliable measurement of tissue levels of bradykinin peptides to be obtained, it is necessary to prevent contact of blood with tissue factor. As indicated by the lack of effect of a 60-second time delay before homogenization on kidney bradykinin peptide levels, the levels of bradykinin peptides in blood remain very low while blood within an organ remains intravascular and not in contact with tissue factor. In the present study, we chose to kill the rats by decapitation to exsanguinate the animals and minimize the risk of contact of blood with tissue factor.

The present estimates of bradykinin peptide levels in blood are lower than recent reports of immunoreactive bradykinin in blood of conscious rats (—35 pg/ml) and in plasma of ether-anesthetized rats (—15 pg/ml). Previous studies have emphasized that measured levels of circulating immunoreactive kinins are dependent on the method of sample collection and on the antiserum used for RIA. Our finding that ether anesthesia was without effect on renal bradykinin peptide levels is evidence that the measurement of bradykinin peptides in blood of ether-anesthetized rats may give a reliable estimate of circulating levels in conscious rats. The present results indicate that BK-(1-9) is the major circulating bradykinin peptide. Although there is evidence for the formation of bradykinin peptides in the vascular wall, apparently very little of these escape into the circulation. That all tissues contain bradykinin peptide levels higher than those in blood is direct evidence for the predominant formation of these peptides at a local tissue site.

Neither Ile,Ser-BK nor Met,Ile,Ser-BK was detected in either kidney or blood, indicating that T-kininogen makes no detectable contribution to either renal or circulating bradykinin peptides. This result is in agreement with the study by Gardes et al., in which it was found that T-kininogen was without effect when introduced into the perfusate of the isolated perfused rat kidney, in contrast to the marked effects of K-kininogen. These data indicate that K-kininogen is the predominant, if not the sole, precursor of bradykinin peptides in the rat.

The measurement of individual bradykinin peptides provides important information on the metabolism pathway of BK-(1-9). In all tissues, BK-(1-7) and BK-(1-9) were the most abundant bradykinin peptides and were present in approximately equal amounts. Thus, BK-(1-7) represents a major pathway of metabolism of BK-(1-9). A number of peptidases have been implicated in the metabolism of BK-(1-9) (Figure 4). BK-(1-7) may result from the action of ACE, prolyl endopeptidase, or EP 24.11. All tissues contained low levels of BK-(1-8), indicating a role for carboxypeptidases in the metabolism of BK-(1-9). In kidney, we also identified low levels of BK-(4-9), which may result from the action of either prolyl endopeptidase or the sequential action of aminopeptidase P and dipeptidylaminopeptidase IV. Further experiments using specific inhibitors will be necessary to delineate the relative contribution of each of these peptidases to bradykinin metabolism.

Previous studies have shown that ACE inhibitors may increase circulating kinin levels and urinary kinin excretion, although EP 24.11 may have a greater role in renal metabolism of BK-(1-9) than ACE. Our ability to simultaneously measure the tissue levels of both angiotensin and bradykinin peptides has given valuable information concerning the effects of perindopril on these two peptide systems in the kidney. Whereas both doses of perindopril reduced renal angiotensin II levels, only the highest dose (4.2 mg/kg per day) of perindopril increased BK-(1-9) and BK-(1-8) levels. That BK-(1-9) and BK-(1-8) responded to only the higher dose may be because BK-(1-9) has a higher affinity for ACE than angiotensin I. Moreover, the effects of 4.2 mg/kg per day perindopril may not be maximal, and it will be necessary to study the effects of higher doses of ACE inhibitor to determine the maximal contribution of ACE to BK-(1-9) metabolism in the kidney. Nevertheless, the present data clearly indicate that ACE plays an important role in BK-(1-9) metabolism in the kidney and that increased renal BK-(1-8) and BK-(1-9) levels may contribute to the renal effects of ACE inhibitors. The failure of BK-(1-7) levels to fall with ACE inhibition is indicative of the operation of alternative pathways, such as EP 24.11 and prolyl endopeptidase, in the formation of this peptide from the twofold higher levels of BK-(1-9). Similarly, the increased BK-(1-8) levels are most likely the direct consequence of increased BK-(1-9) levels.

It is of interest that a number of ACE inhibitors have recently been shown to also inhibit aminopeptidase P. Whether perindopril inhibits aminopeptidase P is not known. However, the significant fall in the BK-(1-7)/BK-(1-9) ratio seen in the present study indicates that
the effect of perindopril on renal bradykinin peptides is mediated predominantly by ACE inhibition.

In conclusion, we have applied HPLC-based RIA to the characterization of bradykinin peptides in kidney, blood, and other tissues. We have shown that tissue-mediated predominantly by ACE inhibition. The characterization of bradykinin peptides in kidney, site. Ile,Ser-BK and Met,Ile,Ser-BK were below the limits of detection in both kidney and blood, indicating that T-kininogen makes no detectable contribution to either renal or circulating bradykinin peptides. Of peptides derived from K-kininogen, BK-(1-9) is the predominant bioactive peptide in all tissues, and a major pathway of BK-(1-9) metabolism involves the formation of BK-(1-7). In kidney, BK-(1-9) levels increase with ACE inhibition, demonstrating an important role for ACE in renal metabolism of BK-(1-9) and providing direct support for the hypothesis that bradykinin peptides mediate, in part, the renal effects of ACE inhibitors.

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