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ORIGIN AND CONTENT OF METHIONYL-LYSYL-BRADYKININ, LYSYL-BRADYKININ AND BRADYKININ IN HUMAN URINE

VALDEMAR HIAL,* HARRY R. KEISER and JOHN J. PISANO
Hypertension-Endocrine Branch and Laboratory of Chemistry,
National Heart and Lung Institute, National Institutes of Health, Bethesda, MD. 20014, U.S.A.

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Abstract—When human urine is collected in acid, three kinins, bradykinin (BK), lysyl-bradykinin (LBK, kallidin) and methionyl-lysyl-bradykinin (MLBK), are found. The identification of the MLBK was based on: (1) a chromatographic procedure which clearly separated it from BK and LBK, (2) its conversion by dipeptidyl aminopeptidase I to BK which was established by chromatographic and bioassay procedures, and (3) its amino acid composition. When the potent pepsin inhibitor, pepstatin, was added to the collection bottle in addition to the usual acid (to inhibit kininases), MLBK was either undetectable or reduced by 90 per cent. Pepstatin did not alter the excretion of BK or LBK. Addition of purified kininogen to acidified urine from a subject with a congenital absence of kininogen led to the formation of MLBK but no BK or LBK. In urine acidified after excretion, it is highly probable that MLBK is formed by uropepsin.

The urinary kinins, kallidin (lysyl-bradykinin, LBK) and badykinin (BK), probably arise in the kidney [1, 2]. LBK is the product of urinary kallikrein [3]. BK is formed by an aminopeptidase which cleaves the amino-terminal lysine from LBK [3, 4]. The third kinin, methionyl-lysyl-bradykinin (MLBK) was found in pooled urine (normal men) which had been processed as soon as possible after collection but not in urine stored frozen before extraction [5]. Since human urinary kallikrein forms only LBK [3] from human kininogen, the origin of MLBK is unknown. The occurrence of MLBK in urine and its source are the subjects of this report.

EXPERIMENTAL PROCEDURE

Materials. The following reagents were obtained from commercial sources: Amberlite IRC-50 (100–200 mesh), Mallinckrodt; SP-Sephadex C-25, Pharmacia; BK, LBK and MLBK, Protein Research Foundation, Osaka, Japan; and dipeptidyl aminopeptidase I (DAP I) from Schwarz/Mann. Pepstatin was kindly provided by Professor P. Umezawa and human kininogen B 3.2α by Drs Jack V. Pierce and Jorge A. Guimaraes [6].

Urine collections. Twenty-four-hr urine specimens were obtained from 17 normal volunteers, 10 men and 7 women, ages 18–48 yr. Urine was collected in plastic bottles containing 20 ml of 6 N HCl (final pH, 2–3) and stored at 4°.

Adsorption to IRC-50 (H^+). The following steps were performed at room temperature. A 200-ml sample of urine was diluted with 200 ml of distilled water in a 500-ml plastic bottle. After adjustment to pH 4 with 1 N NH_4OH, 400 mg IRC-50 was added [5] and the bottle was shaken for 1 hr on a mechanical shaker. The resin was allowed to settle for a few min and the bulk of the supernatant was removed by suction. The residue was vacuum filtered on a Nalgene polyethylene filter, 0.45 micropore (Cat. No. 245-0045), and washed with deionized water until the conductivity of the filtrate was 10–15 μS. Kinins were recovered by transferring the resin to a polyethylene test tube with 20 ml of 9–10 M acetic acid and shaking the tube for 1 hr. The suspension was filtered as above and the filtrate was freeze-dried.

Chromatography on SP-Sephadex C-25. The three kinins, BK, LBK and MLBK, were separated at room temperature on a SP-Sephadex C-25 column, 0.6 × 12.5 cm, by a minor modification of the method of Sampaio *et al.* [7]. The resin was suspended in 0.9 M acetic acid for 24 hr, filtered and equilibrated with 0.05 M Tris–HCl buffer, pH 8.0, containing 0.08 M NaCl and 0.002% merthiolate as preservative. The freeze-dried sample was dissolved in 0.5 to 1.0 ml buffer and applied to the column. Kinins were eluted at room temperature with a buffer flow rate of 3.5 to 5.0 ml/hr.

Bioassays. The fractions obtained from the SP-Sephadex C-25 column were bioassayed with either the isolated guinea pig ileum or rat uterus against standard BK, LBK and MLBK.

The distal 10 cm of the ileum was removed from guinea pigs weighing 150–180 g and placed in Tyrode's solution for at least 1 hr at 4° to reduce spontaneous activity and provide better dose–response curves. A 2- to 3-cm segment of the terminal ileum was then suspended in a 10-ml bath containing aerated Tyrode's solution at 32°. Initially, about 200 ug chymotrypsin [8] was added to the bath for 1–2 min to obtain higher sensitivity and to avoid subsequent spontaneous contraction of the tissue. Assays were made every 3 min and the height of the maximal isotonic contraction was recorded with a Phipps & Bird linear motion transducer.

* Present address: Departamento de Microbiologia e Imunologia, Faculdade de Medicina do Triangulo Mineiro, 38.100 Uberaba M.G., Brasil.

The rat uterus was used when higher sensitivity was required. The uterine horns were excised from 150- to 180-g rats in induced estrus (injected i.p. with 1 mg/kg of diethylstibesterol in 50% ethanol 20–24 hr prior to sacrifice) and placed in cold (0–5°) De Jalon's solution for 1 hr. A 1-cm segment was suspended in a 1-ml bath containing oxygenated (95% O_2–5% CO_2) De Jalon's solution at room temperature (25°). Test solutions were added at 5-min intervals.

The rat duodenum was used in qualitative tests because bradykinin and analogs characteristically relax the duodenum. The proximal 3 cm of the duodenum was removed from 200-g rats and suspended in a 10-ml bath of aerated Tyrode's solution at 32°.

Digestion with DAP I. Aliquots (0.2 ml) of standard BK, LBK and MLBK dissolved in dilute acetic acid and samples from the SP-Sephadex column were adjusted to pH 6.0 by addition of an equal volume of pyridine–acetic acid buffer and incubated at 37° with 10–20 μg DAP I for 5 min. The pyridine–acetic acid buffer contained 20 ml H_2O, 0.16 g pyridine, 0.20 g NaCl and 0.10 ml mercaptoethanol. It was adjusted to pH 5.0 with glacial acetic acid.

Pepstatin and kininogen. The effect of pepstatin on urinary kinins was studied in four men. The pepsin inhibitor, 0.2 mg, was added to the urine collection bottles in addition to the usual 20 ml of 6 N HCl. In another experiment, 1 mg kininogen was added to 50 ml urine from a woman with a congenital deficiency of plasma kininogen [9]. The urine (kindly provided by Dr. Robert Colman) was adjusted to pH 2.0 and incubated for 2 hr at room temperature and kept overnight at 4°.

Recovery of internal standards. BK, LBK and MLBK, 2.5 to 10.0 μg, were added to 200-ml aliquots of two 24-hr specimens. The samples were carried through the entire procedure and recoveries determined for each kinin. Recoveries were also determined for the SP-Sephadex C-25 chromatography step alone. The total amount of kinin was determined by summation of the results from the ileum bioassays of each fraction from the column.

RESULTS

Kinin separation and identification of MLBK. Inasmuch as the three urinary kinins, BK, LBK and MLBK, have similar biological properties, but different potencies, their separation is required prior to assay. This separation was achieved by SP-Sephadex chromatography [7] modified by the use of a shorter column and a slightly different buffer. The three kinins added to urine were eluted with essentially the same retention times as the endogenous kinins (Fig. 1, panels A and B). The order of elution and recoveries was BK, 90 per cent; MLBK, 85 per cent; and LBK, 85 per cent. Small losses occurred at the IRC-50 step; kinins added to the SP-Sephadex column were quantitatively recovered.

Further evidence for the occurrence of MLBK in urine was obtained with DAP I. This enzyme converts MLBK to BK (Fig. 2) but does not hydrolyze BK or LBK because it is unable to cleave bonds on either side of a proline residue [10]. Upon digestion of the three peaks of kinin activity in human urine with DAP I, only the peak with the retention time corresponding to authentic MLBK was affected. Bioassay of the digests (after freeze-drying to remove inhibitory mercaptoethanol) showed the 9- to 10-fold increase in activity expected when MLBK is converted to BK. Chromatographic analysis of the digest revealed that MLBK was completely converted to BK (Fig. 1, panel C). All three urinary kinins, BK, LBK and MLBK, relaxed the rat duodenum before and after treatment with DAP I. Relaxation of this tissue is characteristic of BK and its analogs.

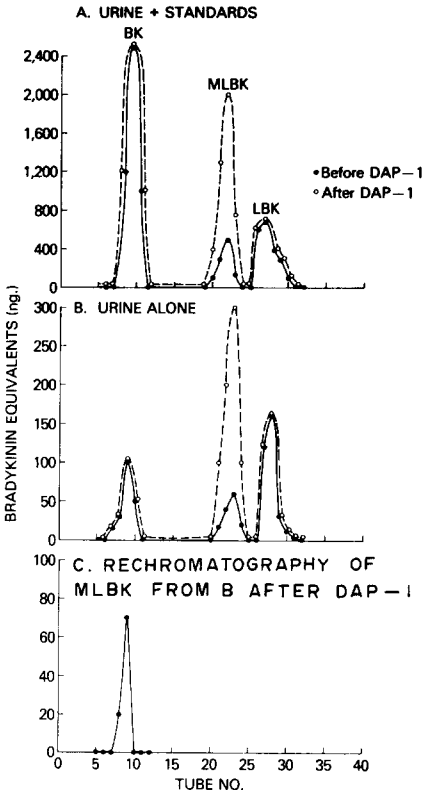

Fig. 1. Chromatography or urine kinins bradykinin (BK), lysyl-BK (LBK) and methionyl-LBK (MLBK). Panel A: urine kinins plus standards before and after treatment of each kinin with DAP I. Panel B: urine alone before and after DAP I treatment. Panel C: rechromatography of urinary MLBK after DAP I treatment. Column: SP-Sephadex C-25, 0.6 × 12.5 cm. Buffer: 0.05 M Tris–HCl, pH 8.0, containing 0.08 M NaCl. Flow rate: 3.5 to 5.0 ml/hr. Fractions: 2.0 ml. Bioassay: guinea pig ileum.

Amino acid analysis of the MLBK isolated from 2 liters urine was somewhat compromised by contaminants eluting from the SP-Sephadex column. However, the molar ratios found in a hydrolysate of 0.01 μmole of sample (and theoretical ratios) were: Met 0.73 (1.0), Arg 2.0 (2.0), Pro 3.0 (3.0), Phe 2.0 (2.0), Lys 1.5 (1.0, high because of an unresolved contaminant peak), Ser 2.4 (1.0) and Gly 3.0 (1.0). Note the correct ratios found for Arg, Pro and Phe (eight of the eleven residues). Methionine was low since some was oxidized during hydrolysis. On the other hand, the high molar ratios for Gly and Ser

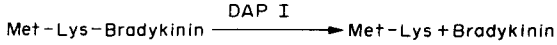

Fig. 2. Formation of bradykinin from methionyl-lysyl-bradykinin through the action of DAP I.

Table 1. Urinary kinins

Subject	Bradykinin (μg/24 hr)	Lysyl-bradykinin (μg/24 hr)	Methionyl-lysyl-bradykinin (μg/24 hr)
Mean:			
1	2.7	11.6	22.0
2	5.2	10.6	7.6
3	1.2	5.7	7.9
4	1.4	13.0	13.9
5	4.1	4.0	5.4
6	4.3	4.5	4.3
7	0.9	8.8	6.3
8	7.5	9.6	9.9
9	2.9	4.2	10.3
10	4.0	4.0	7.2
Mean ± S.E.M.	3.4 ± 0.6	7.6 ± 1.1	9.5 ± 1.6
Women:			
1	0.4	5.0	0.8
2	2.9	8.9	2.6
3	1.7	1.3	0.3
4	1.9	3.1	<0.1
5	1.0	6.5	0.7
6	4.0	4.2	1.3
7	5.6	6.7	2.8
Mean ± S.E.M.	2.5 ± 0.7	5.1 ± 1.0	1.2 ± 0.4

were not unexpected since these amino acids are the most common contaminants in hydrolysates of micro samples recovered from cellulose or Sephadex columns and they are the most abundant amino acids in human fingerprints [11].

Kinin excretion. Urine collected in acid (to inhibit kininases) contained all three kinins. Mean values in urines from men were: BK, 3.4 ± 0.6 μg/day (mean ± S.E.M.); LBK, 7.6 ± 1.1; and MLBK, 9.5 ± 1.6. In women, the excretion of MLBK was significantly lower (1.2 ± 0.4 μg/day), but excretion of BK (2.5 ± 0.7) and LBK (5.1 ± 1.0) was only slightly reduced (Table 1).

A dramatic decrease in MLBK excretion was observed in male urine when the pepsin inhibitor, pepstatin, was added to the collection bottles in addition to the usual acid (Table 2). Subjects 2, 3 and 4 had no detectable MLBK, and in subject 1, pepstatin reduced the excretion by 90 per cent. Two of the subjects were studied on three other days; again, MLBK could not be detected. The pepsin inhibitor did not alter the excretion of BK and LBK (compare Tables 1 and 2).

Approximately one-thousandth the normal level of kinin (BK + LBK + MLBK) was found in the urine of a woman with a congenital deficiency of plasma kininogen [9]. However, when 1 mg kininogen was added to 50 ml of her acidified (pH 2.0) urine, and the mixture allowed to stand for 2 hr at room temperature and then overnight at 4°, 6.0 μg MLBK but no BK or LBK was found. The MLBK found is 37 per cent of the theoretical yield of kinin from the kininogen.

DISCUSSION

MLBK is the kinin formed when porcine pepsin is incubated with highly purified human plasma kininogens [12]. Although the maximal rate of kinin release occurs around pH 1.0, half this rate occurs at pH 5.0. At pH 2.0, pepsin has a higher catalytic activity (k_{cat}/k_m) with kininogen than either trypsin or plasmin and has kinetic constants similar to those found for the kallikreins [12].

The undecapeptide MLBK was discovered over 10 yr ago in a pseudoglobulin fraction of bovine plasma. The plasma was first acidified, then dialyzed against 0.01 N HCl and finally incubated at pH 7.5 for several hr [13]. Another peptide, possibly BK, was also found. In similar studies in equine plasma but with acid treatment for only 10 min, BK was the major peptide along with a smaller amount of another peptide which was probably MLBK [14]. Although the studies with horse plasma clearly indicated that plasma prekallikrein was activated, it now appears likely that acid-activation of plasma pepsinogen also occurred.

Table 2. Effect of pepstatin on urinary MLBK

Subject*	Bradykinin (μg/24 hr)	Lysyl-bradykinin (μg/24 hr)	Methionyl-lysyl-bradykinin (μg/24 hr)
1	4.5	9.0	2.0
2	3.8	12.0	<0.1
3	1.4	5.5	<0.1
4	1.1	12.6	<0.1

* The same men 1–4 as in Table 1.

Before these reports, it was known that a kinin-like peptide, pepsitocin, was produced when pepsin was incubated with serum [15]. Later it was shown that pepsin added to partially purified bovine kininogen releases a low molecular weight peptide, which upon incubation with trypsin becomes nine times more active on the guinea pig ileum and four times more active in lowering rabbit blood pressure [16]. These data suggest that MLBK or a very similar peptide had been produced by pepsin. Independent work at about the same time indicated that pepsin acting on highly purified bovine kininogen produced MLBK-Ser-Val-Gln and MLBK-Ser-Val-Gln-Val-Met [17]. With Cohn fractions IV-1 and IV-4, pepsin produced Gly-(or Ser)-Arg-MLBK [18]. One laboratory has also reported that partially purified human plasmin produces MLBK in addition to smaller amounts of BK and LBK when incubated with partially purified horse kininogen [19]. However, others using highly purified bovine plasmin and bovine kininogen [20] or purified human plasmin and human kininogen [21] found only BK. An enzyme in leukocytes is also suspected of producing a peptide similar or identical to MLBK [22]. Although it is now widely accepted that in a given species plasma kallikrein produces BK and glandular kallikrein, LBK, it has been shown recently that horse urinary kallikrein acting on synthetic substrates produces MLBK, LBK and/or BK depending on the synthetic substrate [23].

The first report of MLBK in human urine contained the apparently paradoxical statement that the undecapeptide was not found in fresh frozen urine [5]. In unpublished studies we have observed that this kinin is stable in frozen urine. Strong evidence for the occurrence of MLBK in human urine and its probable origin is contained in the present report. The urinary kinin is indistinguishable from standard MLBK on SP-Sephadex columns under conditions which clearly separate BK, LBK and MLBK. After digestion with DAP I an 8- to 10-fold increase in activity on the guinea pig ileum was observed. This increase could occur only when MLBK is converted to BK. Further evidence for this conversion was obtained when the digests were chromatographed on SP-Sephadex. The column showed that all the MLBK had been converted to BK. Amino acid analysis of the native peptide supported this conclusion.

Men excrete significantly more uropepsin and more MLBK per day than women [24,25]. The excretion of LBK and BK, on the other hand, is only slightly lower in women. This small difference would not be seen if the data were expressed per unit of urinary creatinine. When the potent pepsin inhibitor, pepstatin [26], was added to the collection bottles (in addition to the usual acid), MLBK was either undetectable or reduced by 90 per cent. The levels of LBK and BK were unaltered.

Thus, it is highly probable that most of the urinary MLBK observed in this study was formed after the urine had been voided into the acid-containing bottles. The urinary substrate for uropepsin is unknown. However, urine concentrates contain protein which forms a precipitin line (Ouchterlong method) with antibody to human plasma kininogens (J. V. Pierce, private communication). Furthermore, no kinins (<0.1 per cent normal) were found in the urine of a subject with a congenital deficiency of kininogens [9]. When kininogen is added to that subject's acidified urine, MLBK (but no BK or LBK) was produced. Presently unanswered is the question: How can kininogen and kallikrein coexist in unacidified urine? Perhaps the urinary kininogen is an immunologically active fragment which is a poor substrate for kallikrein. Alternatively, the activity of urinary kallikrein on native kininogen may be markedly reduced in urine, which is normally mildly acidic. Urine may also contain "kallikrein inhibitors".

The product of human urinary kallikrein is LBK [3]. The other urinary kinin, BK, is probably derived from LBK through the action of an aminopeptidase [3,4]. BK injected into the renal artery is rapidly hydrolyzed. Virtually none appears in renal venous blood or in urine [27]. The mean excretion of kinin stemming from the action of kallikrein (i.e. LBK + BK) was 11.0 and 7.6 μg/day for men and women respectively.

Others have reported similar values for total kinin excretion [28,29], but a direct comparison of the results cannot be made because the kinins were not separated and different bioassay and standards were used. Although the pharmacological actions of the three urinary kinins are qualitatively similar, they differ quantitatively [30]. The original report on MLBK in urine contained data on pooled urine from men [5]. It was estimated that 1 liter of fresh pooled urine contained 10–36 μg BK, 6–7 μg LBK and 13–25 μg MLBK. We found that the BK was usually the least abundant urinary kinin.

It is apparent that most of the MLBK which we observed was generated in acidified urine by uropepsin. Generation of MLBK after voiding could explain its occurrence in urine collected in weak acid (pH 4.5) but not in fresh frozen urine [5]. Nonetheless, the possibility exists that low levels (see Table 2, subject 1) of MLBK are normally produced inasmuch as: (1) urine is weakly acidic, (2) urine contains the appropriate substrate, and (3) pepsin is a highly active kinin-forming enzyme. The possible pathophysiological significance of the uropepsin–urokininogen–MLBK system remains to be determined.

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