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Degradation of Ile-Ser-Bradykinin by enzymes in human serum and ascites

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Previous studies have shown that Ile-Ser-Bradykinin (Ile-Ser-BK) can be found in abundance in human malignant exudates [1, 2]. Prior to this Ile-Ser-BK has only been known to occur in inflammatory diseases in the rat [3]. Because it appeared to be released by trypsin, this new peptide was called T-Kinin. In the rat a T-Kininogen has also been found [4], but in humans the synthetic modus of T-Kinin is not yet defined.

Since the physiological half-life of kinins *in vivo* is only in the range of seconds [5] it is surprising that Ile-Ser-BK has been reported to be present in large amounts in the ascites in some cases of, for example, ovarian carcinoma.

A further point of interest is the finding that, in some ascitic fluids, Ile-Ser-BK cannot be detected. Relevant to this is the finding that, during storage of Ile-Ser-BK-containing samples the peptide concentration decreased or even became undetectable.

If it is thus assumed that Ile-Ser-BK is catabolized in ascites and blood, the cases in which Ile-Ser-BK persists must be explained. Three possible mechanisms may be considered: firstly, degrading enzymes are absent in these cases, secondly, the degrading enzymes in the Ile-Ser-BK-containing ascites are inhibited or thirdly, synthesis of Ile-Ser-BK greatly exceeds degradation.

We here report on incubation studies performed to gain more and detailed information concerning the steps and possibly also the cleaving enzymes responsible for the degradation of Ile-Ser-BK.

Materials and Methods

Potassium phosphate buffer solution 0.1 M, pH 7.5 (PBS) as well as Ile-Ser-BK, BK, desArg-BK, leucine aminopeptidase (EC 3.4.11.1), amastatin and bestatin were purchased from Sigma (Unterhaching, F.R.G.). Captopril was a generous gift by von Heyden GmbH (München, F.R.G.).

Blood was drawn from healthy male and female volunteers, and centrifuged for 10 min at 3000 rpm at 4°. The supernatant serum was separated and stored at −20°.

Ascites was obtained by transperitoneal puncture from patients with metastatic ovarian cancer and centrifuged as above. One aliquot was stored at −20° without, and another with, addition of an inhibitor cocktail containing: Polybrene (0.3 g/L), soy bean trypsin inhibitor (0.075 g/L), aprotinin (40 kI.U./mL), pepstatin 1 μM, EDTA 6 mM, *o*-phenantrolin 80 mM, benzamidine 1 mM. Preliminary experiments showed that kininases in serum and ascites were inhibited by this cocktail.

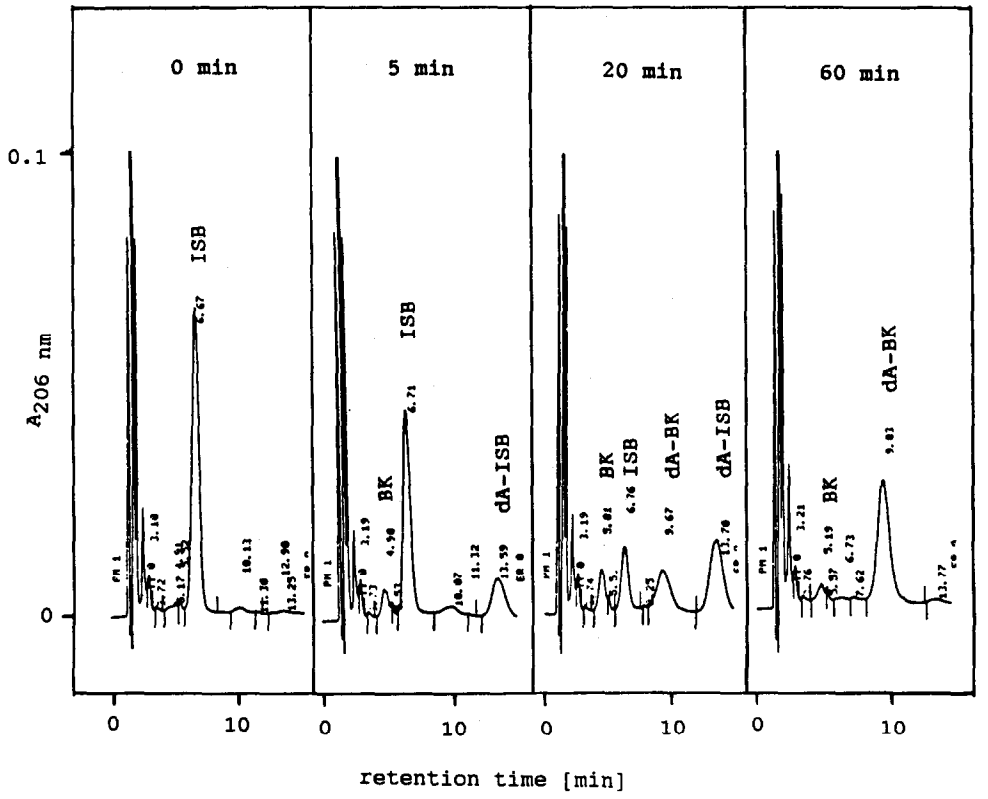

Fig. 1. HPLC chromatograms of Ile-Ser-BK and human serum after incubation for 0, 5, 20 and 60 min at 37°. Ile-Ser-BK (ISB) is degraded to desArg-BK (dA-BK) within 60 min. Intermediary products are desArg-Ile-Ser-BK (dA-ISB) and BK.

Ile-Ser-Bk and its degradation products were measured using a modification [6] of HPLC methods described elsewhere [7–9]. In brief samples were deproteinized by adding 2 mL hot methanol (70°). After centrifugation (10 min, 5000 rpm) the supernatant was lyophilized and subsequently dissolved in 1 mL of 0.1% (v/v) trifluoroacetic acid. This solution was filtered through Millex-Gs 0.22 μm (Millipore S.A. F-678120 Molsheim). One hundred millilitres of the thus prepared sample were separated by HPLC. The separation column (125 mm × 4.6 mm i.d.) and precolumn (20 mm × 4.6 mm i.d.) packed with Shandon Hypersil ODS C_{18}, 5 μm spherical particle size, were obtained from Sykam, D-8000 München. An isocratic elution system was performed with an elution buffer, pH 2.7, containing 0.01 M KH_2PO_4, 0.25 M Na_2SO_4, 0.1% (v/v) trifluoroacetic acid and 19% (v/v) acetonitrile. Adjustment of a flow rate to 1 mL/min resulted in a mean pressure of 70 bar in the system. Using HPLC, several peaks were collected and used subsequently for amino acid analysis [10].

Degradation of Ile-Ser-BK in human serum was studied by using 5 nmol Ile-Ser-BK dissolved in 100 μL PBS and 100 μL of human serum. The mixture was incubated in a water bath at 37°. Incubation was stopped by addition of 2 mL hot methanol (70°) to achieve simultaneously deproteinization of the incubation mixture. Measurements of metabolic products were made after 0, 5, 20 and 60 min of incubation.

In studies in which we tried to prevent Ile-Ser-BK degradation by peptidase inhibitors, 100 μL Ile-Ser-BK 50 mM, 100 μL of the inhibitor solution or PBS as control and 100 μL human serum were incubated for 20 min at 37°. Final concentrations of inhibitors were 25 μM amastatin, 75 μM bestatin, 25 μM captopril and 33 μM HgCl. The samples were processed as described above.

Results

The chromatograms obtained after different incubation times are shown in Fig. 1. After 60 min, Ile-Ser-BK is completely degraded. Amino acid analyses showed three main degradation products: BK, desArg-Ile-Ser-BK and desArg-BK. BK and desArg-Ile-Ser-BK are intermediate metabolites which are further degraded to desArg-BK. The same pattern of degradation was seen when Ile-Ser-BK was incubated with ascites. Ile-Ser-BK could not be detected in ascites which was stored without an inhibitor cocktail even though these probes originally did contain Ile-Ser-BK.

Inhibitor studies showed that the reaction from Ile-Ser-BK to BK was inhibited by amastatin and bestatin. The reaction from Ile-Ser-BK to desArg-Ile-Ser-BK was inhibited by HgCl. Addition of captopril did not influence the degradation (Fig. 2).

Discussion

Two pathways for the enzymatic metabolism of Ile-Ser-BK were found. First, the hydrolysation of Arg at the carboxyl end which occurs also with BK. The enzyme responsible for this process seems to be the carboxypeptidase N or kininase I [5]. The other mechanism is the cleavage of Ile-Ser at the N-terminal site of Ile-Ser-BK to release BK. This has been described by Narayanan and

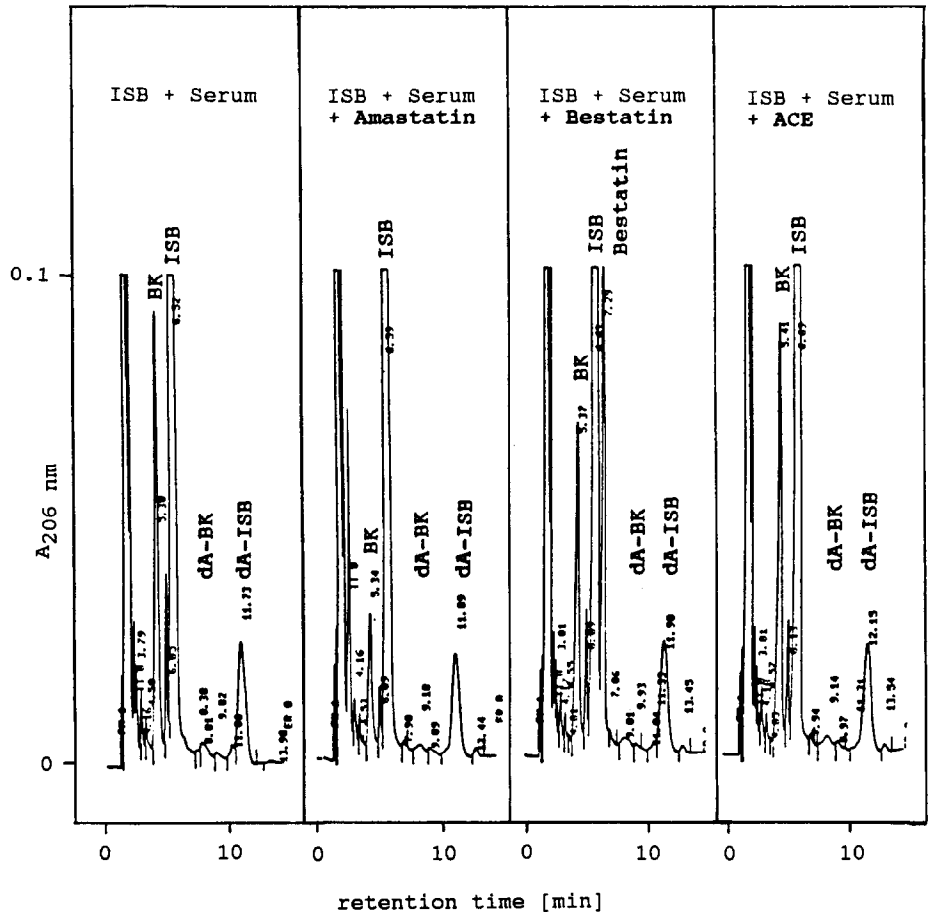

Fig. 2. The activity of leucinaminopeptidase is demonstrated by the degradation of Ile-Ser-BK (ISB) to BK. Amastatin (25 μM) inhibits more than bestatin (75 μm). Captopril (25 μm) has no influence. Mixtures were incubated for 20 min at 37°.

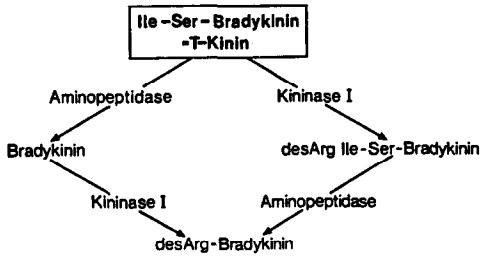

Fig. 3. Proposed pathways for the degradation of Ile-Ser-BK.

Greenbaum [11] to occur after incubation of T-Kinin with aminopeptidase M. As both enzymes (kininase I and aminopeptidase M) occur in human serum it seems likely that they are responsible for the degradation of Ile-Ser-BK. Results of studies in which kininase I was inhibited by HgCl and aminopeptidase M by amastatin and bestatin support this assumption. No change is seen after captopril. This is consistent with the results of Sheik and Kaplan [12] who showed that BK is not a substrate for angiotensin converting enzyme *in vitro*.

For the degradation of Ile-Ser-BK we suggest the model depicted in Fig. 3.

In our experiments we found evidence of Ile-Ser-BK degrading enzymes both in serum and in ascites. In some of the probes we also could detect Ile-Ser-BK in up to micromolar concentrations [6]. Thus, synthesis of Ile-Ser-BK has to exceed degradation considerably. Further studies are needed to clarify how Ile-Ser-BK is generated in human fluids. It is reasonable to assume that Ile-Ser-BK is derived from a third kininogen, different from high- and low-molecular weight kininogens. Whether this third kininogen is a product of the tumor cells or is induced by mediators of the tumor cells is a subject of speculation.

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Effects of calmidazolium and other calmodulin antagonists on adrenal glomerulosa cells

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Angiotensin II (AII*) stimulates aldosterone synthesis by a mechanism that involves alterations in calcium and phosphoinositide metabolism [1–6]. Increases in cytosolic calcium have been observed in adrenal cells exposed to AII, and this calcium may influence steroidogenesis by stimulating calmodulin-dependent enzymes, such as protein kinases [5–8]. Calmodulin is present in the adrenal cortex, and there is evidence to support a role for calmodulin in steroidogenesis [7–13]. A very specific calmodulin antagonist would be useful in defining the calcium–calmodulin-dependent events involved in AII-stimulated steroidogenesis, such as phosphorylation of key proteins. Such a compound would be expected to block a discrete calmodulin-dependent step(s) in AII-stimulated steroidogenesis, but not to have a broad inhibitory effect against adrenal cell function. Several reports have indicated that traditional calmodulin antagonists inhibit steroidogenesis, including AII-stimulated aldosterone synthesis [14–17]. Compound R24571 (calmidazolium) is an

* Abbreviations: AII, angiotensin II; ANP, atrial natriuretic peptide; and W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.