Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 280-286

T-KININOGEN - THE MAJOR PLASMA KININOGEN IN RAT ADJUVANT ARTHRITIS

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Received April 22, 1985

Total kininogen in plasma of Freund's adjuvant treated rats increased 20-fold 7 days following the injection. Analysis of the kininogens demonstrated that increases in T-kininogen was the major reason for the rise in kininogen. High molecular weight and low molecular weight kininogens showed little or no change. The increase in T-kininogen paralleled the inflammatory condition. Anti-inflammatory agents which reduced paw swelling also reduced plasma T-kininogen levels. Unidentified peaks on HPLC of kinin following plasma treatment by trypsin were shown to be oligopeptides containing T-kinin (Ile-ser-bradykinin). The relationship of T-kininogen to the inflammatory response is discussed.

Elevated plasma "total" kininogen levels have been reported in rats following experimentally induced inflammation by a variety of agents which include acetic acid, croton oil, turpentine (1-4) and Freund's adjuvant (5). Recently, Okamoto and Greenbaum (6,7) reported the discovery of T-kininogen in rat plasma which differs from the well known HMW and LMW kininogens in that Ile-Ser-Bradykinin is present in T-kininogen but Lys-bradykinin is not. T-kininogen is cleaved by an excess of trypsin to release Ile-Ser-Bradykinin (T-kinin). T-kininogen is not a substrate for kallikreins, but is a substrate for trypsin and cathepsin D (8).

While inflammatory agents cause an increase in total plasma kininogen, information does not exist on which of the kininogens increases. This is of particular importance since bradykinin has

Abbreviations

HMW for high molecular weight; LMW for low molecular weight and KGN for kininogen.

0006-291X/85 $1.50

always been thought to be the major kinin released in inflammation. The discovery of T-kininogen in rat plasma provides alternate possibilities in terms of the kinin released (T-kinin) and its role in inflammation. In the present study, an inflammation (adjuvant arthritis) was produced in rats with an injection of Freund's adjuvant. The plasma total HMW and LMW kininogen and T-kininogen levels were measured and related to the period of inflammation as well as to the extent of the inflammation. Kininogen levels were also measured following the use of anti-inflammatory agents.

Materials and Methods

Animals and drug administration - Six week old male Sprague-Dawley rats (160-180 g) were used. For arthritic experiments, 0.1 ml of Freund's complete adjuvant (suspension of 10 mg of mycobacterium in 1.0 ml paraffin oil) was injected intradermally into the left hind-paw of rats. Swelling of the paw was measured with a micrometer across a sagittal section (9). On each of days 1,7,14,21, 28 and 42 following the injection, five rats were sacrificed and their blood pooled and assayed for kininogens (see below). Control rats were given injections of paraffin oil without mycobacterium. In experiments using the anti-inflammatory agents, indomethacin (1.0 mg/kg) or dexamethasone (0.25 mg/kg), these drugs were injected subcutaneously 30 min prior to adjuvant and at 24 hour intervals thereafter. Control experiments were carried out substituting paraffin oil for complete adjuvant.

Collection of plasma - Blood was collected from the abdominal aorta as described previously (8) and centrifuged at 800 x g for 15 min at room temperature. Plasma was transferred to a plastic tube and kept at -70°C until use.

Assay of plasma kininogen levels - Plasma kininogen levels were determined by assaying the total amount of kinin (total kininogen), bradykinin (HMW kininogen plus LMW kininogen) and T-kinin (T-kininogen) released by the treatment with an excess amount of trypsin (8). 100 (µl) of plasma was mixed with 900 (µl) of 0.03 N HCl and incubated for 15 min at 37°C to inactivate plasma kininases and aminopeptidases. Following the addition of 25 (µl) of 1 N NaOH, the sample was incubated with 250 (µl) of trypsin solution (5 mg/ml in 0.2 M Tris-HCl, pH 7.8) for 1 hr at 37°C. The reaction was terminated by heating in a boiling water bath for 10 min. Twenty (µl) of this sample was assayed for the total amount of kinin released (total kininogen). The remaining sample was mixed with an equal volume of 30% trifluoroacetic acid (TFA) and centrifuged at 2,000 x g for 10 min. The supernatant was applied to an octadecyl-extraction column (1 x 2 cm), previously primed with 5 ml of methanol and 5 ml of 1% TFA. After washing the column with 1% TFA, kinins were eluted with 3 ml of 50% acetonitrile in 1% TFA. The eluate was evaporated and dissolved in 0.5 ml of distilled water. The kinins in the extract were separated by a reverse-phase HPLC as described previously (6,10,11). Each fraction that corresponded to the time for elution of bradykinin and for T-kinin was

evaporated and dissolved in buffer and subjected to radioimmunoassay (12,13). HMW-plus LMW-kininogen levels and T-kininogen levels were calculated as follows:

$$\text{Total amt of kinin} \times \frac{\text{Amt of kinin in bradykinin fraction}}{\text{Total amt of kinin eluted}} = \text{HMW \& LMW KGN}$$

$$\text{Total amt of kinin} \times \frac{\text{Amt of kinin in the T-kinin fraction}}{\text{Total amt of kinin eluted}} = \text{T-KGN}$$

Materials - The following materials were obtained from commercial sources: trypsin (type XII, TPCK-treated from bovine pancreas, Sigma Corp.); Freund's incomplete adjuvant, Mycobacterium tuberculosis (Difco); bradykinin, [Tyr^1]-kallidin (Peninsula Corp); Na^{125}I (carrier free, New England Nuclear); Octadecyl-extraction column, (J.T. Baker). T-kinin (Ile-Ser-bradykinin) was synthesized and supplied by courtesy of Dr. J.M. Stewart (Department of Biochemistry, University of Colorado Health Science Center).

Results

Figure 1 is a comparison of the immunoreactive kinins generated by trypsin from normal and adjuvant-treated rat plasmas. As chromatographed by HPLC it may be seen that in both the normal and the adjuvant-treated plasma, four different kinin-containing peptides are obtained. The

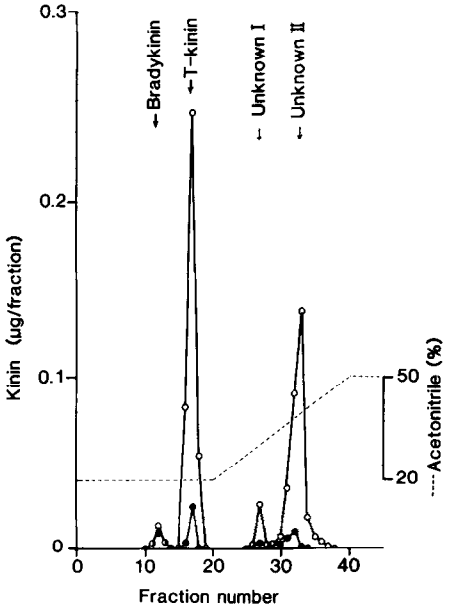

Fig. 1. Reverse-phase HPLC of immunoreactive kinins released by trypsin from the plasma of normal (●—●) and adjuvant-treated (o—o) rats. Kinin in each fraction was assayed by RIA using bradykinin as a standard. UI and UII refer to unknown immunoreactive kinins.

first two fractions are bradykinin and T-kinin respectively. In addition, two peptides, Unknown I and Unknown II (UI & UII) are obtained which do not correspond in their elution time with other known kinins (e.g. Lys-bradykinin or Met-lys-bradykinin). It is obvious from figure 1, that two peptides T-kinin and UII were released in much greater quantities from arthritic plasma than from normal plasma, indicating that the kininogens that increased in these plasmas included T-kininogen and the UII-containing kininogen.

The possibility existed that UI and UII were larger fragments than bradykinin or T-kinin which were not broken down completely to these kinins by the original trypsin digestion. In order to test this possibility, UI and UII were lyophilized and dissolved in a small quantity of buffer and subjected again to trypsin degradation. As seen in figure 2, the original UI and UII immunoreactivities are converted completely by the second trypsin treatment into T-kinin.

As also seen in figure 1, the plasma collected from rats 7 days after the injection of adjuvant released much greater quantities of T-kinin than from normal animals. Bradykinin liberation does not change from that of

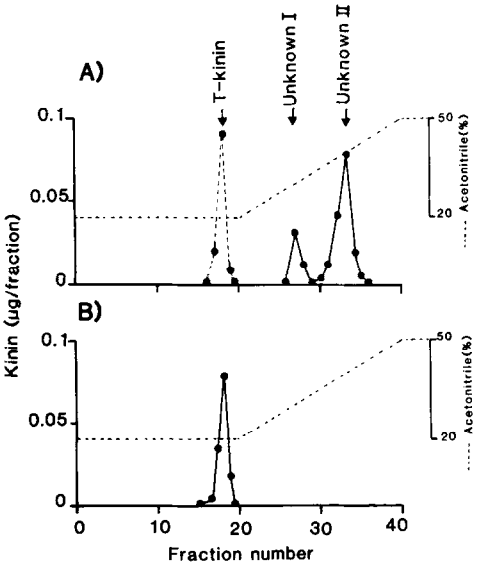

Fig. 2. Conversion of unknown immunoreactive kinins to T-kinin by trypsin. UI & UII immunoreactive kinin fractions (see figure 1) were lyophilized and solubilized (A) as directly chromotographed on HPLC (B) following trypsin incubation and chromatography on HPLC.

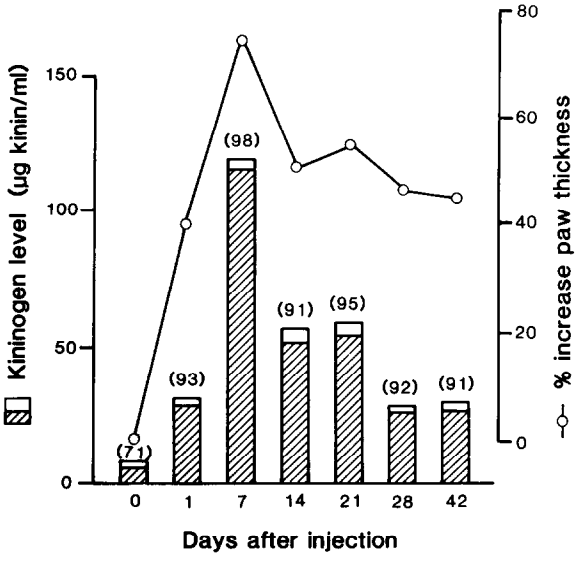

Fig. 3. Increases in paw thickness and plasma kininogen levels of rats after the injection of Freund's complete adjuvant. Total kininogen, HMW-plus LMW-kininogen (open column) and T-kininogen (shaded column) are indicated for the mean values of 5 rats at each day. Numbers in the parentheses indicate the percent of T-kininogen of the total kininogen levels. Each point in paw thickness represents the mean of measurements in 5 rats converted to percent increase over the day zero.

normal rat plasma. Thus T-kininogen is the major factor in the elevation of kininogen levels in rat plasma following adjuvant treatment.

Figure 3 shows the time course changes in paw swelling and plasma kininogen levels following the injection of complete adjuvant. Swelling of the injected paw was observed 24 hrs after the injection and reached maximum at day 7. Plasma total kininogen levels also increased maximally at day 7, and a high level of total kininogen was still observed after 6 weeks. Levels of HMW-plus LMW-kininogen showed little change from normal plasma levels. T-kininogen levels, however, increased markedly; it made up more than 90% of the total kininogen levels over the period of the experiment. Total plasma protein concentration (not shown) also rose somewhat 24 hours after the adjuvant injection (52.8 to 65.8 mg/ml) but not nearly to the extent of the kininogen increase. After 24 hours, there was not additional increase in plasma protein concentration up to 6 weeks.

Two typical anti-inflammatory drugs, i.e., indomethacin and dexamethasone, were administered to rats following the injection of adjuvant to

TABLE I REDUCTION OF PLASMA KININOGEN LEVELS BY INDOMETHACIN AND DEXAMETHASONE OF RATS TREATED WITH FREUND'S COMPLETE ADJUVANT

	KININOGEN LEVELS (μg kinin/ml)			% INHIBITION OF PAW SWELLING	% REDUCTION OF T-KGN LEVELS
	TOTAL	HMW + LMW	T-KGN		
Normal	6.0 ± 1.2	1.21 ± 0.20	4.8 ± 1.0	-	-
Adjuvant Treated	54.8 ± 6.8	1.41 ± 0.19	53.4 ± 6.9	-	-
+ Indomethacin	36.0 ± 4.9	1.62 ± 0.10	34.4 ± 4.8*	33	35
+ Dexamethasone	24.1 ± 2.6	1.21 ± 0.25	22.9 ± 2.5**	56	58
Indomethacin Alone	5.5 ± 0.2	1.48 ± 0.30	4.0 ± 0.5	-	-
Dexamethasone Alone	5.8 ± 0.9	1.67 ± 0.51	4.1 ± 0.8	-	-

Plasma kininogen levels are measured on day 3 following the injection of Freund's complete adjuvant.
Indomethacin (1 mg/kg) and Dexamethasone (0.25 mg/kg) were subcutaneously injected once a day beginning at day zero.
* $p < 0.05$ as compared to adjuvant-treated group.
** $p < 0.001$ as compared to adjuvant-treated group.

observe their effects on kininogen levels. As shown in table 1, indomethacin and dexamethasone suppressed paw swelling by 33% and 56% respectively (as measured on day 3). Suppression of total and particularly T-kininogen levels were also suppressed by these drugs to the same extent as the paw swelling. The administration of these drugs to normal rats did not change plasma kininogen levels.

Discussion

While several investigators have observed an increase in total kininogen in the plasma of rats as a response to procedures or chemical agents which trigger inflammation, the kininogen has not been identified until now. Our current studies definitively demonstrate that in adjuvant arthritis in rats, the increase in plasma total kininogen is almost solely due to the increase in T-kininogen. Not only does T-kininogen increase some 20 fold at day 7 but the increase clearly parallels the increase in paw swelling. Paw swelling, like the plasma T-kininogen levels, reaches maximum on day 7. This correlation would seem to indicate that T-kininogen and the inflammatory response are closely linked in some way. This is further borne out by

the indomethacin and dexamethasone treatments which reduce both the inflammatory response and the plasma T-kininogen levels. Again, there was a remarkable parallelism in the reduction of paw swelling by the anti-inflammatory drugs and the reduction in plasma T-kininogen levels.

Exactly what role T-kininogen is playing in the inflammatory response in rats is not known at this time. One possibility is that T-kinin is released by an unknown protease from T-kininogen and the T-kinin is a key mediator of inflammation. A second possibility is that T-kininogen is an acute phase protein similar or the same as that identified in plasma of turpentine-produced inflammation in rats. These proteins may inhibit thiol proteases involved in the inflammatory process (14). A third possibility is that T-kininogen is a substrate for lysosomal enzymes such as cathepsin D which release T-kinin (8).

In this report we also clearly demonstrated that in addition to the release of bradykinin and T-kinin by trypsin from plasma, two oligopeptides are released which contain T-kinin.

Our overall findings continue to point to T-kininogen as the major kininogen in normal rat plasma and particularly in adjuvant-arthritic rats.

Acknowledgement : Supported by NIH grants HL-32183.

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