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Kallikrein-like activity in human myometrium, placenta and amniotic fluid

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KALLIKREINS are enzymes which split off biologically active peptides i.e. kinins from the molecules of kininogen. Kinin-forming enzymes are found in some tissues of the human body.[1] Recently attention has been drawn to the fact that kinins may influence the contractility of the uterus and of uterine blood flow (UBF) during delivery[2] as well as the processes determining the adaptation of a newborn to new conditions.[3,4] This paper deals with the results of preliminary investigations on the kinin-forming activity in some tissues of the reproductive tract in the woman.

Tissues extracts. Investigations were carried out in tissue extracts prepared in the following way:

(a) Placentae were perfused immediately after delivery with distilled water or 0·9% NaCl to remove the blood. Then the placentae were homogenized in distilled water (1 g of tissue + 2 ml of water). Homogenates were centrifuged at 4000 *g* for 30 min and the sediments were discarded.

(b) Fresh amniotic fluids were immediately centrifuged at 4000 *g* for 30 min and the sediments discarded.

(c) Myometrial tissues were obtained during Caesarian section (dystocia) and were immediately homogenized (1 g + 4 ml H_2O) and centrifuged at 4000 *g* for 30 min. Sediments were discarded. Blood vessels of the myometrium samples were not perfused and homogenates included a little of the blood because of the technical difficulties.

Up to now we have investigated: 15 placentae, 15 different samples of normal amniotic fluid and 8 different samples of myometria. Kinin-forming activity was investigated in:

(a) fresh tissue extracts,

(b) extracts incubated for 10 min at 37°, pH 2·0 (2 N HCl) and realkalized to pH 7·4 (2 N NaOH).

Assessment of kinin-forming activity. Fresh human plasma was used as a substrate to determine the kallikrein-like activity of the tissue extracts. The plasma was taken from the same, healthy non-pregnant woman. The blood was mixed with 3·8% sodium citrate (9:1) and centrifuged at 2000 *g* for 1 hr. Taking of the blood, centrifugation, storing of the plasma and assessment of kinin-forming activity were carried out in siliconized vessels (Siliclad Clay Adams). Plasma used as a substrate did not have kinin activity during the whole experiment. Biological activity was determined on an isolated guinea-pig ileum in oxygenated Tyrode's solution with the addition of atropine (Polfa) 10^{-7} g/ml, at 37°. Kinin-forming activity was examined in the following setting:

0·1 ml of tissue extract or amniotic fluid in dilutions of: 1:1; 1:10; 1:100; 0·01 ml of EDTA 4%, 0·2 ml of fresh and intact plasma (1:1).

In control settings in the same conditions, 0·2 ml of the intact plasma with 0·01 ml of EDTA 4% and 0·1 ml 0·9% NaCl were incubated. Following the incubation for 60 sec at 37°, the enzymatic reactions were stopped, by heating for 10 min in boiling water. After cooling the quantity of the released kinins was measured in the examined setting. The activity of kinins was expressed as an equivalent of the activity of synthetic bradykinin (ng BRS eq.) and referred to 1 g or 1 ml of the examined tissue.

Placenta. A crude tissue extract of placentae does not elicit the contractile activity of the intestine smooth muscle. Also plasma used as a substrate for examined kallikrein-like activity does not elicit the contraction of the intestine. Contractile activity of the smooth muscle are formed in plasma during incubation with the extract from the placentae tissue. The placentae extracts acidified to pH 2·0 and then readjusted to pH 7·3 have higher kinin-forming activity. Figure 1 illustrates these results. Kinin-forming activity of crude extracts of the placentae tissue amounts to an average value of 50 ng BRS eq. per 1 g of tissues, following acidification and readjustment to pH 7·3, this activity increases to 450 ng BRS eq. per 1 g of tissue (data from 15 different fresh homogenates).

Uterus. Kinin-forming activity in the crude fresh extracts from the uterine muscles amounts to an average value of 120 ng BRS eq. per 1 g of tissue, after acidification and readjustment this activity increases to 1300 ng BRS eq. per 1 g of tissue (data from 8 different fresh uterine homogenates).

Amniotic fluid. Kinin-forming activity in the crude material amounts to an average value of 80 ng BRS eq. per 1 ml of tissue, after acidification to pH 2·0 and realkalization the activity increases to the value of about 1000 ng BRS eq. per 1 ml of tissue (data from 15 different fresh samples).

Pharmacological activity. This appears in the mixture of fresh and intact plasma and tissue extracts and requires a more definite characterization. To this end the following experiments have been done:

(a) *Estimation of kinin-forming activity of tissue extracts incubated with crude kininogen preparation.* Fresh human plasma, used as kininogen, was prepared as follows: 1 hr incubation at 61°, and 30 min incubation at pH 2·0 and 37°, and then realkalization was made to pH 7·3, followed by centrifugation for 30 min at 15,000 *g* and the supernatant was discarded. The plasma prepared in such

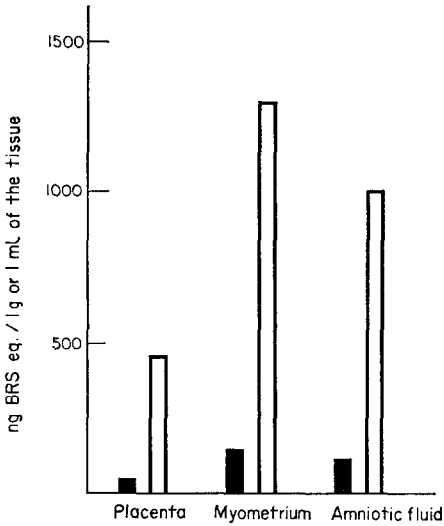

FIG. 1. The behaviour of kallikrein-like activity of extracts from homogenized tissues of placenta and myometrium and from amniotic fluid. Striped rectangles—crude extracts; Open rectangles after acidification to pH 2·0 and realkalization.

a way does not contain prekallikrein, kallikrein or kinins but possesses lowered to a considerable degree kininase activity and includes big enough quantities of kininogen to investigate kallikrein.[5] To compare, I have used the following enzymes which in the reaction with kininogen release kinins: human plasma kallikrein activated with acetone (A) and trypsin 20 μg final concentration in a sample, (B) and tissue extracts of the placenta (C), myometrium (D) and amniotic fluid (E). Crude preparation of human plasma kallikrein were obtained basing on the method of Webster and Pierce.[6] Estimations were done in the system:

0·1 ml of the investigated material (A–E),
0·01 ml EDTA 4%,
0·5 ml of kininogen.

Following incubation (60 sec at 37°), enzymatic reactions were discontinued by heating for 20 min particular samples in boiling water and then centrifugating them for 30 min at 10,000 *g*. One ml of the obtained supernatants in each system contained on an average: A–800, B–80, C–350, D–1000, E–700 ng BRS eq.

(b) *The behaviour of kinin-like activity during incubation with plasma containing kinin-destroying enzymes* (*kininases* Fig. 2). The investigations were performed with pharmacologically active A–E samples obtained as in (*a*), and in addition with the preparation of synthetic bradykinin (F). The estimations were done in the following system:

No. 1. 0·5 ml of the investigated sample (A–E, additionally F),
0·01 ml EDTA 4%,
0·5 ml of fresh plasma (1:10 in 0·9% NaCl).

No. 2. 0·5 ml of the investigated sample (A–E, additionally F),
0·01 ml 0·9% NaCl.
0·5 ml of fresh plasma (1:10 in 0·9% NaCl).

Both systems were incubated for 20 min at 37°, followed by placing in boiling water for 20 min, centrifuged for 10 min at 10,000 *g* and biological activity was later estimated in the supernatant.

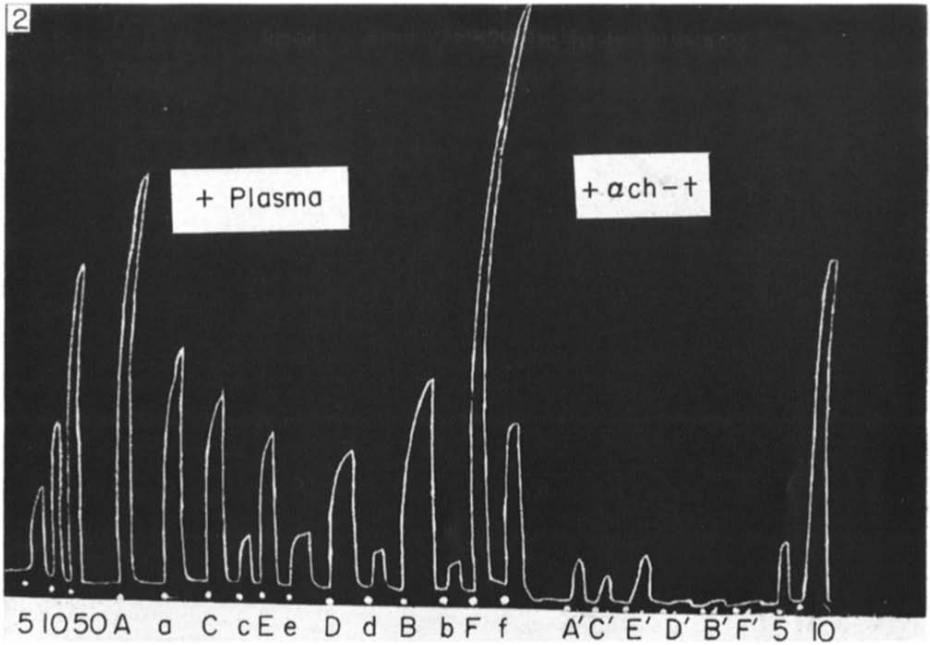

FIG. 2. The behaviour of kinin-like activity during incubation with plasma + EDTA (see text: system No. 1): A; C; E; D; B; F. During incubation with plasma + 0·9% NaCl (see text: system No. 2): a; c; e; d; b; f. During incubation with α-chymotrypsine: A'; C'; E'; D'; B'; F'. Kinin-like activity was obtained in the system containing kininogen and: plasma kallikrein (A), trypsin (B), placenta extract (C), myometrium extract (D), amniotic fluid (E) and synthetic bradykinin (F). At the beginning and in the end of the experiment intestine sensitivity to 5, 10, 50 ng synthetic BRSwas measured.

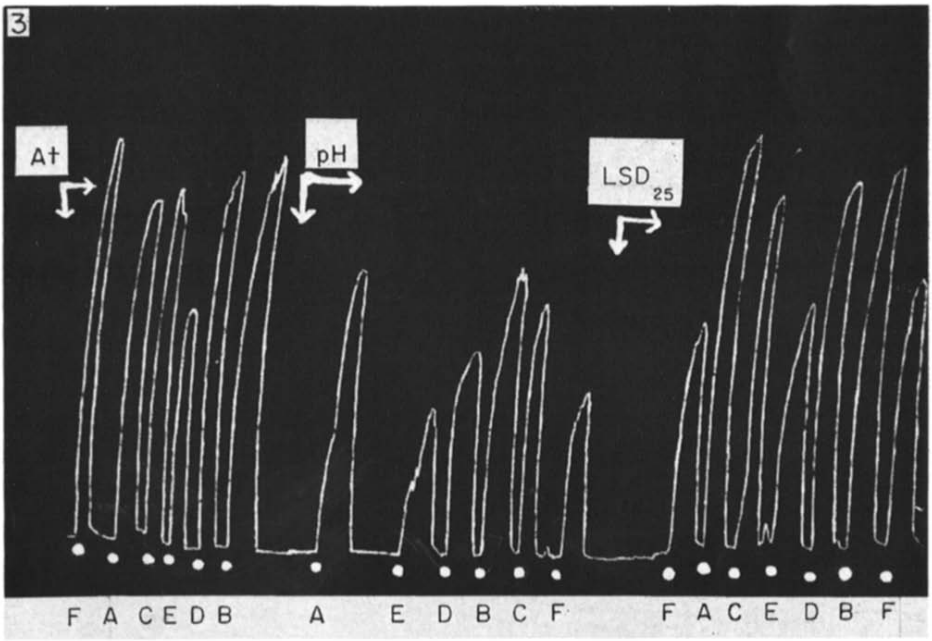

FIG. 3. The behaviour of contractility of isolated guinea pig intestine in Tyrode's solution with atropine (At), phenazoline (pH) and LSD_{25} under the influence of kinin-like activity. Kinin-like activity was obtained in system containing kininogen and: plasma kallikrein (A), trypsin (B), myometrium extract (D), placenta extract (C), amniotic fluid (E). Additionally influence of a constant dose of 10 ng synthetic bradykinin (F) was investigated.

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TABLE 1.

No. exp.	Experiments with plasma kininases			Experiments with α-chymotrypsin		
	1	2	3	1	2	3
A–plasma kallikrein	22%	57·1%	15·5%	5·7%	11·4%	5·1%
B–trypsin	Full inactivity	13·8%	4·3%	Full inactivity	Full inactivity	Full inactivity
C–placenta	3·2%	25%	0·47%	16·2%	14·1%	2·8%
D–myometrium	Full inactivity	30·4%	3·7%	Full inactivity	Full inactivity	Full inactivity
E–amniotic fluid	10·2%	47·6%	11·3%	37·1%	37·5%	4·3%
F–synthetic bradykinin	Full inactivity	28%	1·2%	Full inactivity	Full inactivity	Full inactivity

Biological activity of control samples without plasma and samples from the system No. 1 were practically similar. In the system No. 2, there was always a decrease of the biological activity. Table 1 illustrates biological activity in No. 2 samples. Final activity was expressed as a per cent of the initial activity.

(c) *The behaviour of kinin-like activity during incubation with α-chymotrypsin* (Koch Light Lab., Ltd.) (Fig. 2). Investigations have been carried out with pharmacologically active samples (A–E) obtained as in (*a*), and furthermore with the preparation of synthetic bradykinin (F). Estimations were made in the following system: 0·5 ml chymotrypsin (100 μg final concentration in a sample), 0·5 ml of the investigated sample (A–F).

Following incubation for 90 min at 37°, enzymatic reactions were discontinued by heating of the sample 20 min in boiling water and then centrifugation was performed for 10 min at 10,000 *g*. Supernatant was used for biological investigation. It has been found that in the samples B, D and F total disappearance of the biological activity had always occurred (Table 1). In the remaining samples inactivation had always occurred. Final biological activity following incubation of the samples with chymotrypsin was expressed in per cent. Biological activity of samples before the incubation with chymotrypsin was accepted as 100 per cent.

(d) *An attempt to pharmacologically analyse of kinin-like activity.* (Fig. 3). Investigations were performed on an isolated guinea pig ileum in Tyrode's solution supplemented in succession by: atropine 2 μg/ml, phenasoline (Polfa) 0·5 μg/ml and LSD_{25} (Delysid–Sandoz) 0·1 mg/ml.

Pharmacological analysis excludes the possibility of appearance of acetylcholine, histamine and serotonin in the mixture of kininogen and tissue extracts.

DISCUSSION

The presented investigations show that extracts from placentae, myometrial tissues and amniotic fluids do not have kinin activity but are able to release kinin-like substances from the intact fresh plasma, as well as from acidified (pH 2·0) and warmed (61°) plasma. Intact fresh plasma as a substrate used in investigated kallikrein-like activity had no contractile activity in the control samples. This kinin-forming activity arises from the extracts from placenta, myometrium and amniotic fluid following acidification to pH 2·0 and re-alkalization. It might be suggested that the process of acidification of the tissue extracts elicits releasing of the active enzymes from subcellular structures or removes from the examined environment some inhibitors of kinins or kinin-forming activity.

In the investigations it has been found that:

(a) during incubation of the mixture of the two pharmacologically inactive substances: kininogen and the extract of one of investigated tissues, distinct pharmacological activity appears,

(b) pharmacological activity expressed in releasing contractions of the isolated, guinea pig small intestine in Tyrode's solution with atropine, phenasoline and LSD_{25},

(c) the activity is not altered in the course of sample heating for 20 min in boiling water,

(d) the pharmacological activity distinctly declines or disappears during the incubation of the investigated material with α-chymotrypsin or fresh plasma,

(e) the pharmacological activity remains practically unchanged during the incubation of samples with plasma supplemented with EDTA.

From the obtained results it appears that active biological substances present during the incubation of extracts from placental tissues, myometrium and amniotic fluid might be kinins.[7,8] According to Ratnoff and his co-workers,[9] it seems reasonable to use a more cautious term: "kinin-like agents".

Basing on the presented material one cannot conclude definitely that the level of tissue kallikrein has been assessed but rather kallikrein-like activity. This short report shows that both myometrial and placental tissues as well as amniotic fluid present kinin-forming activity.

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A microsomal amidase which cleaves chloramphenicol*

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ONE OF THE major excretory products of the antibiotic chloramphenicol is chloramphenicol base [D(−)-*threo*-1-(*p*-nitrophenyl)-2-aminopropane-1,3-diol], a metabolite which has lost a dichloroacetyl group and which has no antibacterial activity.[1,2] We report here the presence of an enzyme in the microsomal fraction of mouse liver which cleaves chloramphenicol, liberating dichloroacetate.

Chloramphenicol (dichloroacetyl-2-^{14}C), with a specific activity of 8·5 mCi/m-mole, was purchased from Calatomic, Los Angeles, Calif. The sample contained no detectable radioactive impurities. Microsomes and mitochondria were prepared from the livers of female DBA/2 mice. The animals, weighing about 25 g, were killed by cervical dislocation; the livers were excised, cooled and homogenized in 3 vol. of 0·25 M sucrose. After centrifuging the homogenate for 5 min at 900 *g*, the supernatant fraction was removed and centrifuged at 9000 *g* for 15 min to obtain the mitochondrial fraction, which was washed twice by centrifugation in 0·25 M sucrose. Microsomes were prepared from the 9000 *g* supernatant fraction by centrifuging at 100,000 *g* for 45 min and were washed once in 0·25 M sucrose.

The standard reaction system for chloramphenicol cleavage contained the following: microsomes equivalent to 40 mg liver; sodium glycinate buffer (pH 9·0), 12 μmoles; ^{14}C-chloramphenicol, 87 nmoles; and water in a total volume of 175 μl. The reaction was initiated by addition of the microsomal preparation and was stopped by streaking a 50-μl portion on paper strips. Incubation of the preparation was for 30 min at 37°. Substrate and metabolites were separated by paper chromatography with isopropanol–NH_3–H_2O (85:5:15, by vol.) and were measured with a Packard 7201 radiochromatogram scanner. The R_f-value for chloramphenicol was 0·96 and that for the microsomal metabolite was 0·67.

The electrophoresis experiments involved a buffer of 10% pyridine adjusted to pH 7 with glacial acetic acid and a Savant flat-plate apparatus. A potential of 40 V/cm was applied for 1 hr. Mass spectra were obtained with a Hitachi high-resolution, double-focusing mass spectrometer (RMU-6-D-3). Protein was determined by the method of Lowry *et al.*[3]

The pH optimum for the reaction was 9·0 with sodium glycinate as buffer. Higher pH values of this buffer were less effective, as were pH values 6·2–7·6 with phosphate buffer and pH values 6·9–8·9 with Tris–chloride. No detectable chemical hydrolysis was observed up to pH 9·8. Within the limits employed, the reaction was dependent upon the amount of microsomes present and the time of incubation and followed Michaelis–Menten kinetics (Fig. 1). K_m values for two separate determinations were 0·77 and 0·70 mM. Concentrations of NADPH up to 5 mM had no effect on the rate of reaction.

On the basis of protein added to the reaction system, microsomes had more than six times the activity found in either mitochondria or the 100,000 *g* supernatant fraction. Addition of the 100,000 *g* supernatant fraction to the system containing microsomes resulted in the further metabolism of the microsomal product, with new unidentified peaks appearing at R_f-values of 0·03, 0·10 and 0·47.

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