SHORT COMMUNICATION/KURZMITTEILUNG

Glandular Kallikrein Content in Tissues of Diabetic and Hypertensive Rats Measured by Enzyme Immunoassay

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Summary: The contents of glandular kallikrein in the submaxillary gland and pancreas of normal, diabetic and hypertensive rats were compared using a specific enzyme immunoassay. The kallikrein levels in the submaxillary gland and pancreas of the diabetic rats were significantly lower than those of normal rats. On the other hand, the submaxillary and pancreatic levels in hypertensive rats tended to be higher than in normal rats.

Bestimmung von Drüsen-Kallikrein in Geweben diabetischer und hypertensiver Ratten mit dem Enzymimmunassay
Zusammenfassung: Drüsen-Kallikrein in der Gl. submaxillaris und im Pankreas normaler, diabetischer und hypertensiver Ratten wurde mit einem spezifischen Enzymimmunassay verglichen. Der Kallikreingehalt beider Drüsen diabetischer Ratten war signifikant geringer als bei normalen Ratten; umgekehrt tendierte er bei hypertensiven Ratten zu höheren als bei normalen Ratten gefundenen Werten.

Introduction
Glandular kallikrein (EC 3.4.21.35) is said to be involved in the regulation of the exocrine glands and the kidney blood flow, in water and electrolyte balance, in blood pressure regulation, in the mediation of insulin action on glucose uptake of the skeletal muscle, and in the pathogenesis of experimental and clinical hypertension (1—3). Recently, Powers et al. and Jaffa et al. reported on the relationship between glandular kallikrein and the pathogenesis of experimental hypertensive and diabetic rats (4, 5), using synthetic substrate-hydrolysis and radioimmunoassay techniques for the determination of glandular kallikrein. However, the glandular kallikrein contents of the submaxillary gland and pancreas in normal, diabetic and hypertensive rats have not yet been compared. This paper describes the determination of kallikrein levels in rat submaxillary gland and pancreas in diabetic and hypertensive rats, using an enzyme immunoassay, in an attempt to clarify the relationship between the submaxillary gland kallikrein and blood pressure regulation, and the mediation of insulin action on glucose uptake into responsive cells.

Materials and Methods
A spontaneously hypertensive rat strain of male Wistar rats and the Wistar-Kyoto strain were purchased from Institute for animal experiment, School of Medicine, Hokkaido University. Rats were made diabetic by a single intravenous injection of streptozotocin (70 mg/kg, dissolved in 50 mmol/l citrate buffer, pH 4.5). The pancreas and the submaxillary gland were carefully removed from each animal, washed in saline to remove any traces of blood, and immediately frozen at —30 °C to be stored until use. The levels of blood glucose and systolic pressure of rats were measured by the glucose oxidase method (6) and tail cuff sphygmography, respectively. Protein was determined by the method of Lowry et al. (7), using bovine serum albumin as the standard.

Rat submaxillary gland kallikrein was isolated by the method of Brandzaeg et al. (8). Antiserum to glandular kallikrein was prepared in albino rabbits (weighing about 2.5 kg; males) by subcutaneous injection in the back using an emulsion of Freund's complete adjuvant (Difco Lab. Inc., U.S.A.) and purified kallikrein (1 + 1, by volume). One ml of emulsion containing 0.2 mg kallikrein was injected 4 times at 7-day intervals. Seven days after the final injection the animals were bled.

β-Galactosidase-labeled kallikrein was prepared using a heterobifunctional reagent, N-succinimidyl-3-(2-pyridyldithio)propionate (Pharmacia Fine Chemicals, Sweden) and β-galactosidase from Escherichia coli (Boehringer Mannheim, West Germany), according to the manual of Pharmacia Fine Chemicals, Sweden.

A competitive enzyme immunoassay of kallikrein was carried out using test tubes with special rubber caps (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) as described in table 1. One hundred μl of 1:1000 antiserum dilution, 100 μl of various concentrations of standard kallikrein or sample, and 300 μl of enzyme immunoassay buffer (10 mmol/l sodium phosphate buffer, pH 7.0, containing 100 mmol/l NaCl, 1 mmol/l MgCl2, and 1 g/l NaN3) were mixed with 100 μl of β-galactosidase-labeled kallikrein (5.2 mU β-galactosidase). After incubation at 0 °C for 30 min, 200 μl of protein A-linked Staphylococcus aureus Cowan cell walls (adsorbing capacity for rabbit
The homogenates of the submaxillary gland and the pancreas were added to separate the antibody-bound β-galactosidase-labeled kallikrein. The rubber caps were placed on all test tubes, which were then incubated at 0 °C for 30 min. The reaction mixture was centrifuged at 3000 min⁻¹ for 10 min at 0 °C, with the rubber caps downwards. After centrifugation, the tubes were taken out gently from the centrifuge rack, placed upright in the test tube rack and the rubber caps were removed. The liquid phase, antibody-unbound form of β-galactosidase-labeled kallikrein, was measured for β-galactosidase activity by adding 100 μl of 25 mmol/l p-nitrophenyl-β-D-galactoside (Boehringer Mannheim, West Germany) at 30 °C. After 20 min of incubation, the reaction was stopped with 2.5 ml of 100 mmol/l glycine-NaOH buffer (pH 10.3). The absorbance due to the liberated p-nitrophenol was read on a Hitachi photometer, Type 320, at 405 nm. One unit of β-galactosidase was defined as the amount cleaving 1 μmol of the substrate per minute.

The homogenates of the submaxillary gland and the pancreas were prepared in 10 mmol/l sodium phosphate buffer (pH 6.0), containing 100 mmol/l NaCl (1 g of tissues per 9 ml of buffer), using a polytron homogenizer. Homogenates were centrifuged for 20 min at 10000 min⁻¹. Aliquots of supernatant diluted 1:3 to 1:2500 with the enzyme immunoassay buffer were assayed.

**Results and Discussion**

A typical standard curve for the competitive enzyme immunoassay for the kallikrein is shown in figure 1. Figure 1 also shows that the plots of response against dilution are parallel for rat submaxillary gland homogenates, standard kallikrein and pancreas homogenates. The recovery of added purified rat glandular kallikrein was determined by using the homogenates of the submaxillary gland and pancreas containing 800 μg/l and 700 μg/l, respectively. When 880 μg/l and 1600 μg/l of the purified kallikrein were added to the homogenates, the respective recoveries were: 99.6 ± 2.57 and 104.7 ± 2.85% (submaxillary gland; n = 5), and 107.4 ± 3.07 and 100.9 ± 4.02% (pancreas homogenates; n = 5), thereby demonstrating the high precision of the assay. The intraassay coefficient of variation (n = 5) was less than 5% for rat submaxillary gland kallikrein, and less than 5% for the pancreatic kallikrein (range 550 to 2400 μg/l). The interassay coefficient of variation for the assay of submaxillary gland kallikrein (n = 5; 800 and 2400 μg/l, respectively) was less than 5%; the corresponding value for pancreatic kallikrein (n = 5; 700 and 2280 μg/l, respectively) was also less than 5%. Therefore, in the present study, glandular kallikrein was quantitatively and specifically measured by enzyme immunoassay.

Next we determined the kallikrein contents in rat submaxillary gland and pancreatic homogenates of normal, diabetic and hypertensive rats by using the enzyme immunoassay. Twenty eight days after injection of streptozotocin, the treated rats were diabetic as shown by the clinical state and the high levels of blood glucose (21.7 ± 2.44 mmol/l). As shown in table 2, the content of kallikrein in submaxillary gland homogenates in normal rats was 73.5 ± 27.2 μg/mg protein, which was a value similar to those reported using radioimmunoassay (9). The induced diabetes rats contained less kallikrein (17.4 ± 9.9 μg/mg protein) in the homogenates of their submaxillary gland than the control rats, with the significant difference of p < 0.005. The concentration of kallikrein in pancreatic homogenates of diabetic rats was less than that of normal rats, with a difference of p < 0.005. The reduction of kallikrein in the submaxillary gland of diabetic rats was in good agreement with the results of Jaffa et al. (5), but the results for pancreas were different from those of Jaffa et al. (5). The discrepancy seems to be due to the more prolonged treatment with streptozotocin, namely 28 days, i.e. a difference of 14 days. Thus, it may be suggested that glandular kallikrein is implicated in the pathogenesis of diabetic rats.
Tab. 2. Kallikrein contents in rat submaxillary gland and pancreas of control and streptozotocin-diabetic rats.

<table>
<thead>
<tr>
<th></th>
<th>Kallikrein (µg/mg protein)</th>
<th>Blood glucose (mmol/l)</th>
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<tbody>
<tr>
<td>Submaxillary gland</td>
<td></td>
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<tr>
<td>Control (n = 11)</td>
<td>73.5 ± 27.2*</td>
<td>0.608 ± 0.126*</td>
</tr>
<tr>
<td>Diabetic (n = 10)</td>
<td>17.4 ± 9.9</td>
<td>0.422 ± 0.085</td>
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* Standard deviation

On the other hand, submaxillary gland homogenates from male hypertensive rats (86 mm Hg elevation in systolic blood pressure compared to male, normotensive Wistar-Kyoto strains), contained 78.4 ± 8.8 µg/mg protein of kallikrein. The kallikrein content of submaxillary gland from the normotensive rats was 66.3 ± 7.0 µg/mg protein. The difference between them was significant (p < 0.01). In pancreas homogenates, the level in hypertensive rats tended to be higher than that of normotensive rats, but the difference was not significant, as shown in table 3. In contrast, urinary kallikrein and the level of kallikrein in the anterior pituitary are decreased in hypertensive rats in comparison with normal rats (10, 4). In order to clarify the discrepancy, the interrelation of the kallikrein-kinin and renin-angiotensin systems in the submaxillary gland and kidney will be further investigated.

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References
