Change in Blood Kinin and Plasma Porcine Pancreatic Kallikrein Concentrations after Oral Administration of Kallikrein Formulation in Dog

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Oral formulation of tissue kallikrein consists primarily of porcine pancreatic kallikrein (PPK) and is used to improve peripheral circulation, menopausal symptoms, and impaired chorioretinal circulation. Although gastrointestinal absorption of tissue kallikrein after oral administration has been reported in nonclinical and clinical studies, the increase in the concentration of pharmacologically active kinins, which are produced from kininogen by tissue kallikrein, has not been investigated. In this study, kallikrein formulation was orally administered to dogs and an increase in PPK in plasma was confirmed, along with an increase in the blood kinin level. After oral administration of kallikrein formulation (10 U/kg or 20 U/kg PPK) to dogs, PPK concentration in plasma reached maximum 3 h after administration, and then decreased time-dependently. The maximum concentration was 6.01±1.44 pg/ml in the 10 U/kg group and 10.88±3.59 pg/ml in the 20 U/kg group (mean±S.E.M., n=5). After oral administration of kallikrein formulation (40 U/kg PPK) to dogs, the blood kinin concentration in the PPK-treated group was significantly increased 2 h after administration as compared to the purified water-treated group (before administration: 48.8±2.1 ng/ml vs. 48.1±1.9 ng/ml, 2 h after administration: 55.5±1.6 ng/ml vs. 49.6±1.4 ng/ml; mean±S.E.M., n=4, p<0.05). In conclusion, PPK was considered to be absorbed after oral administration and to exert its pharmacological action via kinins produced by kininogen degradation in dogs.

Key words tissue kallikrein; absorption; kinin; oral administration; enzyme-linked immunosorbent assay

Tissue kallikrein (EC 3.4.21.35) is a serine protease with kininogenase activity. It is also called as kallidinogenase and is found in many tissues including kidney, blood vessels, pancreas, prostate, intestines, salivary gland, sweat gland, spleen, brain, adrenal gland, and neutrophils. Tissue kallikrein selectively acts on the substrate, low-molecular-weight kininogen which is a plasma protein that belongs to beta-globulin fraction and releases vasoactive peptides, bradykinin and Lys-bradykinin (kallidin). Produced kinins are inactivated through degradation by various peptidases with kininase activity; however, intact kinins bind to B2 receptors in cell membrane and exert a peripheral vasodilatation effect by facilitating the production of nitric oxide and prostaglandins. They also increase local blood flow and improve tissue circulation.

Oral formulation of tissue kallikrein contains porcine pancreatic kallikrein (PPK) as a primary component and is mainly used in Japan as a medication with the following indications: improvement of peripheral circulation in hypertension, Meniere’s syndrome, and thromboangiitis obliterans (Buerger’s disease) in addition to improvement of menopausal symptoms and impaired chorioretinal circulation. Since tissue kallikrein is a glycoprotein with a molecular weight of approximately 30,000, some investigators have questioned the absorption after oral administration. On the other hand, some groups have reported gastrointestinal absorption of kallikrein in rats, rabbits and human. However, the kallikrein doses and administration methods or the methods used to measure its concentration in blood varied in each report. Few studies have reported absorption of PPK after oral administration at around the clinical dose. Furthermore, it is unclear whether kinins as pharmacological active substances of kallikrein increase after oral administration of kallikrein formulation.

The present study was conducted to confirm the increase in PPK concentration after oral administration of kallikrein formulation to dogs as well as to investigate the increase in endogenous kinin concentration in blood and to confirm the pharmacological effect of absorbed PPK.

MATERIALS AND METHODS

Materials Carnaculin 50 enteric-coated tablet (Sanwa Kagaku Kenkyusho, Nagoya, Japan) was used as the kallikrein formulation. It contains 50 units (U) of PPK per tablet.

Disodium ethylenediaminetetraacetic acid (EDTA) and o-phenanthroline were purchased from Dojindo Laboratories (Kumamoto, Japan), ethanol and ether from Wako Pure Chemical Industries (Osaka, Japan), pepsin, Tween 20, and N,N′-o-phenylenedimaleimide from Sigma-Aldrich (St. Louis, MO, U.S.A.), galactosidase from Boehringer Mannheim (Mannheim, Germany), H-d-Val-Leu-Arg-p-nitroanilide·2HCl from Sekisui Medical (Tokyo, Japan), and Block Ace from Dainippon Pharma (Osaka, Japan). All other reagents used were of special grade.

Animals All animal experiments were approved by the Ethical Committee for Animal Research of Sanwa Kagaku Kenkyusho, and carried out in accordance with the institutional and national guidelines for the care and use of laboratory animals. Male beagles were purchased from Kitayama Labs (Nagano, Japan). All dogs were housed in individual cages at room temperature of 23±2°C, humidity of 55±10% and ventilation of 14.2 times per hour, and allowed food once daily (laboratory chow diet, DS-A; Oriental Yeast, Tokyo, Japan) and ad libitum access to water.

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Sample Collection for Pancreatic Kallikrein Measurement
One hundred units of PPK was orally administered to 5 male beagles (10 kg) after fasting for approximately 20 h, and 5 ml of blood was collected before and 1, 2, 3, 4, 5, 6, 8, and 10 h after administration. EDTA was used as an anticoagulant. After a 1-week washout, 200 U of PPK was orally administered, and blood was collected in the same way. Blood was subjected to plasma separation after centrifugation (2000×g, 10 min, 4 °C) and cryopreserved at below −70 °C until measurement.

Sample Collection and Pretreatment for Blood Kinin Measurement
Four hundred units of PPK was orally administered to 4 beagles (10 kg) after fasting for approximately 20 h, and 2 ml of blood was collected before and 2 h after administration. In addition, purified water was administered to the control group. Samples for measurement of kinin concentration were collected and pretreated by the following the method by Nakajima et al.9) with some modifications. Briefly, blood was immediately added to 8 ml of ethanol with o-phenanthroline (1 mmol/l) which was cooled with dry ice. The mixture was stirred for 1 min and the supernatant was fractionated after centrifugation (2000×g, 30 min, 4 °C). The residue was stirred after adding ethanol and the supernatant obtained after centrifugation was added to the first supernatant. Collected supernatant was evaporated to dryness at room temperature under nitrogen air flow. Dried samples were redissolved with 1 ml of purified water and pH was adjusted to 3.0 with 0.1 mol/l HCl. The solution was left stand after mixing 2 ml of ether and the aqueous phase was collected. This procedure was repeated twice and pH of the solution obtained was adjusted to approximately 7, which was then lyophilized. Samples were cryopreserved at below −70 °C until measurement.

Measurement of PPK Concentration
PPK concentration in dog plasma was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using the chemiluminescent method. Anti-PPK immunoglobulin G (IgG) purified from rabbit antiserum, which was obtained after subcutaneous injection of purified PPK was used for the capture antibody in ELISA. For the detection antibody, enzyme labeled specific anti-PPK Fab’ which was affinity-purified on antigen column from rabbit antiserum, digested with pepsin and labeled with galactosidase using [m14C]-galactosidase from rabbit antiserum, digested with pepsin and labeled with galactosidase using [m14C]-galactosidase from rabbit antiserum, digested with pepsin and labeled with galactosidase using [m14C]-galactosidase from rabbit antiserum, which was obtained after subcutaneous method by Nakajima et al.9) with some modifications. Briefly, blood was immediately added to 8 ml of ethanol with o-phenanthroline (1 mmol/l) which was cooled with dry ice. The mixture was stirred for 1 min and the supernatant was fractionated after centrifugation (2000×g, 30 min, 4 °C). The residue was stirred after adding ethanol and the supernatant obtained after centrifugation was added to the first supernatant. Collected supernatant was evaporated to dryness at room temperature under nitrogen air flow. Dried samples were redissolved with 1 ml of purified water and pH was adjusted to 3.0 with 0.1 mol/l HCl. The solution was left stand after mixing 2 ml of ether and the aqueous phase was collected. This procedure was repeated twice and pH of the solution obtained was adjusted to approximately 7, which was then lyophilized. Samples were cryopreserved at below −70 °C until measurement.

Measurement of Blood Kinin Concentration
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Dog plasma was pretreated by the following procedure. Briefly, plasma was diluted to 5-fold with sample diluent (10% Block Ace–Buffer A: 10 mmol/l N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid (HEPES)–150 mmol/l NaCl–1 mmol/l MgCl2–1 mg/ml NaN3, pH 7.5) and added to a 96-well microplate (Nunc, Rochester, NY, U.S.A.) coated with rabbit IgG (Zymed, San Francisco, CA, U.S.A.), which was then allowed to react for 20—24 h at 4 °C. The reaction solution was used as the sample for ELISA and measured by the following PPK ELISA.

One hundred microliters of pretreated dog plasma was added to a 96-well microplate for luminescence measurements (Dynex, Chantilly, VA, U.S.A.) coated with anti-PPK IgG and incubated for 20—24 h at 4 °C. The plate was washed five times with Buffer A containing 0.05% Tween 20, and then 100 μl of galactosidase-labeled anti-PPK Fab’ antibody was added and incubated for 5 h at 25 °C. The plate was washed five times, and then 100 μl of substrate solution containing chemiluminescent substrate for galactosidase (Galacto-Plus, Tropix, Bedford, MA, U.S.A.) was added and incubated for 4 h at 25 °C. One hundred microliters of chemiluminescence enhancer (Light Emission Accelerator-II, Tropix) was added to the reaction solution and luminescence was measured using a luminescence plate reader (Micro-Lumat LB96P, EG&G Berthold, Bad Wildbad, Germany). The lower limit of quantification was 0.6 pg/ml plasma. PPK concentration in plasma was calculated by linear regression analysis by log–log plot using Microsoft Excel.

Measurement of Blood Kinin Concentration
Blood kinin concentration in dogs was measured by a commercially available competitive ELISA kit (Peninsula Laboratories, San Carlos, CA, U.S.A.) using samples after extraction and concentration. The cross-reactivity in ELISA was 100% for Bradykinin, Lys-Bradykinin (kallidin), Biotinyl-Bradykinin, [Des-Arg]-Bradykinin, and Bradykinin (4–9), 40% for Bradykinin (5–9), and 0% for (Des-Arg)-Bradykinin and Bradykinin (7–9). The lower limit of quantification was 0.02 ng/ml.

Lyophilized blood samples after pretreatment were redissolved with 200 μl of Assay Buffer included in the kit. Fifty microliters of sample, 25 μl of primary antiserum and 25 μl of biotinylated bradykinin solution were added to ImmunoPlate and incubated for 2 h at room temperature. The plate was washed with Assay Buffer five times, then 100 μl of diluted streptavidin-HRP solution was added and incubated for 1 h at room temperature. The plate was again washed five times, and then 100 μl of TMB substrate solution was added and incubated for 30 min at room temperature. The reaction was stopped by adding 100 μl of 2 mol/l HCl. Absorbance was measured at the wavelength of 450 nm using an absorbance plate reader (SpectraMax Plus 384, Molecular Device, Sunnyvale, CA, U.S.A.). Blood kinin concentration was calculated by 4-parameter nonlinear logistic curve fit analysis using SoftMaxpro software (version 4.8, Molecular Device).

Statistical Analysis
Data are expressed as means with S.E.M. For comparison of blood kinin concentration between groups, statistical significance of differences was assessed with Student’s t-test using EXSAS software (version 7.1.6, Arm, Osaka, Japan). Differences were regarded as statistically significant when the p-value was less than 0.05.

RESULTS

Change in Plasma PPK Concentration after Oral Administration of PPK
Kallikrein formulation (10, 20 U/kg PPK) was orally administered to dogs, and PPK concentration in plasma was measured up to 10 h after administration (Fig. 1). PPK concentration in plasma reached maximum 3 h after administration in both 10 U/kg- and 20 U/kg-treated
groups, and then decreased time-dependently. The maximum concentrations increased dose-dependently, at 6.01±1.44 pg/ml (n=5) in the 10 U/kg-treated group and 10.88±3.59 pg/ml (n=5) in the 20 U/kg-treated group, respectively.

Change in Blood Kinin Concentration after Oral Administration of PPK Kallikrein formulation (40 U/kg PPK) was orally administered to dogs and blood kinin concentration was measured before and 2 h after administration (Fig. 2). Blood kinin concentration in the PPK-treated group was 48.8±2.1 ng/ml (n=4) before administration and 55.5±1.6 ng/ml (n=4) 2 h after administration, respectively. Similarly, blood kinin concentration in the purified water-treated group was 48.1±1.9 ng/ml (n=4) before administration and 49.6±1.4 ng/ml (n=4) 2 h after administration, respectively. Blood kinin concentration in the PPK-treated group increased significantly 2 h after administration compared to that in the purified water-treated group.

**DISCUSSION**

There was a concern over absorption of kallikrein (kallidinogenase) formulation after oral administration because major component PPK is a high-molecular-weight protein. Miska et al.\(^7,8\) developed a highly sensitive assay for PPK using the bioluminescence method (the limit of detection: 1 pg/ml serum) and measured PPK concentration in serum after oral administration of 4500 and 600 biological kallikrein units (KE) of kallikrein formulation to healthy adults. PPK concentration in serum reached maximum 2—6 h and 4—12 h after administration in the 4500 KE- and 600 KE-treated groups, respectively. Thus PPK was reported to be absorbed after oral administration. We developed a highly sensitive assay for PPK (the lower limit of quantification is 0.6 pg/ml plasma) using the chemiluminescence method and found that the maximum concentration reached 3 h after oral administration of 100 and 200 U of kallikrein formulation to dogs (Fig. 1). Although the percent of absorbed PPK protein in a dose was unclear in this study, it was thought to be below about 1% from the results of some previous reports.\(^5,7\)

In the reports by Miska et al.,\(^7,8\) since the kallikrein activity of 1 U is converted to that of 2 KE, the doses of 600 and 4500 KE were 5 and 37.5 U/kg, respectively, assuming the body weight of an adult to be 60 kg. The maximum PPK concentration in blood was 1.0—2.25 ng/ml in the 5 U/kg-treated group and 0.5—7.0 ng/ml in the 37.5 U/kg-treated group, respectively. In our results, however, the maximum concentration after administration of kallikrein formulation to dogs was 6.01±1.44 pg/ml (n=5) in the 10 U/kg-treated group and 10.88±3.59 pg/ml (n=5) in the 20 U/kg-treated group, respectively. There was a large discrepancy in the PPK concentration between both studies. It is assumed that the discrepancy is due to the differences in species (human and dogs), the type of sample (serum and plasma), the quality of the standard for calibration curve, and the specificity and/or selectivity of anti-PPK IgG, but the details are unknown.

To verify whether PPK detected in dog plasma by ELISA has pharmacological activity, blood kinin concentration was measured 2 h after administration when the PPK concentration in dog plasma had clearly increased. In a preliminary study, change in blood kinin concentration showed about the same process as that in plasma PPK concentration after oral administration of kallikrein formulation in dog (data not shown). As a result, the increase in blood kinin concentration was confirmed (Fig. 2). While blood kinins are degraded rapidly by some peptidases with kininase activity, they are simultaneously produced from kininogen by kallikrein.\(^1\) Therefore, immediately after blood collection in this study, metal ion chelating agent o-phenanthroline was added to inhibit kininase activity in blood and ethanol was used to de-proteinize blood samples. These pretreatment procedures are considered to have minimized the degradation and production of kinins after blood collection and resulted in successful reduction of the effect on measurement. Although it was unclear whether blood kinins increased after oral administration of kallikrein formulation, we demonstrated that blood kinins increased in association with the increase in PPK concentration in plasma. In this study, the increasing level of blood kinin concentration from the basal level was about 7 ng/ml (10% Increases) at 2 h after administration. Some researchers have reported that the vasoactive effects of bradykinin were detected in a concentration-dependent manner from at least 1×10\(^{-9}\) mol/l (1 ng/ml) when bradykinin was applied to isolated blood vessels\(^9,14\) or when it was infused to rats.\(^9,14\) Thus, the increasing level of blood kinins in this study is considered sufficient to show pharmacological effect of PPK after oral administration.

In clinical studies for oral administration of kallikrein formulation, various pharmacological effects have been re-
ported. For example, in a double-blind study in which 600 KE/d of PPK was orally administered to the patients with essential hypertension for 4 weeks, blood pressure in the patients with hypertension was significantly reduced compared to the placebo group. In a double-blind study in which 600 KE/d of PPK was orally administered to the infertile male patients for 3 months, the rates of motile and morphologically normal sperm were increased in the infertile patients. In addition, chorioretinal blood flow was significantly increased after oral administration of 150 U/d of PPK to the healthy adults for 4 weeks. Furthermore, after oral administration of 150 U/d of PPK to the patients with simple diabetic retinopathy for 6 months, a-wave latency and oscillatory potential latency (O2) on the electroretinogram (ERG) after administration were significantly shortened compared to those before administration in the PPK-treated group, suggesting the improvement of choroidal and retinal circulation. Based on a number of these evidences, orally administered PPK is assumed to be absorbed in the gastrointestinal tract and to exert its pharmacological action through the production of kinins.

In conclusion, PPK was considered to be absorbed after oral administration and to exert its pharmacological action via kinins in dogs.

REFERENCES