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A VASOPRESSOR FACTOR PARTIALLY PURIFIED FROM HUMAN PARATHYROID GLANDS

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Recently, a parathyroid hypertensive factor was postulated to play a role in the pathogenesis of hypertension in genetically hypertensive rats. Therefore it was examined, whether in human parathyroid glands a vasopressor substance can be detected. For this purpose, homogenates of hyperplastic parathyroid glands from 20 patients with tertiary hyperparathyroidism were deproteinized and fractionated by gel chromatography. The fractions obtained were tested for vasopressor activity in isolated perfused rat kidneys. A vasopressor fraction containing substances of 0.6 - 2.5 kDa was identified in the parathyroid glands. The responsible product was heat sensitive, peptidase-, trypsin- and carboxypeptidase y- sensitive and hydrophilic, as it did not bind to hydrophobic reversed-phase gel. These results suggest that parathyroid glands contain a hydrophilic peptide-like vasopressor substance different from the parathyroid hormone.

In the pathogenesis of essential hypertension yet unidentified circulating vasopressor agents are being discussed. A so-called digoxin-like factor is postulated, which, according to recent findings, may be identical with ouabain and may be produced in adrenal tissue (1). Pang's group described the purification of a circulating substance from plasma of spontaneously hypertensive rats, which may be produced in the parathyroid glands (2-5). Bachmann et al. (6) demonstrated the existence of a vasoconstrictor humoral factor, which was shown to be elevated in the plasma of hypertensive patients. In this study fractions from the parathyroid tissue were screened for vasopressor substances.

MATERIALS AND METHODS

A. Parathyroid glands

The parathyroid gland samples stemmed from 20 patients, which underwent parathyroidectomy because of tertiary hyperparathyroidism, which was a consequence of end-stage renal failure. 14 patients were hypertensive. Serum Ca was

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3.07 ± 0.21 mmol/l. Hyperplastic parathyroid tissue was proven histologically in each case. The mean weight of the parathyroid glands was 0.9 ± 0.1 g (mean ± SD).

B. Chemicals

HPLC-grade water, HPLC-grade acetonitrile and HPLC-grade trifluoracetic acid were from J.T.Baker (Phillipsburg, N.J., USA). Trypsin and carboxypeptidase y were obtained from Boehringer Mannheim (Germany). Buffer substances were purchased from Merck, Darmstadt, Germany. All other substances used were obtained from Sigma (Deisenhofen, Germany).

C. Homogenization and precipitation

The parathyroid glands (5 samples per each purification procedure) were put into 20 ml ice cold water and were homogenized 10 times in intervals of 30 sec in a blade homogenizer, which was cooled in an ice bath. The homogenate was centrifuged to remove crude material. Then perchloric acid was added till a concentration of 1 M was reached. The solution was centrifuged (2000 g, 10 min) and the supernatant was titrated to pH 7 with 1 M KOH. After another centrifugation the precipitate was discarded and the supernatant was lyophilized. The preparation of parathyroid glands was done at 4° C.

D. Chromatographic purification of the parathyroid gland extract

The dried material was resuspended with 2 ml bidestilled water. After removal of the particulate with centrifugation and filtration (0,2 μm filter, Millipore, Eschborn, Germany) the solution was chromatographed with a gelfiltration column (2 cm x 120 cm; Bio-Gel P2, Biorad, München, Germany). Bidestilled water was used as eluent. The flow rate was 20 ml/h. Absorbance and conductivity were monitored continuously (Uvicord S II, Pharmacia, Freiburg, Germany; LE 191, WTW, Germany). Each fraction contained 10 ml (RediFrac, Pharmacia, Freiburg, Germany). The column was calibrated with substances of known molecular weight (insulin from bovine, 6000 Da, cyanocobalamin 1355 Da). The fractions obtained from gel filtration were lyophilized. Then the residues were reconstituted in Tyrode's solution to a volume of 100 μl. In addition, Na^+ and K^+ content, pH and osmolality of the final samples were monitored in order to avoid unspecific effects on perfusion pressure due to deviations of these parameters from the physiologic range.

E. Assay for vasoactivity

The perfusion procedure generally followed the description given by Hofbauer et al. (7). Male normotensive Wistar Kyoto rats weighing 300-400 g were anesthetized with 1.4 g/kg urethane. The abdomen was opened by a midline incision and the abdominal aorta, renal artery and left kidney were exposed. Ligatures were placed around the renal artery and the abdominal aorta below the renal artery. The renal artery was cannulated through the aorta with a flexible polyethylene catheter without interruption of flow. The catheter was flushed with heparinized saline (100 U/ml). Perfusion was started immediately after insertion of the catheter into the renal artery. After excision, the kidney was isolated and placed immediately in a warmed plexiglas chamber. The kidney was perfused with Tyrode's solution at a constant flow rate of 9 ml/min provided by a peristaltic pump. The perfusion medium was maintained at a temperature of 37 ^{O}C and equilibrated with 5 % CO_2 / 95 % O_2. The composition of the

perfusion medium was (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 12, NaH_2PO_4 0.42, D-(+)-glucose 5.6. Perfusion pressure was monitored with a pressure transducer (Gould P23) connected to a bridge amplifier (Hugo Sachs, Freiburg, W. Germany) and recorded on a penwriter. Since the flow rate was kept constant, changes in perfusion pressure reflected alterations in renal vascular resistance. An equilibration period of 30 min was allowed before starting experiments. Fractions and vasoactive substances were administered as bolus injections in a volume of 100 μl. After perfusion of 5 h, perfused kidneys weighed 108.4 $\pm$ 7.2 % (n=34) of the nonperfused, contralateral kidney, indicating that no significant edema formation occurred during this period. After the equilibration period, the duration of the experiments in one kidney did not exceed 2 h. A dose response curve was recorded using different concentrations of angiotensin II (Fig. 2). In 17 experiments the response to angiotensin II at the end of the perfusion was 123 $\pm$ 19 % of the initial response.

F. Binding experiment with the vasoconstrictive factor

For the binding studies self assembled solid-phase extraction cartridges and a vacuum manifold column processor (Burdick & Jackson, Muskegon, MI, USA) connected to a water-driven vacuum pump were used.
Siliconized glass columns (2 ml, J.T.Baker, Phillipsburg, N.J., USA) with teflon frits (J.T.Baker, Phillipsburg, N.J., USA) were used. The columns were filled with 1 g reversed-phase C_{18} silica material from J.T.Baker (Phillipsburg, N.J., USA).
The solid phase extraction was preceded by conditioning the columns with 20 ml acetonitrile. Then the reversed-phase column was washed with 20 ml of solution consisting 0.1 % trifluoraceticacid in water. The eluate was collected in siliconized glass tubes and concentrated to dryness in a Speed Vac concentrator (Savant, Framingdale, NY, USA).

G. Inactivation experiments with the vasopressor fraction

The vasoactive fraction (0.5 g tissue-aliquot), dissolved in 400 μl Tyrode's solution was heated half an hour in a boiling water bath and after cooling to 37° C tested in the isolated perfused kidney assay. Further three 0.5 g tissue-aliquots (dissolved in 400 μl) were incubated overnight at 37° C with peptidase (0,01 U, from porcine intestinal mucosa), trypsin (1 U, sequencing grade) and with carboxypeptidase y (0,1 U, sequencing grade). The reactions were stopped after centrifugation of the mixture with ultrafiltration devices (Ultrafree-MC, 5 kDa exclusion limit, Millipore, Eschborn, Germany). The filtrate was tested for vasoactivity.

H. Analysis of the vasoactive fraction by MALDI-MS [2]

The vasoactive material was examined with the MALDI-MS. A reflector-type time-of-flight mass spectrometer equipped with a nitrogenlaser (337 nm, pulselength 4 ns) was used for ion generation and mass analysis. Details of the MALDI-MS have been reported elsewhere (8). Samples were dissolved in 5 μl water. 1 μl of this solution was mixed with 1 μl of an aqueous solution of 10 g/l 2,5-dihydroxybenzoic acid representing the UV-absorbing matrix. 1 μl of the final solution was dripped and dried onto a metallic substrate. Desorption of analyte ions was achieved by laser shots of irradiances in the 10^6 to 10^7 W/cm^2 focused to spot sizes of typically 200 μm in

[2] MALDI-MS : Matrix-assisted laser desorption/ionization mass spectrometry.

diameter. The spectra were registered by a LeCroy 9400 transient recorder and typically accumulated from 10 single laser shots. Total time of measurements including preparation was 10 minutes. The results were expressed as molecular weight/electrical charge of the substance (M_r/z in Fig. 5). Since with this form of mass spectrometry substances with a single charge are produced, M_r/z is identical with the molecular weight (9).

RESULTS

Fig. 1 shows the effect of fractions obtained by gel chromatography from extracts of parathyroid glands on the perfusion pressure to an isolated perfused rat kidney. In one fraction labeled as no. 2 in Fig. 1 a vasopressor compound was detected. Less vasoactivity was detected in a fraction appearing later in the eluate. Repeated administrations of the active parathyroid fraction showed similar responses, indicating that no significant tachyphylaxis occurred (data not shown). Due to the relatively small number of experiments a correlation between blood pressure of the patients and vasopressor activity could not be established. After gel filtration 62 $\pm$ 16 % of the total vasopressor activity of the perchloric acid extract was recovered (4 experiments with samples of 5 patients each).

Parathyroid hormone was tested in a dose of 10^{-6} M and did not change perfusion pressure. Fig. 2 shows dose-response curves for angiotensin II in the same preparation.

In Fig. 3 a typical gelfiltration chromatogram of the Biogel P2 column with the corresponding profile of the vasopressor activity is depicted. According to the calibration of the column (elution time of insulin: 190 min; elution time of cyanocobalamin: 500 min), the molecular weight of the vasoactive substance can be estimated at 1 - 5 kDa.

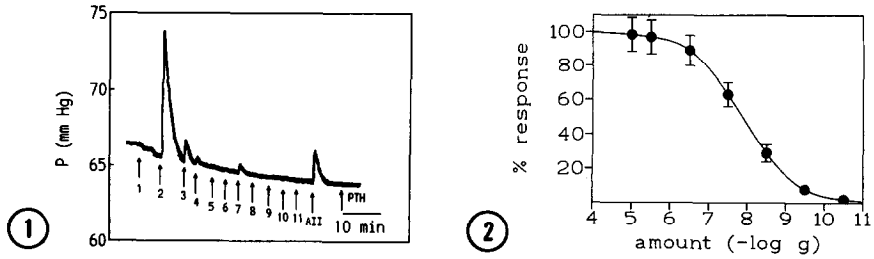

Fig. 1. Changes in perfusion pressure (P, mm Hg) in the isolated perfused kidney at constant flow after administration of samples of 1 g aliquots of pooled parathyroid glands, dissolved in 0.1 ml physiological buffer solution, obtained by gel chromatography of extracts from parathyroid glands. AII = $5 \cdot 10^{-12}$ mol angiotensin II (total amount). PTH = 10^{-6} M parathyroid hormone (1-84).

Fig. 2. Dose-response curve for angiotensin II in the isolated perfused rat kidney. Abscissa: dose (-log g). 100 % response of the kidney stimulated with angiotensin II: 93 $\pm$ 6 mmHg.

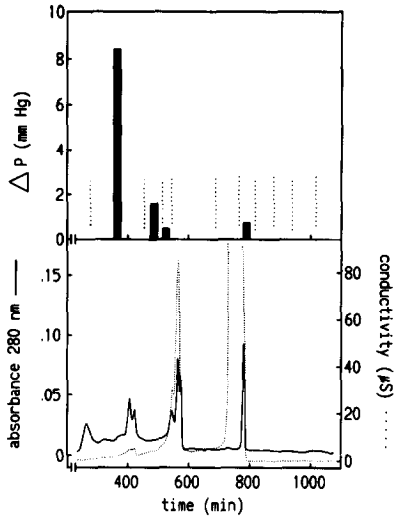

Fig. 3. Size-exclusion-chromatography (Biogel P2) of extracts from parathyroid glands. Changes in perfusion pressure (Δ P, upper panel) after administration of samples of 0.5 g aliquots of pooled parathyroid glands, dissolved in 0.1 ml physiological buffer solution, to the isolated perfused rat kidney; UV absorbance at 280 nm and conductivity (lower panel). Abscissa: elution time. The fractions formed from the eluate are marked by dotted lines in the upper panel.

With the reversed-phase gel no absorption could be noticed. All the activity (57 ± 19 % of that in the perchloric acid extract) was found in the water fraction.
The inactivation experiments (Fig. 4) revealed that the vasopressor agent is not heat resistant. The proteases trypsin, peptidase and carboxypeptidase y abolished the vasopressor action (Fig. 4).

As shown in the mass spectrum in Fig. 5, the active fraction contained substances in the range of 1.5 - 2.5 kDa in accordance to the result of gel filtration.

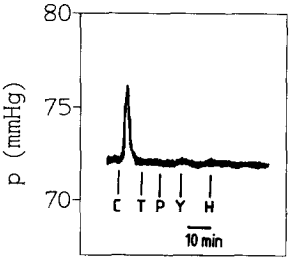

Fig. 4. Changes in perfusion pressure (P, mm Hg) in the isolated perfused kidney at constant flow after administration of samples of 0.5 g aliquots of the vasoactive fraction obtained by the size-exclusion-chromatography (Figs. 1 and 2, fraction 2) before (**C**) and after incubation with the proteases trypsin (**T**), peptidase (**P**) and carboxypeptidase y (**Y**) and after heating (**H**) (boiling water bath, 1 h).

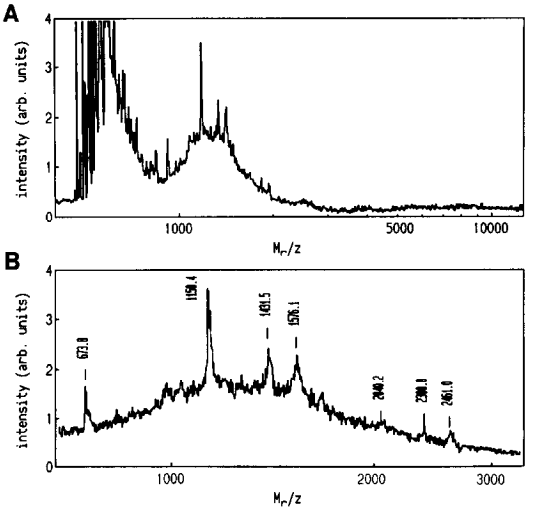

Fig. 5. MALDI-MS spectrum of the vasoactive fraction of a parathyroid gland obtained by gel chromatography (fraction no. 2 in Fig. 1), followed by a reversed-phase cleaning step. Abscissa: mass/electrical charge (M/z), ordinate: relative intensity (arbitrary units).

DISCUSSION

Since experiments in spontaneously hypertensive rats showed that parathyroid glands are necessary for the maintenance of hypertension (10), a parathyroid factor may play a role in the pathogenesis of primary hypertension. This vasopressor agent of parathyroid origin described in spontaneously hypertensive rats has a delayed action on blood pressure (11). Its effect is potentiated by other vasopressors such as angiotensin II and norepinephrine, and it blocks the vasodilator effect of parathyroid hormone (11).

In the present study in parathyroid tissue from patients with tertiary hyperparathyroidism a vasopressor with a molecular weight in the range of 1.5 -2.5 kDa agent was demonstrated. As evidenced by the reversed-phase binding studies the agent has a polar, hydrophilic character. As the vasopressor is instable to several proteases, the substance may be a peptide. Size, hydrophilicity and biological action of the vasopressor, characterized in this study, are different from parathyroid hormone, indicating that these substances are not identical.

Since in the vasoactive fraction MALDI-MS revealed a number of substances of similar molecular weight, further purification will be necessary.

At present, it must remain open, whether it is a factor in the development of increased vascular tone in primary hypertension.

In summary, we have shown that a novel, low molecular weight, hydrophilic, peptide-like vasopressor, which is heat- and peptidase-sensitive, can be extracted from parathyroid glands.

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