Quantitative Determination of Parathyroid Hypertensive Factor by Enzyme-Linked Immunosorbent Assay

Svetlana M. Krylova, Peter K.T. Pang, Jacqueline Shan, Richard Z. Lewanczuk, and Christina G. Benishin

A new competitive enzyme immunoassay for the detection parathyroid hypertensive factor (PHF) in human plasma using a PHF–horseradish peroxidase conjugate and IgM antibody adsorbed on the microtiter plate was established. The antibodies raised against rat PHF could recognize human PHF. Cross-reactivity of anti-PHF antibodies with other serum haptens and proteins was negligible. Conjugation of PHF with horseradish peroxidase did not neutralize the antigen activity. The limit of detection of PHF was 0.02 U/mL in reference units and PHF levels between 0.02 and 1 U/mL could be detected. Within-run coefficient of variation (CV) was less than 10%, and between-run CV was less than 15% for over the dynamic range of the assay. Preliminary clinical studies were performed with plasma samples from hypertensive patients with confirmed diagnosis. Parathyroid hypertensive factor levels, as detected with this immunoassay, were positively correlated with PHF levels detected with the semiquantitative blood pressure (BP) bioassay previously used. Parathyroid hypertensive factor levels detected with the enzyme-linked immunosorbent assay (ELISA) were also correlated with BP in patients. The PHF ELISA provides a selective, simple, and rapid method that can be used for routine determination of PHF in human plasma, and provides useful clinical information. Am J Hypertens 2000;13:1173–1179 © 2000 American Journal of Hypertension, Ltd.

KEY WORDS: Hypertension, parathyroid hypertensive factor, enzyme-linked immunosorbent assay, anti-PHF antibodies.

Parathyroid hypertensive factor (PHF) is a newly described hypertensive factor that may cause elevation of blood pressure (BP) in approximately 40% of essential hypertensive patients. PHF was first discovered in the plasma of several rat models of hypertension as well as in low-renin salt-sensitive hypertensive humans. The occurrence of PHF in humans and various animal models has also been confirmed independently by other investigators. Parathyroid hypertensive factor elicits a unique delayed and prolonged BP response when injected into normotensive rats. Endocrinectomy and glandular re-
placement studies suggested that the parathyroid gland (PTG) was the source of PHF. Subsequently, isolated PTG in organ culture was shown to secrete the substance. Parathyroid hypertensive factor has been purified from three sources of material: plasma of spontaneously hypertensive rats (SHR), culture medium from organ culture of SHR parathyroid glands, and from subcultured SHR PTG cells, and the chromatographic characteristics of PHF from these sources were identical. Polyclonal antisera to PHF that have been raised in mice significantly lower BP in SHR to near-normal levels. Clinically, PHF was found to be positively correlated with salt sensitivity and negatively correlated with elevated renin secretion. The hypothesis that PHF is related to the salt-sensitive and low-renin form of hypertension was further supported by studies with animal models. It was further proposed that PHF alters the ability of cells to regulate and maintain calcium equilibrium. Original studies on PHF focused on the vascular system in relation to hypertension. However, recent data suggest that excessive PHF may be manifested in different individuals with symptoms of other diseases associated with abnormal cellular calcium regulation, such as type II diabetes and some forms of cancer.

This article describes a simple and accurate in vitro diagnostic test to detect and quantify PHF in human plasma. A complex, semiquantitative in vivo bioassay was the only tool available to measure PHF before the development of this assay. This advancement allows for the continued investigation of PHF as a primary causative factor in calcium dysregulation disorders, and elucidation of the metabolic pathway of PHF.

METHODS

Subjects  Heparinized plasma samples were collected from 120 hypertensive and normotensive individuals (men and women, age range 19 to 65 years) by Research Sample Bank, Inc., Pompano Beach, FL. Heparinized plasma was kept frozen at −70°C before assay.

PHF Antigen  Parathyroid glands were harvested from SHR and used to generate PTG cultures. The low calcium-containing medium (Ham’s F-12 medium supplemented with 10% fetal bovine serum, exposed to cells for 3 to 4 days) was the source of rat PHF. Parathyroid glands that were resected from hypertensive hyperparathyroid patients, who were confirmed to have PHF, were also used to generate PTG cultures, in a manner essentially identical to that used for culture of rat PTG cells. Media from either source were ultrafiltered at 10,000 dalton molecular weight cut off to remove large molecules, and then concentrated over a 1000-dalton molecular weight cut off filter, and lyophilized. The lyophilized powder was then reconstituted in water, and dialyzed at 2000 daltons molecular weight cut off for 24 to 72 h against water to thoroughly remove small molecules. After dialysis, the retained dialysate was lyophilized, and used as antigen for the enzyme-linked immunosorbent assay (ELISA) studies. Biologic activity of all fractions was verified by BP bioassay in normotensive male Sprague-Dawley rats. Parathyroid hypertensive factor samples were prepared in a stock standard solution of 10 mg/mL, and then diluted. The highest concentration of PHF, in 1:100 dilution of PHF stock standard solution, was defined as 1 U/mL of PHF. The concentration of the PHF samples from subsequent preparations was calculated using calibrators prepared from the stock standard solution.

ELISA Reagents  The coating and the dilution buffers were phosphate-buffered saline (PBS) (0.15 mol/L NaCl, 1.5 mmol/L KH2PO4, 8.5 mmol/L K2HPO4, pH 7.4); the washing buffer was PBS containing 0.05% Tween 20 (ICN Biomedicals Inc., Aurora, OH); the incubation buffer for the PHF–horseradish peroxidase (HRP) conjugate was PBS containing 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and 0.05% Tween-20, 0.05 mol/L phosphate-citrate buffer containing 0.03% sodium perborate, pH 5.0 (Sigma Chemical Co.) was used as the substrate buffer. The chromogenic substrate was 3',3',5',5'-tetramethylbenzidine (TMB, Sigma Chemical Co.). Antimouse polyclonal (IgG, IgM, IgA) immunoglobulins HRP were obtained from Sigma Chemical Co. Mouse clonotyping system/HRP ELISA kit for isotype determination of antibodies was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL).

Anti-PHF Specific Antibodies  Mixed hybridoma cell lines (oligoclonal) were injected into Pristane-primed BALB/C mice. Ascites fluid was collected 10 to 14 days after injection of cells. Ascites were chromatographed on a column of Sephacry G-200 (100 × 2.6 cm). The column was eluted with PBS buffer containing 0.1% (w/v) NaN3 at a flow rate of 30 mL/h. Eluants were monitored at 280 nm, and anti-PHF activity was determined by ELISA. Anti-PHF containing fractions were pooled, and concentrated using a membrane ultrafiltration system (Amicon Co., Danvers, MA) with a pore size of 30,000 daltons, and stored at 4°C until use. The pooled antibody has been termed oligoclonal to indicate that antibody is derived from multiple cell lines.

Specificity of anti-PHF antibodies was verified by inactivation of PHF biologic activity, detected with the bioassay method described previously. Each sample of antibody was mixed with PHF standard solution (1 U/mL) ex vivo before injection into a normotensive bioassay rat, and the BP monitored.

Anti-PHF antibodies were tested for cross-reactivity with bovine and rat parathyroid hormone (1-34); hu-
man parathyroid hormone (1-84) (Allelix Biopharmaceuticals, Mississauga, ON, Canada); cholecystokinin, human and rat calcitonin gene-related peptide, salmon calcitonin (Bachem Inc., Torrance, CA), angiotensin I, angiotensin II (Peninsula Lab., San Carlos, CA); calcitriol (Roussel UCLA SF, Paris, France); creatine phosphate (Boehringer Mannheim GmbH, Mannheim, Germany); lysophosphatidyl choline, lysophosphatidyl ethanolamine, lysophosphatidyl inositol, lysophosphatidyl serine (Avanti Polar-Lipids Inc., Alabaster, AL); α-melanocytostimulating hormone, adenosine triphosphate, atrial natriuretic peptide, vasopressin, bradykinin, chonic gonadotropin, cytochrome C, dehydroisoandrosterone, hemoglobin, 5-hydroxytryptamine, histamine, insulin-like growth factor-I, insulin-like growth factor-II, insulin, nerve growth factor, neurotensin, ouabain, phosphatidyl serine, prostaglandin E$_2$, prostaglandin I$_2$, secretin, somatostatin, thrombin, thyroglobulin (Sigma Chemical Co.). Each hapten was added to the wells of microplates (at the concentration of 100 µg/mL) and titrated in parallel with rat PHF, which was the positive standard. The percentage of cross-reactivity was calculated as the ratio of the concentrations of the antigen and rat PHF, which provided the same level of inhibition of the reaction of rat PHF–HRP conjugate with anti-PHF antibodies.

**Labeling of Rat and Human PHF With Peroxidase**

Ten milligrams of horseradish peroxidase, grade 1 (Boehringer Mannheim, Laval, PQ, Canada) were dissolved in 1 mL of H$_2$O and mixed with 50 µL of freshly prepared 0.5 mol/L Na$_2$IO$_4$ (Sigma Chemical Co.) solution and incubated at room temperature for 30 min in the dark. The reaction mixture was loaded into a plastic microcolumn and a dry powder of Sephagen G25 (Pharmacia Biotech Inc., Baie d’Urfe, Quebec, Canada) was added in the amount required for preparation of a light suspension. The reaction mixture was incubated until the color changed from green to brown. Then, activated HRP was eluted with H$_2$O and dialyzed against H$_2$O for 1 h at room temperature. The optical density was measured at 403 nm to determine the concentration of HRP (ε = 1.02 × 10$^5$ mol/L/cm). One hundred microliters of 2.2 × 10$^{-4}$ mol/L activated HRP and 10 µL of 0.5 mol/L NaHCO$_3$ pH 9.5, were added to 10 µg of PHF dissolved in 100 µL of 0.1 mol/L sodium bicarbonate at pH 9.5. The reaction mixture was incubated at room temperature for 2 h. Ten microliters of freshly prepared 8 mg/mL NaBH$_4$ solution in 10 mmol/L NaOH was added and incubated for 15 min. Conjugated PHF–HRP was dialyzed against PBS for 24 h at 4°C. HRP activity was measured using 2,2'-azido-bis(3 ethylbenzthiazoline-6-sulfonic acid) (ABTS) and H$_2$O$_2$ as HRP substrates. The color development was read at 405 nm. PHF–HRP solution was stored at −70°C until use.

**Competitive ELISA Protocol**

Polystyrene flexible microtiter plates (Falcon brand, No. 3911; VWR, Mississauga, ON, Canada) were coated, incubated overnight at 4°C, and then incubated further for 2 h at 37°C with 2 µg/mL purified anti-PHF antibodies (100 µL/well) in coating buffer. The plates were washed five times with washing buffer using an Ultrawash Plus microplate washer (Dynex Technologies Inc., Greensey, Great Britain). Triplicates of each PHF standard in plasma, controls, or unknown samples (50 µL/well, 1:20 dilution with the dilution buffer) were pipetted, and then PHF–HRP conjugate (50 µL/well, 1:2000 dilution with the incubation buffer) was added, and the mixture was incubated for 2 h at 37°C. Bound PHF–HRP was measured using the peroxidase reaction with TMB as the substrate. Microplates were washed five times with washing buffer and chromogenic substrate (50 µL/well) was added and incubated for 30 min in the dark. The color reaction was stopped by addition of 2 mol/L H$_2$SO$_4$ (50 µL/well). Oxidized substrate was measured at 450 nm with a Labsystem Multiskan MCC ELISA microplate reader (MTX lab system Inc., McLean, VA). The calibration curve and results were analyzed with the Microplate Manager/PC software (Bio-Rad Laboratories, Mississauga, ON, Canada). The correlation of BP versus PHF concentration was analyzed using Origin Software (MicroCal Software, Inc., Northampton, MA). The correlation between PHF detected with ELISA and PHF detected with bioassay was analyzed using GraphPad Prism Software (San Diego, CA).

**Blood Pressure Bioassay**

Biologic activity of all anti-PHF antibodies, all fractions and PHF-containing plasma samples was verified by BP bioassay in normotensive male Sprague-Dawley rats (300 to 350 g) anesthetized with intraperitoneal pentobarbital (50 mg/kg).

**RESULTS**

**Antibody Specificity and Selectivity**

Partially purified rat PHF was used to raise specific antibodies in mice. Spleens harvested from those mice were used to create hybridoma cell lines. After subcloning, 18 hybridoma clones were selected for further investigation. Isolated anti-PHF antibodies were tested for cross-reactivity with human PHF by the method of ELISA. Four clones (not monoclonal) that reacted with human PHF as well as rat PHF were selected for use in ELISA for detection of PHF in patient plasma samples. Figure 1 shows human PHF cross-reactivity with a pool of these purified antibodies.

Bioassay experiments were conducted to verify that antibodies were specific to PHF. Mixing PHF with antibodies before injection into normotensive rats led
to an inactivation of the BP elevation response that is characteristic of PHF (data not shown).

Several experiments were designed to further establish the selectivity and specificity of anti-PHF antibodies. The first experiment was the conjugation of the control samples with HRP label using the same method as we used for labeling PHF. The control samples were media that had not been exposed to cells, or media harvested from other cell types. It was prepared by the same methods of filtration and dialysis that was used for isolation of rat PHF. Further immunoassay did not show binding of any of these “conjugates” with anti-PHF antibodies (data not shown).

Antibody specificity was confirmed by study of antibody cross-reactivity with other antigens including rat, human, and bovine parathyroid hormone, shorter parathyroid hormone fragments, different vasoactive and circulating substances, and human plasma proteins (see anti-PHF specific antibodies in Methods). In all cases, little (less than 1%) or no cross-reactivity was observed.

ELISA Optimization  Each step of the competitive ELISA for detection of PHF in human plasma was optimized. The concentration- and time-dependence for the adsorption of the anti-PHF antibodies on the polystyrene surface were studied. The rate of saturation increased by increasing the antibody concentration. Saturation of antibody adsorption to the microtiter plate was reached after overnight incubation at 4°C followed by a 2-h incubation at 37°C. The concentration of anti-PHF antibodies (Fig. 2a) necessary to achieve optimum sensitivity of the standard curve was determined experimentally at a fixed concentra-
tion of labeled antigen. Higher amounts of antibody bound to the solid phase increased the level of PHF antigen needed for competition to be detected. The concentration of PHF–HRP conjugate was also optimized (Fig. 2b). A high concentration of conjugate increased the background of the assay, and a low concentration led to a loss of sensitivity. The optimum concentrations were 2 μg/mL of anti-PHF antibody (Fig. 2a) and 1:2000 dilution of PHF–HRP conjugate (Fig. 2b). Various incubation times for the immunoreaction were also examined. Incubation for 2 h incubation at 37°C was optimal for immunoreaction.

**Standard Curve, Assay Range, and Detection Limit**

An example of the standard curve obtained for ELISA for PHF determination (as described in the Methods section) is shown in Fig. 3. The mean values of triplicates (±SD) were plotted against the known concentration of each PHF calibrator. The standard curve was linear in the range of PHF 0.03 to 1 U/mL when plotted on a log-linear basis. The limit of detection of PHF was defined as the smallest single value that can be distinguished from zero. A statistical estimation of the minimal detectable PHF concentration was calculated as 0.02 PHF U/mL (the concentration at the mean ± 2 SD from 20 determinations of the zero calibrator).

**Precision**

The precision of the ELISA was estimated with three different plasma pools of samples containing PHF. The intraassay precision was determined from the mean of triplicates measured 18 times in the same assay. The interassay precision was determined from the mean of the average of triplicates for 10 separate runs by three operators. The coefficient of variation for and intra- and interassay series ranged from 5.3 to 9.4% (n = 18) and 9.4 to 13.2% (n = 8), respectively.

**Accuracy and Linearity**

Human plasma samples with three known concentrations of PHF were used. Recovery levels were determined by comparing the expected concentration versus measured concentration and these levels ranged from 86.9 to 110.9%. Three human samples were diluted with a pool of normal human plasma and recoveries ranged from 84.1 to 108.6%.

**Correlation Between Bioassay and ELISA Results**

Plasma samples from 10 hypertensive and normotensive individuals were assayed by competitive ELISA, and by BP bioassay for determination of PHF. Linear regression of the data revealed a positive correlation between the value of PHF measured with the ELISA and the BP bioassay (Fig. 4). The correlation coefficient was 0.64 (n = 10). These data also indicate that the minimal level detected with the bioassay is approximately 0.6 U/mL.

**Correlation Between ELISA Results and Clinical Data**

Plasma samples from 120 hypertensive and normotensive individuals were assayed by competitive ELISA.
for determination of PHF. Data were plotted as mean arterial pressure versus PHF concentration (Fig. 5). Linear regression of the data revealed a positive correlation between BP and PHF concentration; correlation coefficient (r) was 0.40 (n = 120). The slope of the fitted line is equal to 49 ± 11 mm Hg (U/mL).

**DISCUSSION**

The study reported here was undertaken to develop a competitive ELISA method for the detection and quantitation of PHF in plasma, a potential marker for salt-sensitive hypertension. Our method for measurement of PHF in human samples involved simple and easy-to-prepare reagents, such as anti-PHF IgM antibodies immobilized on microtiter plates, and PHF labeled by HRP incubated simultaneously with each patient sample. Parathyroid hypertensive factor–horseradish peroxidase was allowed to bind to a limited amount of antibody in the presence of the antigen to be measured, and the amount of PHF to be measured was assessed from the decrease in the amount of PHF–HRP bound to the antibody. Direct conjugation of enzymes to antigen has greatly simplified the development and performance of our PHF immunoassay. Horseradish peroxidase was chosen as a stable and inexpensive enzyme label. Horseradish peroxidase has been well studied and successfully used as a label in immunoassays. However, the method of enzyme labeling of PHF was not reported before. The conjugation reaction between PHF and HRP involved a covalent linkage between carbohydrate residues of the enzyme and amino groups of PHF, and did not alter HRP activity. Competitive ELISA indicated that the binding site for the antigen was not damaged by the conjugation with enzyme.

Four oligoclonal cell lines that produced anti-PHF IgM antibodies were developed in the process of developing antibodies against rat PHF. We investigated the cross-reactivity of the oligoclonal antibodies raised against rat PHF with human PHF. The data showed that human PHF and rat PHF had the same ability to displace the enzyme-labeled rat PHF in competitive ELISA. It is evident that anti-PHF oligoclonal antibodies can recognize both rat PHF and human PHF. Our bioassay and cross-reactivity studies demonstrate high specificity of the oligoclonal antibodies. Specificity and selectivity of anti-PHF antibodies used in ELISA were tested three different ways: the first two were the tests of the components of media for cross-reactivity with antibodies and the third was study of cross-reactivity of vasoactive and human plasma circulating substances. All studies concluded that anti-PHF antibodies are specific and selective to rat and human PHF. A pool of purified antibodies was used for the ELISA development.

Anti-PHF antibodies have been used to develop a simple method of competitive ELISA to determine PHF concentration in human plasma. To validate the ELISA method, we compared the PHF concentration determined by ELISA and by the bioassay method. We found that the ELISA was positively correlated with the bioassay (correlation coefficient of 0.64), and the sensitivity of ELISA was 30-fold higher than the sensitivity of bioassay.

The potential of the ELISA was evaluated by estimation of PHF in plasma of hypertensive and normotensive patients. Data we observed are in agreement with previous studies demonstrating that although PHF was detected in both groups of patients, the PHF levels were higher in hypertensive patients. We observed a positive correlation between the level of PHF measured in human plasma by ELISA and mean arterial pressure in our study, but the correlation coefficient was not equal to unity. There are several possible reasons for this result. First, PHF levels may be elevated in some normotensive individuals before the onset of hypertension symptoms. Second, according to our hypothesis, elevated PHF may lead to expression of other diseases associated with abnormal calcium handling. Third, high BP has different causes, therefore in some cases high BP may not be associated with an elevated concentration of PHF. The salt-sensitive population of hypertensives, who previously have been reported to have PHF, make up about 40% of the hypertensive population. To evaluate the first possibility, it will be necessary to conduct a more extensive multidimensional analysis with at least two extra variables.
parameters involved, age and gender with subjects studied over a long time. The second issue will be clarified as more information is collected regarding the relationship between PHF and other pathologies associated with abnormal calcium handling. The third possibility will be clarified as larger populations of hypertensive patients are screened. Further clinical studies on the level of PHF in a larger population of hypertensive patients are now in progress and will be reported elsewhere.

The method described is simple, rapid, and suitable for adapting to test a large number of samples in research or clinical laboratories with standard equipment for enzyme immunoassay. Only 10 μL of plasma samples are needed for measurement of human PHF, which is very convenient, and consistent with the clinical laboratory practices where several determinations are usually performed on each patient sample. It is important to identify patients with excessive levels of PHF and determine those that are likely to benefit from therapies targeted at reducing or inhibiting PHF. This method of ELISA for detection and quantification of PHF in patient plasma samples will provide a clinical diagnostic kit for detection of PHF, and will furnish the basis for specific therapeutic regimens by blocking PHF production or actions to treat the cause of disease and alleviate the symptoms.

ACKNOWLEDGMENTS

We thank Teresa Labedz, Meili Zhang, and Yin-Qi Wu for excellent and skillful technical assistance.

REFERENCES


