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ORIGINAL ARTICLE

Mass Spectrometric and Immunologic Detection of Prolactin-Derived Vasoinhibin in Human Serum

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SUMMARY

Background: **Circulating levels of the antiangiogenic protein, vasoinhibin, derived from the proteolytic cleavage of prolactin (PRL), in prolactinoma are unknown, as is the molecular nature of its isoforms. Dimerization of recombinant vasoinhibin has been reported.**
Methods: **Vasoinhibin in a human serum sample was identified by using preparative electrophoresis with subsequent SDS-PAGE and Western blot analysis, as well as mass spectrometry (MS) and ELISA.**
Results: **MS identified a partial vasoinhibin sequence in a 14-kDa protein band from human serum, which eluted in the 28-kDa fraction from the preparative electrophoresis. Measurement of vasoinhibin levels by ELISA identified a concentration of 284 ng/mL at a PRL level of 9,850 ng/mL. Recombinant human vasoinhibin demonstrated dimerization and multimerization when analyzed directly by SDS-PAGE and Western blot analysis under reducing and non-reducing conditions, as well as after immunoprecipitation.**
Conclusions: **The vasoinhibin sequence was identified in a higher molecular weight fraction, corroborating experimental evidence showing the dimerization and aggregation of recombinant human vasoinhibin. This report is significant, regarding the higher risk of cardiovascular disease and mortality in male patients with hyperprolactinemia as well as emerging reports of linking PRL and vasoinhibin levels in patients with prolactinoma with left ventricular dysfunction and Takotsubo syndrome.**
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INTRODUCTION

The antiangiogenic protein, vasoinhibin, which is generated by the proteolytic cleavage of prolactin (PRL), is of interest in the context of various diseases, such as retinopathies [1-3], peripartum cardiomyopathy [4,5], preeclampsia [6-8], inflammatory arthritis [9,10], and cancer [11,12]. Vasoinhibin, previously designated as 16 kDa PRL or 16K PRL [13], is usually detected by immunoprecipitation followed by SDS-PAGE and Western blot analysis. However, this method has several dis-

advantages, including limited sensitivity, semi-quantitative information, and dependence on polyclonal antibodies with limited specificity. Recent studies have suggested the presence of vasoinhibin dimers that retain bioactivity [14,15]. PRL, which is the precursor molecule of vasoinhibin, exists as dimers, multimers, and immunoglobulin complexes, and PRL is aggregated in secretory granules [16,17]. Therefore, alternative techniques are required to detect vasoinhibin in human blood samples and to investigate aggregated forms of vasoinhibin. Excessive vasoinhibin levels have been reported to be causally linked with peripartum cardiomyopathy [5], and emerging case reports link circulating PRL and vasoinhibin levels in patients with prolactinoma with left ventricular dysfunction [18] and Takotsubo syndrome [19]. Given that males with prolactinoma and/or hyperprolactinemia have an increased risk of cardiovascular disease and cardiovascular mortality [20,21], the conversion of PRL to vasoinhibin, as well as the endogenous isoforms and levels of vasoinhibin, require exploration.

MATERIALS AND METHODS

Human blood sample

The patient in the present study was diagnosed with a prolactinoma several years before, and he received medical therapy. Due to persistent hyperprolactinemia and incomplete regression of the adenoma, the patient was referred to Nuremberg General Hospital for evaluation of neurosurgical treatment options. A blood sample from this patient was collected and centrifuged, and serum was recovered and stored at -80°C until further analysis. Routine laboratory analyses revealed a normal blood count and serum PRL levels of 9,850 ng/mL (Elecsys Prolactin II Assay, Roche Diagnostics GmbH, Mannheim, Cat. no. 07027737190). A polyethylene glycol (PEG) precipitation was performed to exclude macroprolactinemia. The patient did not have any cardiac diseases.

High-resolution continuous elution electrophoresis

The serum sample (200 µL) was separated by a preparative electrophoresis system as described previously [22]. Briefly, the sample was mixed with 200 µL of Laemmli buffer, denatured, and loaded on a PrepCell 491 device (Bio Rad, Cat. no. 170-2925) equipped with a 14% acrylamide separating gel and a 4% stacking gel. The PrepCell was placed into a chromatography apparatus at 4°C and electrophoresed at 150 VDC, with a maximum of 40 mA and 20 W. After 5 hours, the elution was started with a flow rate of 1 mL/minute. Consecutive 13 mL fractions were collected, which were loaded into 15 mL centrifugal filters (Vivaspin 15R, Sartorius) and centrifuged until the concentrate volume was < 200 µL.

SDS-PAGE, Western blot, immunoprecipitation, and MS analyses

The sample was loaded into a SDS-PAGE gel and electrophoresed for 40 minutes at 200 V. The proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (0.2 µm pore size, Cat. no. LC2002, Invitrogen) using the Trans-Blot Turbo Transfer System (Cat. no. 170-4155, Bio-Rad) at 25 V and 1.3 A for 5 minutes. The membrane was blocked with 35 mL of blotting grade blocker (Cat. no. 170-6404, Bio-Rad) for 45 minutes at room temperature (RT). After washing with tris-buffered saline with Tween (TBST), the membrane was incubated with anti-PRL polyclonal antibodies diluted in blocking buffer (1:3,500; AA47-76 N-term epitope, Cat. no. ABIN954307, Antibodies Online, no longer available) for 12 hours at RT. The membrane was washed with TBST, followed by incubation with a secondary antibody diluted in blocking buffer (1: 60,000; Cat. no. 111035144, peroxidase-conjugated goat anti-rabbit, Jackson Immunoresearch Laboratories Inc., Ely, UK, RRID: AB_2307391) for 3 hours at RT. After repeated washing, 4 mL of a development solution (SuperSignal West Dura Extended Duration Substrate, Cat. no. 34075, ThermoFisher Scientific) was added to the membrane for 5 minutes at RT. Chemiluminescence single- and multi-channel (chemiluminescence and fluorescence) images were acquired using the Chemi Doc MP Imaging System (Cat. no. 1708280, Bio-Rad). The molecular weights of the bands were determined in the fluorescence image by using Image Lab Software (version 5.2.1, build 11, Bio-Rad). NanoLC-ESI-MS/MS was performed by Proteome Factory (Berlin) on the indicated band as previously reported [23]. Standard reducing conditions were applied using Laemmli buffer with 5% β-mercaptoethanol for 5 minutes at 95°C, and prolonged denaturation was applied with the addition of 1 M dithiothreitol (DTT) for 5 hours at 95°C where indicated. For the immunoprecipitation, 25 µg of biotinylated vasoinhibin monoclonal antibody was incubated with 22.5 µg of recombinant vasoinhibin in 100 µL of phosphate-buffered saline (PBS) overnight (OVN), under rotation at RT. Streptavidin-coated magnetic beads (Dynabeads MyOne Streptavidin T1, Thermo Fisher, Cat. no. 65601) were used for the precipitation according to the manufacturer's instructions. The elution was performed with 2 x Laemmli buffer at 95°C, under shaking for 30 minutes. The beads were separated by magnetization, and the precipitate was recovered.

ELISA

PRL levels were determined using the Elecsys Prolactin II Assay (Roche Diagnostics GmbH, Mannheim, Cat. no. 07027737190), and vasoinhibin levels were measured by using a previously described ELISA [24]. Briefly, a microplate was coated with 4 µg/mL anti-vasoinhibin monoclonal antibody diluted in carbonate buffer (100 µL/well) OVN at 4°C. The plate was then washed three times with TBST, and 300 µL of a bovine

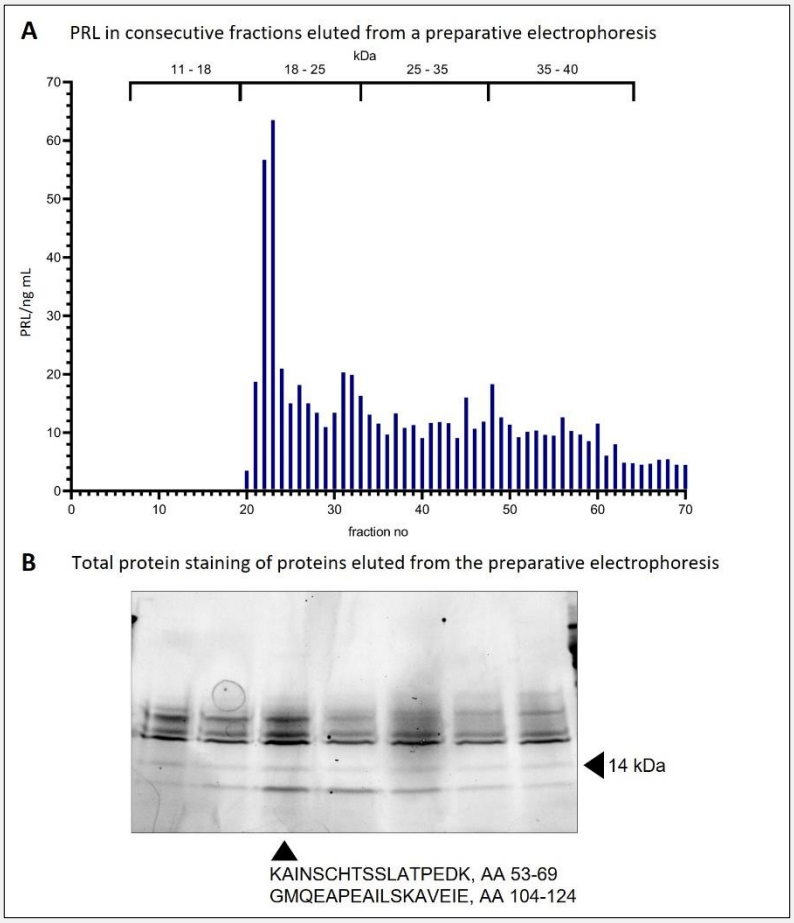

Figure 1. Preparative electrophoresis.
A) PRL content as measured by ELISA in consecutive fractions eluted from preparative electrophoresis. The majority of PRL eluted in the 18 - 25 kDa fractions, but PRL was also found in consecutive fractions containing proteins up to 40 kDa and higher. B) A 14 kDa band appeared in the total protein staining of the 28 kDa fraction. This band was subjected to MS analysis, which identified a vasoinhibin sequence.

serum albumin (BSA) blocking solution (Thermo Fisher, Cat. no. 37520) was added to each well for 1 hour at RT. After washing with TBST, 100 µL of sample (1:16) or standard preparation was added to the wells and incubated for 2 hours at RT. After washing the plate with TBST, a biotinylated anti-PRL polyclonal antibody (Assay Pro, Cat. no. 33110-05121, diluted 1:1,000 in blocking buffer) was added to the wells and incubated for 2 hours at RT. After washing the plate with TBST, 100 µL of streptavidin poly-horseradish peroxidase (HRP) (Thermo Fisher, Cat. no. 21140, 1:3,000 diluted in blocking buffer) was added to the wells and incubated for 1 hour at RT. After washing the plate with TBST, 100 µL of One Step Ultra TMB ELISA substrate solution (Thermo Fisher, Cat. no. 34028) was added to the wells and incubated for 15 minutes. Then, 100 µL of stop solution (Invitrogen, Cat. no. SS04) was added to the wells, and the absorbance was read at 450 nm.

RESULTS

The preparation of the serum sample using the high-resolution continuous elution electrophoresis demonstrated effective fractionation of the serum proteins. As expected, PRL eluted primarily in the fractions around 23 kDa, as shown by a PRL ELISA performed with the fraction content, but it also eluted in consecutive fractions up to 40 kDa and higher (Figure 1A). In addition, a 14 kDa protein band appeared in the SDS-PAGE of the fraction eluted at 28 kDa from the preparative electrophoresis (Figure 1B). Although the band was not im-

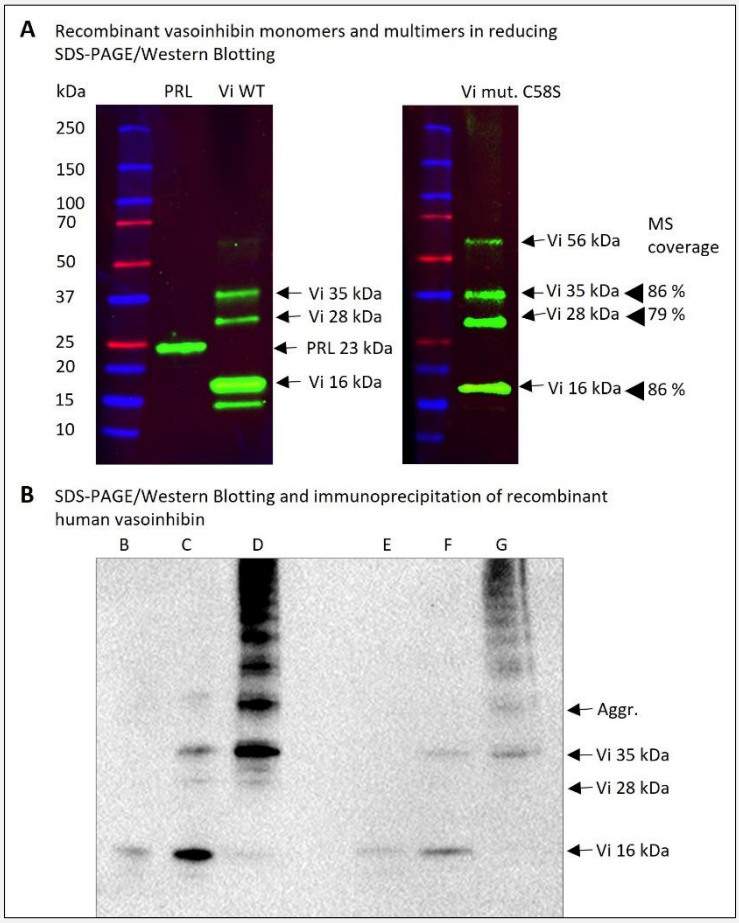

Figure 2. A) SDS-PAGE and Western blot analysis of WT and mutant (C58S) vasoinhibin identified monomers at 16 kDa and multimers at 28 kDa, 35 kDa, and 56 kDa; B) Prolonged denaturation and reducing conditions showed only a monomer; C) Standard reducing conditions showed a monomer and multimers; D) Non-reducing conditions showed only multimers; (E - G) Immunoprecipitation with a vasoinhibin monoclonal antibody prior to SDS-PAGE and Western blot analysis resulted in a similar pattern.

munoreactive in Western blot analysis (data not shown), it was subjected to MS analysis due to its unexpected appearance and molecular weight of 14 kDa, which is consistent with a vasoinhibin monomer. In total, 17 and 21 consecutive residues of the vasoinhibin sequence were detected by MS, demonstrating the presence of vasoinhibin (Figure 1B). Of note, C-terminal PRL sequences, which are not present in vasoinhibin, were not detected. As determined by ELISA, the vasoinhibin level in the serum sample from the patient was 284 ng/mL. Recombinant human vasoinhibin demonstrated dimerization and multimerization when analyzed directly by SDS-PAGE and Western blot analysis under standard reducing conditions (Figure 2A) as well as under prolonged reducing, standard-reducing, and non-reducing conditions (Figure 2B - D). A similar pattern was identified after immunoprecipitation with an anti-vasoinhibin monoclonal antibody (Figure 2E - G). The 16-kDa (vasoinhibin monomer), 28-kDa (vasoinhibin dimer), and 35-kDa bands were subjected to MS, which demonstrated 86% and 79% coverage of the total vasoinhibin sequence (Figure 2A). Some of the multimer bands may represent fractions of vasoinhibin containing the his-tag from the production procedure. Vasoinhibin dimers and multimers were observed in both the vasoinhibin wild-type (WT) and mutant (C58S) preparations (Figure 2A).

DISCUSSION

The present study eluted endogenous vasoinhibin from a preparative electrophoresis at a higher molecular weight (in this case: 28 kDa) than what would be expected for a free monomer (5 - 18 kDa). This finding

may be due to an endogenous vasoinhibin dimer present in the serum sample, which dissociated during the second application of SDS-PAGE reducing conditions, or due to a vasoinhibin molecule bound to an unknown protein from which it was separated, such as immunoglobulin G. In the present study, recombinant human vasoinhibin aggregated into dimers and multimers, suggesting that endogenous vasoinhibin may be present in dimers. Dimerization of endogenous vasoinhibin is also consistent with proposed models of vasoinhibin dimers [15] and bioactive recombinant human vasoinhibin dimers [14]. In contrast to the present results, a previous study has reported that C58S mutants cannot dimerize [25]. The presence of high-stability vasoinhibin dimers is consistent with high-stability dimers of growth hormone [26] and a PRL dimer resistant to β-mercaptoethanol [27].

The endogenous vasoinhibin, which was eluted at 28 kDa and found at 14 kDa in SDS-PAGE, may have eluted from cleaved PRL, in which the PRL molecule is cleaved but held together by a disulfide bridge. Cleaved PRL has been reported to migrate at a higher molecular weight due to the disruption of the amino acid chain with a less compact molecule structure and a concomitant increase in the Stokes radius [27,28]. However, the corresponding C-terminal fragment at approximately 8 kDa was not identified in SDS-PAGE. In the present study, self-aggregation of recombinant human vasoinhibin was observed. Therefore, these findings suggested that circulating vasoinhibin may be present in monomers, dimers, or self-aggregates of higher order.

The concentration of circulating vasoinhibin was 284 ng/mL at a concomitant PRL level of 9,850 ng/mL. It is unknown whether this is a representative ratio between vasoinhibin and PRL, indicating that quantitative vasoinhibin measurements in additional patients are required. Because the patient did not present any clinical signs of cardiac dysfunction, the vasoinhibin levels in the present case did not negatively impact the cardiovascular system. The hypothesis regarding the involvement of vasoinhibin in cardiac dysfunction proposes a two-hit model, in which high PRL levels coincide with another factor facilitating the conversion of PRL to vasoinhibin [29], which may have been absent in the present case. In general, however, vasoinhibin levels should be higher than normal preceding the onset of vasoinhibin-related cardiac damage, suggesting that measurement of circulating vasoinhibin levels in patients with prolactinoma is reasonable for risk stratification. Determining vasoinhibin levels in patients with various circulating PRL levels under physiological and pathological conditions will help to validate vasoinhibin as a clinically meaningful biomarker.

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Ethical Approval Statement:
The study was conducted in accordance with the ethical standards of the WMA Declaration of Helsinki and its ethical principles for medical research involving human subjects. Written informed consent has been obtained from the patient. An ethics committee approval was waived by the institutional review board of the Paracelsus Medical University Nuremberg.

Data Availability Statement:
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Interest:
The anti-vasoinhibin monoclonal antibody is subject of a pending European patent application (application number: EP23209080.3) owned by J.T. and T.B. The content of this manuscript has been presented in part at the 22nd European Congress of Endocrinology, e-ECE Online, September 5-9-2020, and is published in Endocrine Abstracts 2020, DOI: 10.1530/endoabs.70.AEP 543.

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