HUMAN PLACENTAL TISSUE CONTAINS A PLACENTAL LACTOGEN DERIVED VASOINHIBIN

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ABSTRACT

Hormonal factors impacting the vascular adaptions of the uteroplacental unit in non-complicated and complicated pregnancies are of interest. Here, 4 human placentas from women with and without preeclampsia were investigated for the presence of placental lactogen derived, antiangiogenic vasoinhibin. Western blotting and mass spectrometry of placental tissue revealed the presence of a 9 kDa placental lactogen derived vasoinhibin, the normal 22 kDa full-length placental lactogen, and a 28 kDa immunoreactive protein of undetermined nature. The sequence of the 9 kDa vasoinhibin includes the antiangiogenic determinant of vasoinhibin and could constitute a relevant factor in normal pregnancy and preeclampsia.

Keywords: vasoinhibin, placental lactogen, preeclampsia, placenta running vasoinhibin in human placenta

INTRODUCTION

Pregnancy is accompanied by vascular adaptations in the placenta, the uterus, and the systemic vascular physiology. The vasculature in healthy adult tissues is quiescent, the uterine vessels during the menstrual cycle and during pregnancy are an exception. The vascular adaptations are in part under hormonal control, and a dysbalance of pro- and antiangiogenic factors is contributing to pregnancy complications and disease.[1] One such hormonal factor is vasoinhibin, an antiangiogenic protein hormone which was reported to be elevated in the circulation, urine, and amniotic fluid of patients with preeclampsia and was suggested to contribute to endothelial cell dysfunction in PE[2–5].

Vasoinhibin is generated by the proteolytic cleavage of prolactin (PRL), but also, owing to the phylogenetic relationship between these hormones, by cleavage of placental lactogen (PL) and growth hormone[6–8]. The bioactive site of vasoinhibin resides in the loop connecting α-helices 1 and 2 and is defined by a short HGR-motif at positions 74-76 in PRL, and residues QK at positions 66-67 in PL[9]. Based on the fundamental importance of factors regulating angiogenesis in the normal placenta in the aetiopathology of preeclampsia, the present study aimed to investigate the presence of PL-derived vasoinhibin in the placenta.

METHODS

Human placenta samples. Four placentas were collected from pregnant women presenting at the Department of Gynecology and Obstetrics of the General Hospital Nuremberg either with (n = 2) or without (n=2) preeclampsia. Maternal and neonatal clinical information are summarized in Table 1. Written informed consent has been obtained from the study participants. In agreement with the recommendations of the American College of Obstetricians and Gynecologists Task Force on Hypertension in Pregnancy, the diagnosis of

preeclampsia was established when blood pressure was ≥ 140/90 mmHg and proteinuria was ≥ 300 mg/24 h [10].

SDS-PAGE and Western blotting. Placentas were thawed, washed with cold PBS, and cut into slices of 2-3 cm thickness. A tissue sample was obtained from the parenchyma of a full thickness sample between the chorionic plate and the basal plate, where the villous tree including syncytiotrophoblast cells, the source of PL, are located [11,12], placed into a 2 ml lysis tube (innuSpeed Lysis Tube E, Analytik Jena, Jena, Germany) and homogenized in cold PBS using the SpeedMill Plus (Analytik Jena) homogenizer. The homogenates were centrifuged for 10 minutes at 4°C and 10,000 rpm (Eppendorf centrifuge 5424R, rotor FA-45-24-11), and supernatants were recovered and frozen at -80°C until analysis. Total protein was determined with a Bradford total protein assay (Quick Start Bradford Protein Assay Kit, cat. no. 5000202, Bio-Rad Laboratories GmbH, Feldkirchen, Germany) according to the manufacturer's instruction. Samples containing 400 µg of total protein and 1 µl of the PL standard (purified human placental lactogen, cat. no. PHP157G, Bio-Rad) were mixed with 25 µl sample buffer (2x Laemmli buffer (cat. no. 1610737, Bio-Rad) containing 5% ß-mercaptoethanol (v/v) (cat. no. 1610710, Bio-Rad)), heated at 95°C for 10 minutes and centrifuged for 2.5 minutes at 4°C and 3,500 rpm (Eppendorf centrifuge 5424R, rotor FA-45-24-11). Electrophoresis was performed for 40 minutes at 200 V. The proteins were transferred onto a PVDF-membrane with 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, 1.3 A for 5 minutes. The membrane was blocked with 35 ml of 1x TBS with 1% Casein blocking buffer (cat. no. 1610782, Bio-Rad) for 45 minutes at RT. After washing with TBST, the membrane was incubated with anti-CSH1 polyclonal antibodies diluted in blocking buffer (1:3,500; AA42-70 N-term epitope, cat. no. ABIN1881233, antibodies online, RRID: AB_2905465; AA177-204 C-term epitope, cat. no. ABIN654883, antibodies online, RRID:

AB_2905466; full-length epitope, cat. no. PAB5015, Abnova, RRID: AB_1671888) 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 Immunoresarch Laboratories Inc., Ely, UK, RRID: AB_2307391) for 3 hours at RT. After repeated washing, 4 ml of a development solution (Clarity Max Western ECL Substrate, cat. no. 1705062, Bio-Rad, for Figure 1A and 1B & SuperSignal West Dura Extended Duration Substrate, cat. no. 34075, ThermoFisher Scientific for Figure 1C) was added to the membrane for 5 minutes at RT. Chemiluminescence images were taken using the Chemi Doc MP Imaging System (cat. no. 1708280, Bio-Rad). The molecular weights of the bands were determined in a fluorescence and chemiluminescence image of the membranes with the Image Lab Software (Version 5.2.1, build 11, Bio-Rad).

Mass spectrometry. Mass spectrometry (MS) was performed by Proteome Factory Berlin on the indicated bands as follows. For each proteolysis experiment, an SDS-PAGE band was subjected to in-gel proteolysis. The gel bands were prepared for enzymatic cleavage by 3 times swelling/shrinking in 100 mM triethylammonium bicarbonate buffer (TEAB) or 50 mM TEAB, 60% acetonitrile respectively. During consecutive swelling steps the bands were treated with tris (2-carboxyethyl) phosphine (5 mM final) and iodoacetamide (10 mM final) for reduction and alkylation of cysteine residues, respectively. Each step was carried out for 20 minutes at room temperature. After the last shrinking step, the gel slices were dried in open Eppendorf cups for 15 minutes. Subsequently the samples were digested separately by trypsin (200 ng). The Agilent 1100 nanoLC system was coupled to an Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Samples from proteolysis were applied to nanoLC-ESI-MS/MS after acidification. After trapping and desalting the peptides on enrichment column (Zorbax SB C18, 0.3 mm x 5 mm, Agilent) with 0.5% acetonitrile/0.5% formic acid

solution for five minutes, peptides were separated in a Zorbax 300 SB C18, 75 µm x 150 mm column (Agilent, Waldbronn, Germany) using an acetonitrile/0.1% formic acid gradient from 5% to 40% acetonitrile. MS overview spectra were automatically taken in Fourier Transform-mode according to manufacturer's instrument settings for nanoLC-ESI-MSMS analyses. Peptide fragmentation (CID) and detection operated in iontrap-mode. MS/MS data was searched against the SwissProt database or the human subset of UniProt using the Mascot search algorithm. Maximum mass deviation of ±5 ppm for precursor ions and ±0.6 Da for fragment ions were allowed. Carbamidomethylation of cysteines was set as a fixed modification, and oxidation of methionine and deamidation of asparagine and glutamine were considered as variable modifications. A maximum of two missed cleavage sites was assumed.

RESULTS AND DISCUSSION

Western blots probed with an anti-full-length PL polyclonal antibody, showed an immunoreactive band with the expected 22 kDa molecular mass of PL in placenta tissue lysates that comigrates with the PL standard (Figure 1A). The PL standard also contained immunoreactive proteins of 9 and 13 kDa which contain the N- and C-terminal ends of PL, as revealed by Western blots probed with an anti-N-terminal PL polyclonal antibody (Figure 1B) and an anti-C-terminal PL polyclonal antibody (Figure 1C), respectively. The 9 kDa-immunoreactive protein is also present in placental extracts and corresponds to vasoinhibin, as it contains the N-terminal region of PL defining vasoinhibin (Figure 1A & 1B)[7,9]. In addition, the anti-N-terminal PL antibody reacted with a 28 kDa protein in placental tissue lysates (Figure 1B). Because of vasoinhibin corresponds to the N-terminal end of PL and it is known to aggregate[13,14], it is tempting to speculate that the 28 kDa protein could correspond to a vasoinhibin trimer. However, the fact that the 28 kDa protein was not detected by anti-

full-length PL polyclonal antibodies (Figure 1A), its identity and significance remains unknown and requires further studies.

To further assess the vasoinhibin nature of the 9 kDa immunoreactive protein, its sequence was analysed by MS. The collection of peptides obtained after the enzymatic degradation of the 9 kDa protein covered part of the N-terminal sequence of PL with the putative end of the protein (cleavage site responsible for its generation) located at the triple leucine (LLL) motif in position 106-108. Cleavage at this site is consistent with the calculated molecular mass (9 kDa) of the vasoinhibin, as determined by Expasy[15], and leucine is a preferred residue at cleavage sites of enzymes capable of generating vasoinhibin, like cathepsin D[16–19]. Although few sequences detected by MS corresponded to regions C-terminal from the LLL motif (data not shown), their contribution was minor as indicated by their lack of detection in Western blots probed with an anti-C-terminal PL antibody (Figure 1C).

This short communication unveils a novel, 9 kDa, PL-derived vasoinhibin isoform in placental tissue. This vasoinhibin isoform contains the QK-motif which has recently been identified as the antiangiogenic motif of the vasoinhibin derived from PL[9]. The placental vasoinhibin may fulfil physiological functions in the control of blood vessel growth and function in the placenta but may also contribute to placental pathology in preeclampsia. Vasoinhibin produced in peripheral tissues can enter the circulation and cause elevation of systemic blood pressure, as shown in an in vivo mice model using hepatic overexpression of a PRL-derived vasoinhibin[20]. Along this line, it can be speculated that PL-derived vasoinhibin from the placenta can enter the maternal circulation and contribute to endothelial dysfunction and the development hypertension, as seen in preeclampsia. Vasoinhibin generation by cleavage of placental lactogen in the circulation may also occur but is rather unlikely[21]. Testing these hypotheses should include a quantitative evaluation of placental and circulating PL-derived vasoinhibin in a case-control study, as well as functional assays of the placental

vasoinhibin, the evaluation of enzymes responsible for its generation, and the analysis of PL mutations at vasoinhibin-generating cleavage sites[22].

ETHICS 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. The study protocol was reviewed and approved by the ethics committee of the Bavarian Chamber of Physicians, Munich, on August 29, 2019, file number 19033. The study is registered at the German Clinical Trials Register, DRKS-ID: DRKS00017719. Written informed consent has been obtained from the study participants.

AVAILABILITY OF SUPPORTING DATA

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

COMPETING INTERESTS

The authors declare that no conflicts of interest exist.

AUTHORS' CONTRIBUTIONS

Experimental analyses: HMH, LN, JT; data analysis: HMH, JT, LN, LL, CC, TB; patient recruitment and sample collection: NK, SE, LL, CB, CW; conception and design: JT, HMH, TB, CB, CW, TM, OM, PR; supervision: PR, CW, TB, JT; writing of the manuscript: JT, HMH, CC, TB; approval of the final manuscript: all authors.

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FIGURE 1 LEGEND

Western blot analysis of human placental lactogen (hPL) and human placental tissue lysates. Western blot probed with anti-full-length polyclonal PL antibodies (**A**), anti-N-terminal PL polyclonal antibodies (**B**), and anti-C-terminal PL polyclonal antibodies (**C**) showing immunoreactive protein bands (indicated by arrow-heads on left and right sides of blots) in the hPL standard (hPL Std) and in placental tissue extracts from preeclamptic and healthy subjects. The amino acid (AA) sequence and corresponding residue number of the peptides obtained after the enzymatic degradation of the 9 kDa protein from each placental tissue sample revealed by mass spectrometry is indicated in **A** (upward black arrow-heads).

TABLES

TABLE 1

Maternal and neonatal characteristics of the patients

Characteristic	Preeclampsia		Healthy	
Maternal Age (y)	29	29	29	32
Delivery Mode	C.-Section	C.-Section	C.-Section	C.-Section
Gestational Age (w+d)	39+5	36+2	39+0	40+2
Birth Weight (g)	2690	2780 + 2160	2820	3470
Gender	Female	Male + Male	Male	Male
APGAR 5 min.	10	10	9	10

Figure 1