

# Human Placental Tissue Contains A Placental Lactogen–Derived Vasoinhibin

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## Abstract

Hormonal factors affecting the vascular adaptations of the uteroplacental unit in noncomplicated and complicated pregnancies are of interest. Here, 4 human placentas from women with and without preeclampsia (PE) were investigated for the presence of placental lactogen (PL)-derived, antiangiogenic vasoinhibin. Western blotting and mass spectrometry of placental tissue revealed the presence of a 9-kDa PL-derived vasoinhibin, the normal 22-kDa full-length PL, 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 PE.

**Key Words:** vasoinhibin, placental lactogen, preeclampsia, placenta

**Abbreviations:** MS, mass spectrometry; PE, preeclampsia; PL, placental lactogen; PRL, prolactin; RT, room temperature.

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 an imbalance of proangiogenic and antiangiogenic factors contributes to pregnancy complications and disease [1]. One such hormonal factor is vasoinhibin, an antiangiogenic protein hormone that has been reported to be elevated in the circulation, urine, and amniotic fluid of patients with preeclampsia (PE) and has been 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  $\alpha$  helices 1 and 2 and is defined by a short HGR-motif at positions 74 to 76 in PRL, and residues QK at positions 66 to 67 in PL [9]. Based on the fundamental importance of factors regulating angiogenesis in the normal placenta in the etiopathology of PE, the present study aimed to investigate the presence of PL-derived vasoinhibin in the placenta.

## Materials and 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 ( $n = 2$ ) PE. Maternal and neonatal clinical information are summarized in Table 1. Written informed consent was 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 PE was established when blood pressure was greater than or equal to 140/90 mm Hg and proteinuria was greater than or equal to 300 mg/24 h [10].

### Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western Blotting

Placentas were thawed, washed with cold phosphate-buffered saline, and cut into slices of 2- to 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),

**Table 1.** Maternal and neonatal characteristics of the patients

Characteristic	Preeclampsia		Healthy	
Maternal age, y	29	29	29	32
Delivery mode	C	C	C	C
Gestational age, wk + d	39 + 5	36 + 2	39 + 0	40 + 2
Birth weight, g	2690	2780 + 2160	2820	3470
Sex	Female	Male + male	Male	Male
Apgar 5 min	10	10	9	10

Abbreviation: C, cesarean delivery.

and homogenized in cold phosphate-buffered saline 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, catalog No. 5000202, Bio-Rad Laboratories GmbH) according to the manufacturer's instructions. Samples containing 400 µg of total protein and 1 µL of the PL standard (purified human PL, catalog No. PHP157G, Bio-Rad) were mixed with 25-µL sample buffer (2× Laemmli buffer, catalog No. 1610737, Bio-Rad) containing 5% β-mercaptoethanol (v/v) (catalog No. 1610710, Bio-Rad), heated at 95 °C for 10 minutes, and centrifuged for 2.5 minutes at 4 °C and 3000 rpm (Eppendorf centrifuge 5424R, rotor FA-45-24-11). Electrophoresis was performed for 40 minutes at 200 V. The proteins were transferred onto a polyvinylidene fluoride-membrane with a 0.2-µm pore size (catalog No. LC2002, Invitrogen) using the Trans-Blot Turbo Transfer System (catalog No. 170-4155, Bio-Rad) at 25 V, 1.3 A for 5 minutes. The membrane was blocked with 35 mL of 1× Tris-buffered saline with 1% casein blocking buffer (catalog No. 1610782, Bio-Rad) for 45 minutes at room temperature (RT). After washing with TBST, the membrane was incubated with anti-CSH1 polyclonal antibodies diluted in blocking buffer (1:3500; AA42-70 N-term epitope, catalog No. ABIN1881233, antibodies online, RRID: AB\_2905465, [http://antibodyregistry.org/AB\\_2905465](http://antibodyregistry.org/AB_2905465); AA177-204 C-term epitope, catalog No. ABIN654883, antibodies online, RRID: AB\_2905466, [http://antibodyregistry.org/AB\\_2905466](http://antibodyregistry.org/AB_2905466); full-length epitope, catalog No. PAB5015, Abnova, RRID: AB\_1671888, [http://antibodyregistry.org/AB\\_1671888](http://antibodyregistry.org/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; catalog No. 111035144, peroxidase-conjugated goat anti-rabbit, Jackson ImmunoResearch Laboratories Inc, RRID: AB\_2307391, [http://antibodyregistry.org/AB\\_2307391](http://antibodyregistry.org/AB_2307391)) for 3 hours at RT. After repeated washing, 4 mL of a development solution (Clarity Max Western ECL Substrate, catalog No. 1705062, Bio-Rad, for Fig. 1A and 1B and SuperSignal West Dura Extended Duration Substrate, catalog No. 34075, Thermo Fisher Scientific for Fig. 1C) was added to the membrane for 5 minutes at RT. Chemiluminescence images were taken using the Chemi Doc MP Imaging System (catalog No. 1708280, Bio-Rad). The molecular weights of the bands were determined in a fluorescent and chemiluminescent image of the membranes with Image Lab Software (version 5.2.1, build 11, Bio-Rad).

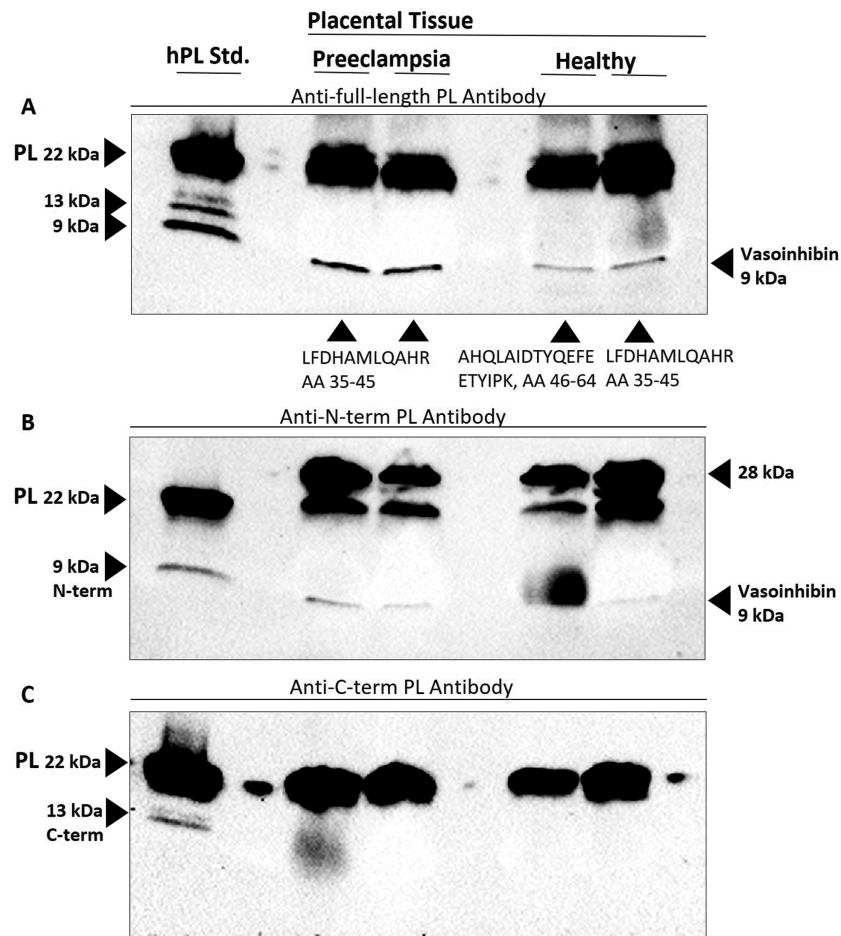
## Mass Spectrometry

Mass spectrometry (MS) was performed by Proteome Factory Berlin on the indicated bands as follows. For each proteolysis experiment, a sodium dodecyl sulfate–polyacrylamide gel electrophoresis band was subjected to in-gel proteolysis. The gel bands were prepared for enzymatic cleavage by swelling/shrinking 3 times 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 RT. 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 × 5 mm, Agilent) with 0.5% acetonitrile/0.5% formic acid solution for 5 minutes, peptides were separated in a Zorbax 300 SB C18, 75-µm × 150-mm column (Agilent) 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 the manufacturer's instrument settings for nanoLC-ESI-MSMS analyses. Peptide fragmentation (CID) and detection operated in iontrap mode. MS/MS data were 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 2 missed cleavage sites was assumed.

## Results

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 (see Fig. 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 (see Fig. 1B) and an anti-C-terminal PL polyclonal antibody (see Fig. 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 (see Fig. 1A and 1B) [7, 9]. In addition, the anti-N-terminal PL antibody reacted with a 28-kDa protein in placental tissue lysates (see Fig. 1B). Because vasoinhibin corresponds to the N-terminal end of PL and is known to aggregate [13, 14], it is tempting to speculate that the 28-kDa protein could correspond to a vasoinhibin trimer. However, because the 28-kDa protein was not detected by anti–full-length PL polyclonal antibodies (see Fig. 1A), its identity and significance remain unknown and require further studies.

To further assess the vasoinhibin nature of the 9-kDa immunoreactive protein, its sequence was analyzed by MS. The collection of peptides obtained after the enzymatic degradation



**Figure 1.** Western blot analysis of human placental lactogen (hPL) and human placental tissue lysates. Western blot probed with A, anti–full-length polyclonal PL antibodies; B, anti–N-terminal PL polyclonal antibodies; and C, anti–C-terminal PL polyclonal antibodies showing immunoreactive protein bands (indicated by arrowheads on left and right sides of blots) in the hPL standard (hPL Std) and in placental tissue extracts from preeclamptic and healthy individuals. 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 arrowheads).

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 to 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 a few sequences detected by MS corresponded to the region’s 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 (see Fig. 1C).

## Discussion

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 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 PE. Vasoinhibin produced in peripheral tissues can enter the circulation and cause elevation of systemic blood

pressure, as shown in an *in vivo* mouse 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 of hypertension, as seen in PE. Vasoinhibin generation by cleavage of PL 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].

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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 participants. The study protocol was reviewed and approved by the ethics committee of the Bavarian Chamber of Physicians, Munich, August 29, 2019, file number 19033.

## Author Contributions

Experimental analyses: H.M.H., L.N., and J.T.; data analysis: H.M.H., J.T., L.N., L.L., C.C., and T.B.; patient recruitment and sample collection: N.K., S.E., L.L., C.B., and C.W.; conception and design: J.T., H.M.H., T.B., C.B., C.W., T.M., O.M., and P.R.; supervision: P.R., C.W., T.B., and J.T.; writing of the manuscript: J.T., H.M.H., C.C., and T.B.; approval of the final manuscript: all authors.

## Disclosures

The authors have nothing to disclose.

## Clinical Trial Information

German Clinical Trials Registration number DRKS-ID: DRKS00017719 (registered December 23, 2019).

## Data Availability

Some or all data sets generated during and/or analyzed during the present study are not publicly available but are available from the corresponding author on reasonable request.

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