The American Journal of Pathology, Vol. 183, No. 5, November 2013

The American Journal of
PATHOLOGY
ajp.amjpathol.org

ANIMAL MODELS

Transthyretin Is Dysregulated in Preeclampsia, and Its Native Form Prevents the Onset of Disease in a Preclinical Mouse Model

Satyan S. Kalkunte,* Stefan Neubeck,† Wendy E. Norris,* Shi-Bin Cheng,* Stefan Kostadinov,* Dang Vu Hoang,‡ Aftab Ahmed,‡ Ferdinand von Eggeling,† Zahir Shaikh,‡ James Padbury,* Goran Berg,§ Anders Olofsson,¶ Udo R. Markert,† and Surendra Sharma*

From the Departments of Pediatrics and Pathology, Women and Infants Hospital—Warren Alpert Medical School of Brown University, Providence, Rhode Island; the Department of Obstetrics,† Institute of Human Genetics, University of Jena, Jena, Germany; the Department of Biomedical and Pharmaceutical Sciences,‡ College of Pharmacy, University of Rhode Island, Kingston, Rhode Island; the Division of Obstetrics and Gynecology,§ Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden; and the Department of Medical Chemistry,¶ Umea University, Umea, Sweden

Accepted for publication
July 22, 2013.

Address correspondence to Surendra Sharma, M.D., Ph.D., Department of Pediatrics, Women and Infants Hospital—Warren Alpert Medical School of Brown University, 101 Dudley St, Providence, RI 02905. E-mail: ssharma@wihri.org.

Preeclampsia is a major pregnancy complication with potential short- and long-term consequences for both mother and fetus. Understanding its pathogenesis and causative biomarkers is likely to yield insights for prediction and treatment. Herein, we provide evidence that transthyretin, a transporter of thyroxine and retinol, is aggregated in preeclampsia and is present at reduced levels in sera of preeclamptic women, as detected by proteomic screen. We demonstrate that transthyretin aggregates form deposits in preeclampsia placental tissue and cause apoptosis. By using *in vitro* approaches and a humanized mouse model, we provide evidence for a causal link between dysregulated transthyretin and preeclampsia. Native transthyretin inhibits all preeclampsia-like features in the humanized mouse model, including new-onset proteinuria, increased blood pressure, glomerular endotheliosis, and production of anti-angiogenic factors. Our findings suggest that a focus on transthyretin structure and function is a novel strategy to understand and combat preeclampsia. *(Am J Pathol 2013, 183: 1425—1436; http://dx.doi.org/10.1016/j.ajpath.2013.07.022)*

Preeclampsia occurs in 5% to 8% of pregnancies worldwide and is a major cause of fetal and maternal morbidity and mortality.[1–3] It is a heterogeneous disease with varied presentations from mild self-limited hypertension and proteinuria to severe forms with significant end-organ dysfunction and HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets).[3] Although the cause of preeclampsia and its appropriate treatment remain elusive, this syndrome has been proposed to reflect at least two stages of complications during pregnancy. These begin with preclinical manifestations at the maternal-fetal interface, followed by systemic clinical symptoms.[1,2] Hypertension, proteinuria, and edema, with a variable degree of fetal growth restriction, are the cardinal features of preeclampsia.[3] Because the placenta is the nutritional and immunological gateway to normal fetal development and pregnancy outcome, placenta-related events are believed to be central to the pathogenesis of this disease. Evidence exists for the release of disease-initiating molecules into maternal circulation that triggers the clinical symptoms.[1,4] Placental and systemic anomalies reflected by circulating placental debris, inflammation, impaired remodeling of spiral arteries, placental hypoxia/ischemia, excess production of anti-angiogenic factors [soluble fms-like tyrosine kinase-1 (sFlt-1)], and soluble endoglin (sEng), and angiotensin receptor autoantibodies have all emerged as contributors to the pathophysiological characteristics of preeclampsia.[2,4–14]

Supported by NIH grant P20RR018728 (J.P.), National Institute of Environmental Health Sciences grant P42ES013660 (S.S.), and Rhode Island Research Alliance Collaborative Research Award 2009-28 (S.S.).

Disclosures: A patent application (US patent 13/147,993) for compositions, formulations, and methods for treating preeclampsia-type disorders of pregnancy was filed in 2010.

http://dx.doi.org/10.1016/j.ajpath.2013.07.022

Preeclampsia has remained enigmatic because of lack of well-defined etiology and animal models. Although normal mice do not develop preeclampsia spontaneously, mouse models have been judged to be particularly useful to uterine diseases and pregnancy complications because many similarities in female reproduction and placentation have been identified between the two species.[15] Moreover, their tractable genetics provide an effective way to probe mechanisms more deeply than many other species.[15–17] We recently showed that sera from preeclamptic women could function as a source of novel causative factors that induced hypertension, proteinuria, and kidney pathological characteristics, as well as intrauterine growth restriction (IUGR), in *IL-10*$^{-/-}$ mice in a pregnancy-specific manner.[18] IL-10 functions as a potent vascular and anti-inflammatory cytokine and has been shown to be present at significantly reduced levels in preeclampsia placental tissue.[19,20] Preeclampsia serum (PES) was found to disrupt endovascular cross talk between trophoblasts and endothelial cells and to induce placental hypoxia and excess production of sFlt-1 and sEng,[18] soluble factors known to precipitate maternal symptoms.[21,22] These results from our serum-based humanized mouse model suggest that the pathophysiological characteristics of preeclampsia are more complex than previously thought and are likely to involve interactions and dysregulation of multiple factors. By using serum proteomic screening by surface-enhanced laser-desorption ionization-time-of-flight (SELDI-TOF), our results suggest that PES contains a reduced abundance of transthyretin, a plasma transport protein for the thyroid hormone, thyroxine, and retinol-binding protein.[23] More important, transthyretin has been widely studied for its role in amyloid diseases associated with protein misfolding and aggregation, resulting in deposits of toxic, fibrillar aggregates in specific organs.[24–26] Dysregulated or reduced transthyretin has also been implicated in Alzheimer disease, and overexpression of a wild-type human transthyretin transgene has been shown to ameliorate the disease in the transgenic murine model of human Alzheimer disease.[27,28] Transthyretin in its native form assumes a homotetrameric quaternary configuration (approximately 14 kDa per monomer). Post-translational modifications of the monomer result in detection of several isoforms.[29] Circulating transthyretin is also a validated marker of malnutrition and has a putative role in oocyte maturation and inflammation.[30–32] Although the presence of transthyretin during implantation in mice and in the placenta and trophoblasts in humans has been reported,[33,34] its functional role in normal pregnancy or adverse pregnancy outcomes has not been recognized. We hypothesize that transthyretin in preeclampsia is structurally and functionally dysregulated and contributes to the onset of this serious pregnancy complication. Herein, we present complementary *in vitro* and *in vivo* approaches, which show that endogenously altered transthyretin is a preeclampsia-causing agent and that native transthyretin has the ability to block the onset of preeclampsia-like features.

Materials and Methods

Human Subjects

Preeclampsia was defined by new-onset systolic and diastolic blood pressures of >140 and 90 mm Hg, respectively, and proteinuria (>300 mg of protein in a 24-hour urine collection or a random urine protein/creatinine ratio of >0.3) after 20 weeks' gestation. Severe preeclampsia was further defined by systolic and diastolic blood pressures of >160 and 110 mm Hg, respectively. For the purposes of this study, we followed the exclusion and inclusion criteria as described elsewhere.[18] Gestational age-matched healthy, normotensive, pregnant women (the normal group) were included as controls. Blood was obtained from pregnant women during 32 to 36 weeks of pregnancy with informed consent, under the approved protocols by the Institutional Review Boards of Women and Infants Hospital of Rhode Island (Providence), Linkoping University Hospital (Linkoping, Sweden), and University of Jena (Jena, Germany). Serum was separated and frozen as aliquots at −80°C until further use. Patient characteristics included for the study are provided in Supplemental Table S1.

Proteomics and Two-Dimensional Gel Electrophoresis

Two different groups of gestational age-matched serum samples were analyzed by SELDI-TOF. In the first group, normal pregnancy serum (NPS) ($n = 16$) or PES ($n = 53$) samples were analyzed by SELDI-TOF using the anionic exchange Q10 chip and analyzed with a ProteinChip Reader (series 4000; Bio-Rad, Munich, Germany). The bioinformatical analysis was performed using Ciphergen Express Client version 3.0 software. Arrays were exposed to 2200 nJ laser energy to detect proteins in the range of 2 to 20 kDa and to 3500 nJ for proteins in the range of 20 to 200 kDa at a pressure of <150 μPa. Calibration was performed externally through the use of a protein molecular weight standard kit (Bio-Rad, Waltham, MA). A similar SELDI-TOF analysis was performed using an additional second group of serum samples from normal pregnancy ($n = 7$) and preeclampsia ($n = 8$) from an independent cohort of subjects to validate the proteomics findings from the first group.

To identify the protein(s) that is altered in PES, we performed two-dimensional gel electrophoresis using both NPS and PES samples. Briefly, 50 μL of serum was precipitated in 50 μL of 20% trifluoroacetic acid and 50% acetonitrile for 30 minutes at −20°C. Protein pellets were washed twice in ice-cold 80% acetone and rehydrated for 48 hours in 125 μL of 2% immobilized pH gradient buffer (0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate, 8 urea, and 0.002% bromophenol blue). Concentrated protein solution was applied on a 7-cm nonlinear Immobiline DryStrip pH 3-11 (GE Healthcare, Boston, MA) through incubation for 14 hours under mineral oil using a Dry Strip reswelling tray. Isoelectric focusing was

performed using a Multiphor II Flatbed Electrophoresis System (Amersham, Piscataway, NJ). Subsequently, the 2% immobilized pH gradient strips were transferred into 10 mL of an equilibration solution consisting of 23% glycerol, 4.6 mol/L urea, 30 mmol/L Tris(hydroxymethyl)aminomethane (Tris), 0.03 mmol/L SDS with 65 mmol/L dithiothreitol (DTT) for 15 minutes, followed by incubation in a 10-mL equilibration solution (135 mmol/L iodoacetamide and Serva Blue G-250). The second dimension was performed using a NuPAGE 4% to 12% Bis-Tris Zomm Gel (Invitrogen, Carlsbad, CA) in a Novex Mini- Cell (Invitrogen). Spots in the appropriate molecular mass range were cut out from the gel, destained, dried, and trypsin digested. The peptide fingerprints obtained from trypsin-digested spots were analyzed using the ProFound database (The Rockefeller University, *http://prowl.rockefeller.edu/prowl-cgi/profound.exe*, last accessed August 20, 2011).

ELISA Data

Serum levels of human transthyretin were measured by using a transthyretin-specific enzyme-linked immunosorbent assay (ELISA) kit (ALPCO kit, Salem, NH), according to the manufacturer's protocol. Briefly, the serum samples were diluted 1:10,000 or 1:20,000 in wash buffer and incubated on a precoated plate for 1 hour at room temperature, washed, and incubated with peroxidase-labeled antibody for 1 hour. Color was developed using TMB substrate for 10 minutes, stop solution was added, and the plate was read at 450 nm. The serum levels of mouse sFlt-1 and sEng were measured by ELISA (R&D Systems, Minneapolis, MN), as per the manufacturer's protocol.

Immunodepletion of Transthyretin

Approximately 10 μL of protein A agarose beads was washed in coimmunoprecipitation buffer and incubated with either 5 μL anti-human transthyretin antibody (whole antiserum; Sigma-Aldrich, St. Louis, MO) or isotype IgG (Dako, Carpentaria, CA). After blocking with 3% milk and being washed, the beads were incubated with serum (1:50 dilution). The supernatants after centrifugations were used for SELDI-TOF analysis or for *in vivo* experiments. Immunodepletion of transthyretin was confirmed by using Western blot analysis.

Immunoprecipitation of Transthyretin

Transthyretin antibody-bound γ bind Plus Sepharose beads were incubated with 100 μL serum overnight and centrifuged, and the immunoprecipitates and supernatant were used for experiments. Western blot analysis using a reducing gel confirmed the depletion of transthyretin (TTR). For protein-protein interaction studies, individual serum samples were spiked with equal amounts of purified sEng protein before immunoprecipitation.

In Vivo Studies

All animal protocols were approved by the Lifespan Institutional Animal Care and Use Committee. *IL-10*$^{-/-}$ mice with a C57BL/6 background were housed and mated in a specific pathogen-free facility under the care of the Central Research Department of Rhode Island Hospital. All mating experiments were repeated at least three times, with at least four to seven mice per treatment. The day of vaginal plug appearance was designated gestational day (gd) 0. Animals received i.p. injections of severe preeclampsia serum (100 μL) per mouse or an equivalent volume of normal pregnancy serum as control on gd 10, as described.[18] For gain-of-function studies, PES was incubated with 20 or 100 μg transthyretin protein purified from human serum (AbD Serotec, Oxford, UK) at 37°C for 15 minutes before injection on gd 10. Similarly, immunoprecipitate or supernatant obtained from serum immunodepleted using transthyretin or isotype antibody (Dako, Glostrup, Denmark), as described later, was injected on gd 10.

Assessment of Proteinuria, Blood Pressure, Fetal Weight, Renal Pathological Characteristics, and ELISA

On gd 16, the animals were transferred to metabolic cages for 24-hour urine collection for assessing proteinuria. Total urinary albumin was measured using Albumin (mouse) ELISA kit (ALPCO Diagnostics, Germany). To normalize the albumin, urinary creatinine was measured using Metra Creatinine Kit (Quidel Corporation, San Diego, CA), according to the manufacturer's protocol. Proteinuria was represented as the ratio of urinary albumin/creatinine and was expressed as μg/mg. On gd17, blood pressure was recoding by the tail-cuff method, as described earlier.[14,18] The animals were then euthanized, and the uteroplacental units were surgically removed, the placenta was separated from the fetus and imaged, and fetal weights were recoded. Kidney tissue was harvested from gd17 mice, fixed in 10% buffered formalin, and stained with H&E and PAS for histopathological examination, as previously described.[14,18] Morphological changes were recorded using SPOT Advanced software (Diagnostic Instruments Inc., Sterling Heights, MI) at ×100 magnification (Nikon Eclipse 80i microscope, Tokyo, Japan).

In Vitro Three-Dimensional Tube Formation Assay

A three-dimensional tube formation assay has been used to mimic *in vitro* the endovascular interaction between trophoblasts and endothelial cells. Briefly, the first-trimester extravillous trophoblast cell line, HTR8,[35] and 2.5×10^4 human umbilical cord endothelial cells were co-cultured on Matrigel in the presence of NPS or PES, as described.[18,36] For gain- or loss-of-function studies, PES used in these experiments was either incubated with a different concentration of transthyretin (AbD Serotec) or immunodepleted

using transthyretin (Dako), or isotype antibody was used for endovascular activity.

Transthyretin Aggregation Studies

Phosphate-buffered serum from normal pregnancy or preeclampsia, 0.4 mg/mL human transthyretin, or 0.4 mg/mL bovine serum albumin (BSA) were diluted 1:1 with a buffer (100 mmol/L KCl and 1 mmol/L EDTA) to achieve the desired pH (sodium citrate for pH <3.2, sodium acetate for pH 3.5 to 5.3, and sodium phosphate buffer for pH >5.3).[37] The solutions were incubated at 37°C in Eppendorf tubes. At measurement, the tubes were vortex mixed for 5 seconds, and turbidity was measured at 330 nm in triplicate using a nanodrop. The percentage of aggregation was calculated, with the 100% value corresponding to the maximum signal of native TTR protein or PES. All measurements were made in triplicate in at least two independent experiments.

Western Blot Analysis

Serum samples or immunoprecipitates were separated on 12% native PAGE (aggregated TTR) or precast 4% to 15% SDS-PAGE gel (TTR monomers) under reducing conditions and electroblotted on a polyvinylidene difluoride membrane. The membranes were subsequently blocked with 5% milk in PBS with Tween 20 for 1 hour at room temperature. Blots were then probed with rabbit anti-human TTR antibody (1:500; Dako, Glostrup, Denmark) in 1% PBS with Tween 20 for 1 hour at room temperature. The bands were visualized using horseradish peroxidase–conjugated secondary antibodies (α Diagnostics, San Antonio, TX), followed by enhanced chemiluminescence (Pierce, Rockford, IL). The protein bands were recorded using a Konica SRX 101A developer (Tokyo, Japan). For protein-protein interaction studies, transthyretin immunoprecipitates from NPS or PES were probed under reducing conditions with biotinylated goat anti-human sEng or biotinylated mouse anti-human retinol binding protein (RBP)-4 (1:2000).

Far Western Analysis

Recombinant retinol binding protein-4, 3.75 μg (Fitzgerald Industries, Acton, MA), 0.75 μg endoglin (R&D Systems), 1.5 μg sFlt-1 (Fitzgerald Industries), and 6 μg vitronectin (Promega, Madison, WI) were separated on a 4% to 15% precast SDS gel (Bio-Rad) and electroblotted on a polyvinylidene difluoride membrane. The proteins were denatured and renatured on the membrane using a modified AC buffer [100 mmol/L NaCl, 20 mmol/L Tris (pH 7.6), 0.5 mmol/L EDTA, 10% glycerol, 0.1% Tween-20, 2% BSA, and 1 mmol/L DTT] by gradually reducing the guanidine-HCl concentration, as per the protocol described.[38] The membranes were subsequently blocked with 5% BSA in PBS with Tween 20 for 1 hour at room temperature and incubated with 10 μg TTR protein (AbD Serotec) in a modified protein binding buffer [100 mmol/L NaCl, 20 mmol/L Tris (pH 7.6), 0.5 mmol/L EDTA, 10% glycerol, 0.1% Tween-20, 2% BSA, and 1 mmol/L DTT] overnight at 4°C. Blots were then probed with 1:500 TTR antibody (Dako) in 1% PBS with Tween 20 containing BSA for 1 hour at room temperature. The bands were visualized using horseradish peroxidase–conjugated secondary antibodies (α Diagnostics), followed by enhanced chemiluminescence (Pierce). The protein bands were imaged (Gel Document System, GDS 8000; Bio-Rad Laboratories, Hercules, CA).

IHC and Thioflavin S Staining

Antigen retrieval of deparaffinized term human placental sections was performed using a citric acid–based unmasking solution (Vector, Burlingame, CA) and microwave oven technique. Sections from normal and preeclampsia specimens were blocked for 1 hour with 20% goat serum and incubated with primary transthyretin polyclonal rabbit antibody (1:400; Dako) in a humidified chamber for 1 hour in room air, followed by biotinylated secondary anti-rabbit antibody (VectaStain Elite Kit, Burlingame, CA) for 45 minutes. Immunolabeling was performed by a standard avidin-biotin technique, as per the manufacturer's protocol. Labeling was developed with 0.05% 3,3′-diaminobenzidine (Sigma), and slides were counterstained with hematoxylin (Fisher Scientific, Kalamazoo, MI). Human transthyretin in the mouse placenta was probed using transthyretin polyclonal rabbit antibody (1:200, overnight at 4°C; Dako) and detected using Cy-3–conjugated anti-rabbit IgG (1:100; Invitrogen). Sections were counterstained with DAPI. Thioflavin S staining was performed as described.[39] Briefly, deparaffinized sections were sequentially treated with 0.25% potassium permanganate solution for 5 minutes, bleaching solution (1% potassium metabisulfite and 1% oxalic acid) for 5 minutes, rinsed with water, and stained with 0.02% thioflavin S (Sigma) for 3 to 5 minutes. Finally, the sections were rinsed twice with 80% alcohol and water, dehydrated, and mounted with coverslips.

TUNEL Staining

TUNEL staining for apoptotic nuclei was performed using the ApopTag Peroxidase *in Situ* Apoptosis Detection Kit (Millipore, Billerica, MA), according to the manufacturer's instructions. Labeling reactions were performed for 60 minutes at 37°C in a humidified chamber. Color development was accomplished using 3,3′-diaminobenzidine for 5 minutes. Sections were counterstained with methyl green.

Statistical Analysis

SELDI-TOF raw data were analyzed using the software Ciphergen express client version 3.0 in two separate parts, in accordance with laser energy. First, peaks were automatically

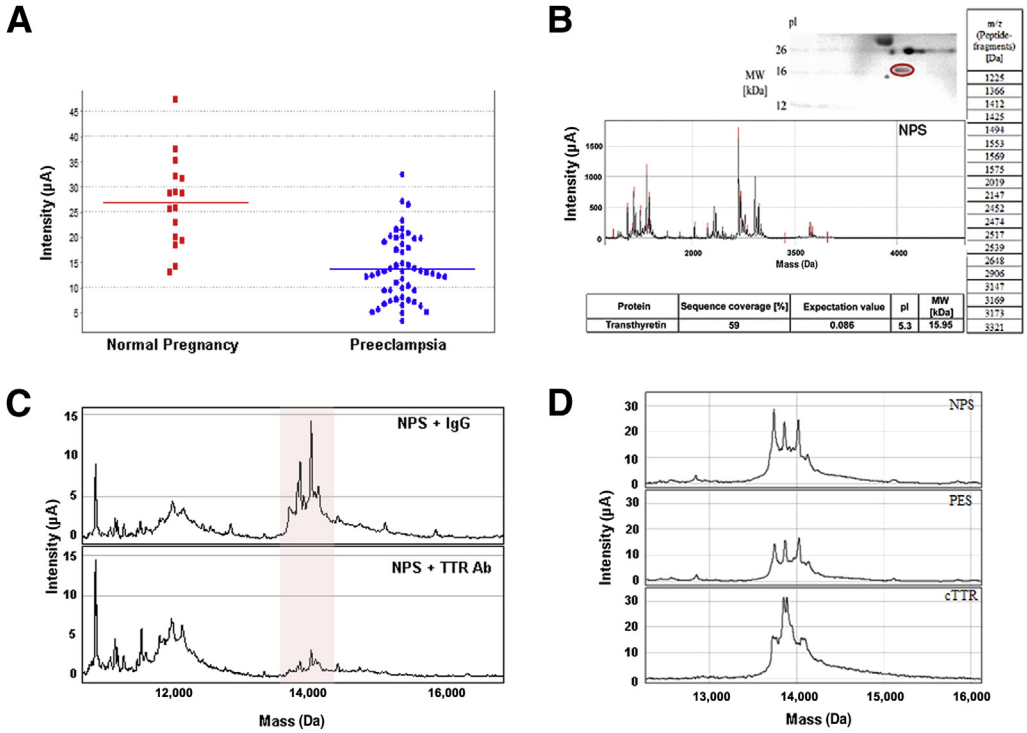

Figure 1 Surface enhanced laser desorption ionization-time-of-flight (SELDI-TOF) and biochemical analyses of transthyretin in preeclampsia serum. **A:** Preeclampsia serum (PES) ($n = 53$) and normal pregnancy serum (NPS) ($n = 16$) were analyzed by SELDI-TOF in the molecular mass range of 2 to 200 kDa. In the entire molecular weight (MW) range, the time of flight scatterplot shows most significant changes (reduction) in the median intensities of a 14-kDa protein group in PES when compared with NPS samples. **B:** Isoelectric point (pI) and molecular weight of a protein spot (approximately 14 kDa) separated by two-dimensional gel electrophoresis from a representative NPS sample are shown. The mass fragmentation pattern of the corresponding trypsin-digested spot and molecular mass (m/z) of the peptide fragments are also shown. The sequence coverage obtained by comparison with the ExPASy database (P02766; Swiss Institute of Bioinformatics, *http://www.expasy.org*, last accessed August 20, 2011) identified the protein spot (approximately 14 kDa) to be transthyretin. **C:** Normalized Protein Chip array profiles are shown of a representative NPS sample immunodepleted with a transthyretin-neutralizing antibody or an isotype-matched antibody. Successful immunodepletion of transthyretin (pink highlighted area) confirmed that the protein with a molecular mass of approximately 14 kDa was transthyretin. **D:** Comparison of transthyretin protein peaks (molecular mass of approximately 14,000 Da) in NPS, PES, and commercial human transthyretin (cTTR) is shown.

detected and manually adjusted, followed by linear normalization to compensate for differences in total protein concentration. Spectra showing normalization factors <0.5 or >3, which shows low quality of the spectra, were excluded from analysis. Subsequently, P values were calculated by using a two-sided t-test ($n = 2$ groups), a paired t-test ($n = 2$ paired groups), or single-factor/one-way analysis of variance ($n = \geq 3$ groups) for normally distributed data, and a U-test ($n = 2$ groups), a Wilcoxon t-test ($n = 2$ paired groups), or a Kruskal-Wallis H test ($n = \geq 3$ groups) for not normally distributed data. For normally distributed animal and ELISA data, two-tailed Student's t-tests were used in our analysis. $P < 0.05$ was considered significant.

Results

Proteomic Analysis Reveals Reduced Levels of Transthyretin in Preeclampsia Serum

We recently showed that PES, but not NPS, induces a full spectrum of preeclampsia-like features in pregnant *IL-10*$^{-/-}$ mice.[18] To identify the potential causative factor(s) in PES, we undertook a comparative proteomic analysis of PES and NPS. PES and control gestational age-matched NPS were analyzed by SELDI-TOF (2 to 200 kDa). A protein of approximately 14 kDa molecular mass was found to be consistently present at reduced levels in PES (Figure 1A). Because the number of NPS samples in initial analysis was comparatively fewer than PES, this observation was confirmed using an independent cohort of 15 serum samples (Supplemental Table S2), where all four isoforms of transthyretin were recorded at reduced intensities in PES. Two-dimensional gel electrophoresis and peptide mass fragmentation of the trypsin-digested spots identified an approximately 14 kDa protein cluster that was determined to be transthyretin by comparison with the ExPASy database (Figure 1B) (P02766; Swiss Institute of Bioinformatics, *http://www.expasy.org*, last accessed August 20, 2011). The identity of the protein was further confirmed by the loss of transthyretin signal after its immunodepletion from NPS (Figure 1C) and by direct comparison of the molecular mass with commercial human transthyretin (Figure 1D). Each peak in the group was assigned to be either native protein or cysteinylated, cysteinglycinylated, and glutathionylated isoforms (Supplemental Figure S1). The identities of these isoforms were based on their molecular mass, as described in

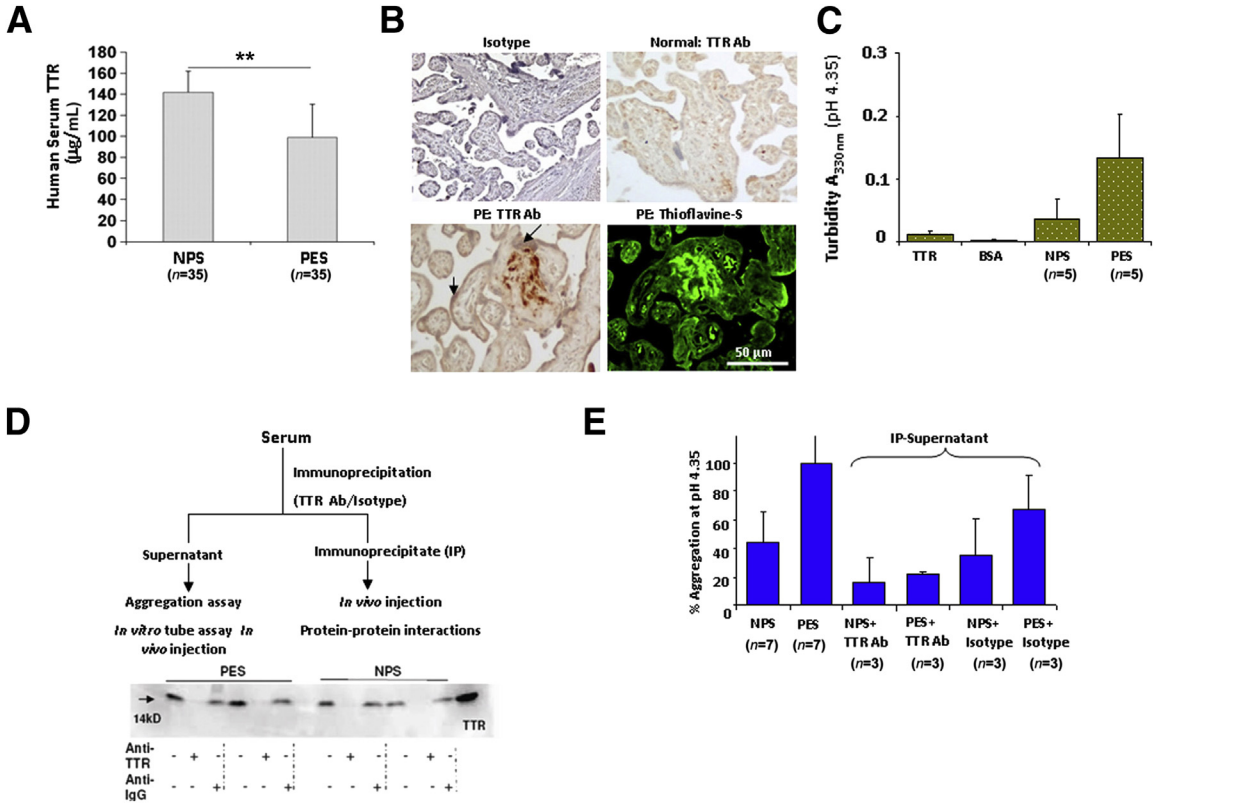

Figure 2 Transthyretin in preeclampsia is dysregulated and forms aggregates. **A**: Analysis of serum transthyretin in normal pregnancy serum (NPS) and preeclampsia serum (PES) by ELISA. Transthyretin is present at significantly reduced levels in PES ($P < 0.001$). **B**: Immunohistochemical (IHC) analysis using transthyretin-specific antibody (Ab) shows strong transthyretin staining in the extravillous domain of human placental section from preeclampsia, not normal pregnancy, in addition to staining in the trophoblast layer (**arrows**) lining the placental villi. Adjacent section of the same preeclampsia placenta shows intense fluorescence for amyloid-specific thioflavin S staining that overlaps with extracellular transthyretin-positive deposits. Additional placental tissue sections are shown in Supplemental Figure S2. **C**: Comparative analysis of aggregation of purified transthyretin, NPS, PES, and albumin indicated by turbidity measurements (330 nm) is shown as average values of multiple serum samples analyzed. All of the serum samples used were normalized to 0.4 mg/mL transthyretin, as determined by ELISA. PES showed a higher propensity to aggregate, as reflected by higher turbidity. On an average, eight different samples of NPS and PES were analyzed. **D**: Experimental flow chart for transthyretin IP and depletion is presented. SDS-PAGE immunoblotting shows the presence or absence of transthyretin monomer in the immunoprecipitate or supernatant of PES or NPS, respectively. **E**: Turbidity (330 nm) of supernatants obtained from transthyretin-depleted NPS or PES samples was measured and expressed as percentage aggregation. Antibody-mediated transthyretin depletion abolished aggregation associated with PES. $^{**}P < 0.01$.

the literature.[40] As evident from the data, the relative abundance of native transthyretin and all of its isoforms was reduced in PES compared with NPS (Supplemental Figure S1). In contrast, the level of albumin was unchanged between PES and NPS samples.

Evidence for the Presence of Transthyretin Protein Aggregates in Preeclampsia

We next examined whether the SELDI-TOF data could be confirmed by ELISA that recognizes all forms of transthyretin. As shown in Figure 2A, the SELDI-TOF data were confirmed by ELISA and a significant reduction ($P < 0.001$) was observed in transthyretin levels between NPS ($n = 35$) and PES ($n = 35$). However, our initial Western blot analysis under reducing conditions provided contrasting data in that when an equal amount of total serum protein was probed for transthyretin between NPS and PES, it resulted in a similar band intensity profile. Because the protein chips used in SELDI-TOF are derivatized to interact and sequester proteins according to their surface interaction potential,[41] it is possible that structurally altered transthyretin in PES showed reduced binding efficiency. Similarly, antibodies used in the ELISA kit may show poor affinity for altered transthyretin. In amyloid diseases, aggregation of transthyretin leads to toxic tissue deposits.[42–45] This prompted us to probe possible aggregation properties of transthyretin in preeclampsia. We first examined human placental tissue from normal pregnancy and preeclampsia deliveries for transthyretin immunostaining and deposits. When probed with a transthyretin-specific antibody, preeclampsia placental tissue, in general, showed intense transthyretin staining that was particularly apparent in the extravillous trophoblast domain regions, as assessed by perinatal pathologists (Figure 2B and Supplemental Figure S2A). Furthermore, staining with thioflavin S, a specific fluorescent dye that detects extracellular deposits,[39] displayed an intense signal that colocalizes with transthyretin-immunoreactive regions from an adjacent placental section from preeclampsia, suggestive of the amyloidal nature of transthyretin deposits (Figure 2B).

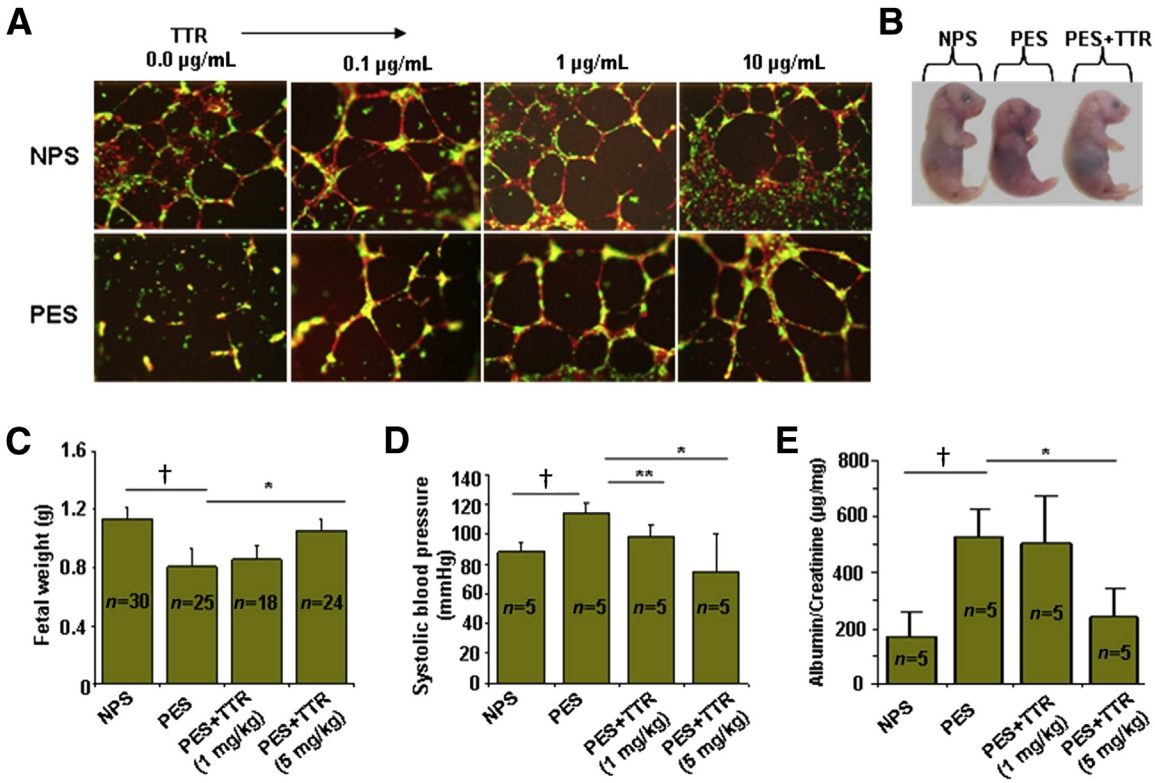

Figure 3 Exogenous transthyretin inhibits preeclampsia-associated features *in vitro* and *in vivo*. **A:** Serum-induced and transthyretin-modified endovascular interaction between endothelial cells (human umbilical cord endothelial cells, cell tracker red) and first-trimester trophoblasts (HTR8, cell tracker green) was analyzed by three-dimensional tube formation on Matrigel.[18] Native transthyretin rescued tube formation disrupted by preeclampsia serum (PES). **B:** Reversal of PES-induced intrauterine growth restriction (IUGR) by exogenous transthyretin in *IL-10*$^{-/-}$ mice. A representative image of gd 17 fetus is shown. A total of five animals were used in each condition. **C:** The average fetal weight from *IL-10*$^{-/-}$ mice receiving different treatments was evaluated. **D:** Systolic blood pressure in pregnant mice on gd 17 was evaluated in response to different treatments. **E:** Proteinuria in pregnant mice was assessed on gd 17 in response to various treatments. The results are expressed as a ratio of albumin/creatinine excretion (μg/mg). All values represent means ± SD of at least 5 to 30 animals per group, depending on the experiment. $^{*}P < 0.01$, $^{**}P < 0.05$ between PES and transthyretin treatment groups; $^{\dagger}P < 0.05$ between NPS and PES groups.

Thioflavin S staining from additional placental tissue sections is shown in Supplemental Figure S2B. These findings support our contention that aggregated transthyretin forms extracellular amyloid deposits in the placenta, which may cause toxic effects and disrupt placental function.

Sera from Preeclampsia Patients Exhibit Increased Propensity for Transthyretin-Specific Protein Aggregation

In solution, transthyretin and its mutant variants are known to undergo pH-dependent monomerization and aggregation.[37,42,43] Transthyretin aggregation in NPS and PES was monitored by measuring solution turbidity at 330 nm, a measurement previously considered valid for quantification of transthyretin misfolding and aggregation.[37,42] Consistent with previous reports,[42] purified (native) transthyretin exhibited weak aggregated characteristics (turbidity) between pH 3.5 and 4.5, with peak aggregation at 4.35, whereas albumin exhibited no turbidity over this pH range (Supplemental Figure S2C). Under identical conditions, we then assessed NPS and PES for intrinsic and transthyretin-mediated aggregation. As observed in Figure 2C, PES exhibited higher aggregation propensity than NPS. To assess transthyretin-mediated aggregation propensity of PES, we performed experiments by immunodepleting transthyretin, as shown in the scheme (Figure 2D). The depletion of transthyretin in serum samples was confirmed by immunoblotting for the protein. Transthyretin antibody, not isotype control IgG, depleted the protein (Figure 2D). In protein aggregation studies at pH 4.35 (Figure 2E), depletion of transthyretin in PES significantly abolished aggregation, confirming its specific contribution in this process. Isotype control antibody had a minimal effect on the aggregation profile of NPS or PES.

Exogenous Transthyretin Rescues PES-Induced Disruption of *in Vitro* Three-Dimensional Tube Formation and *in Vivo* Disease Features

We have previously shown that PES disrupts endovascular cross talk between endothelial cells and trophoblasts,[18,36] a process required for spiral artery remodeling. We next evaluated the effect of native transthyretin on PES-mediated disruption of three-dimensional tube formation between endothelial cells and trophoblasts. Replenishment of PES with exogenous transthyretin restored capillary tube formation in

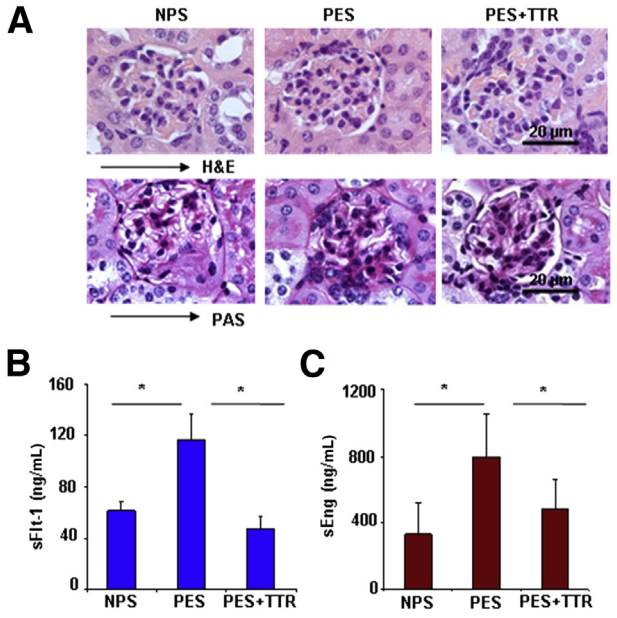

Figure 4 Exogenous transthyretin rescues glomerular integrity and inhibits production of sFlt-1 and sEng in preeclamptic *IL-10*$^{-/-}$ mice. **A:** Histopathological analysis of H&E- or periodic acid schiff (PAS)-stained renal tissues from NPS^{-}, PES^{-}, or PES^{+} transthyretin-treated (5 mg/kg) pregnant *IL-10*$^{-/-}$ mice are shown. Original magnification, ×100. The H&E stain shows the normalization of PES-induced capillary occlusion, enlarged glomeruli, and swollen endothelial cells by transthyretin treatment that is comparable to NPS-treated control mice. PAS-based staining indicates that transthyretin treatment reverses PES-induced inflammation of capillary endothelial cells (endotheliosis). These pathological changes are absent in the NPS-treated mice. A representative image from staining of at least three animals per group is shown. **B:** Serum levels of mouse sFlt-1 from pregnant *IL-10*$^{-/-}$ mice obtained on gd 17 from different treatment groups are shown. Treatment with 5 mg/kg transthyretin reverses the PES-induced excess production of sFlt-1 in *IL-10*$^{-/-}$ mice. **C:** Serum levels of mouse sEng from pregnant *IL-10*$^{-/-}$ mice obtained on gd 17 from different treatment groups are shown. Treatment with transthyretin reverses the preeclampsia serum (PES)-induced excess production of sEng to levels comparable to normal pregnancy serum (NPS) treatment control group. All values are expressed as means ± SD obtained from at least five animals per treatment group. *$P < 0.05$ represents the significance between different treatment groups.

a dose-dependent manner, whereas PES disrupted this interaction (Figure 3A). We next examined whether exogenous human transthyretin could inhibit the PES-induced onset of preeclampsia pathological characteristics in *IL-10*$^{-/-}$ mice. Co-administration of a single dose of 5 mg/kg transthyretin and PES on gd10 prevented the PES-induced IUGR (Figure 3, B and C), reversed PES-induced hypertension (Figure 3D), and restored proteinuria (Figure 3E) to normal values. These observations clearly suggest that exogenous (normal) transthyretin has the potential to counter the effects of altered transthyretin present in PES.

We have demonstrated that PES causes renal pathological characteristics and induces production of anti-angiogenic factors, sFlt-1 and sEng.[18] Next, we evaluated whether exogenous transthyretin inhibited production of sFlt-1 and sEng and protected against glomerular endotheliosis in response to PES. As shown in Figure 4A, human transthyretin prevented the PES-induced glomerular endotheliosis in pregnant *IL-10*$^{-/-}$ mice, as suggested by H&E and PAS staining. We assessed mouse serum levels of sFlt-1 and sEng in response to treatment with NPS, PES, or PES spiked with transthyretin. As shown in Figure 4, B and C, exogenous human transthyretin significantly inhibited the PES-induced increase of circulating sFlt-1 and sEng to levels comparable to the NPS-treated group, suggesting that dysregulated transthyretin affects overall preeclampsia pathological characteristics and that native protein can blunt all these contributing factors.

Evidence that Transthyretin Immunoprecipitated from PES Induces Disease Features in Pregnant *IL-10*$^{-/-}$ Mice

To obtain direct evidence that dysregulated transthyretin from PES contributes to preeclampsia-like features in *IL-10*$^{-/-}$ mice, we evaluated the effect of transthyretin immunoprecipitated from NPS (NPS-transthyretin) or PES (PES-transthyretin), as shown in the flow chart (Figure 2D). The presence of approximately equal amounts of transthyretin in immunoprecipitates was confirmed by using Western blot analysis (Figure 2D). The administration of PES-transthyretin on gd 10 resulted in IUGR, as indicated by reduced fetal weight (Figure 5A), elevated hypertension (Figure 5B) and proteinuria (Figure 5C), and induced production of sFlt-1 (Figure 5D). Furthermore, as shown in Figure 5E, administration of PES-transthyretin resulted in signature features of glomerular endotheliosis, including inflammation of endothelium and swelling, as indicated by H&E and PAS staining. In contrast, NPS-transthyretin did not cause significant changes in all of the parameters monitored. These findings provide direct evidence that dysregulated transthyretin contributes to preeclampsia-like features in pregnant *IL-10*$^{-/-}$ mice.

Does PES transthyretin form toxic deposits in the mouse placenta? We evaluated the placenta from NPS^{-} and PES-transthyretin−treated *IL-10*$^{-/-}$ mice for human transthyretin. As shown in Supplemental Figure S3, immunoprecipitated transthyretin from both NPS and PES reached the placenta (Supplemental Figure S3, C and D), whereas saline control (Supplemental Figure S3A) or isotype-matched IgG-mediated immunoprecipitates from PES showed no evidence of human transthyretin (Supplemental Figure S3B). More important, transthyretin antibody-mediated immunoprecipitates from PES formed heavy deposits (Supplemental Figure S3D) and caused apoptosis (Supplemental Figure S3F) in the placental region, as assessed by TUNEL staining. In contrast, although NPS-transthyretin reached the placenta (Supplemental Figure S3C), it did not cause cell death (Supplemental Figure S3E). These results suggest that PES-transthyretin elicits toxic effects in the mouse placenta.

We also screened flow through from transthyretin-immunodepleted serum samples for their ability to cause preeclampsia-like features in *IL-10*$^{-/-}$ mice. The basic premise was that depletion of dysregulated transthyretin from PES

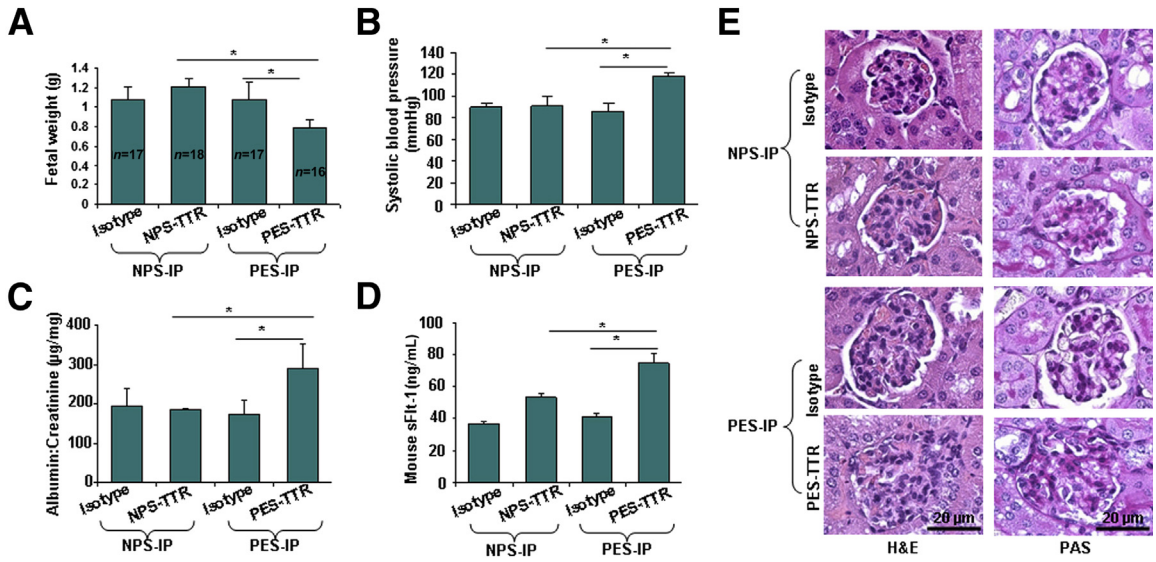

Figure 5 Immunoprecipitated (IP) transthyretin from preeclampsia serum (PES) induces disease features in *IL-10*$^{-/-}$ mice. Pregnant mice were injected (i.p.) on gd 10 with transthyretin immunoprecipitate obtained from 100 µL normal pregnancy serum (NPS) or PES each, as described in *Materials and Methods*. **A**: The average weight of fetal units derived from pregnant mice on gd 17 receiving different treatments is shown. **B**: The systolic blood pressure of pregnant mice on gd 17 was evaluated in response to different treatments. **C**: Proteinuria (albumin/creatinine ratio, in µg/mg) is shown in response to various treatments. PES-transthyretin caused significant proteinuria compared with NPS-transthyretin. Isotype-matched IgG-mediated immunoprecipitation from PES resulted in values similar to NPS, confirming the validity of transthyretin-specific antibody. **D**: Transthyretin immunoprecipitate from PES induced excess production of mouse sFlt-1 in pregnant *IL-10*$^{-/-}$ mice. **E**: Histological analysis using H&E or periodic acid schiff (PAS) staining of renal tissue from pregnant *IL-10*$^{-/-}$ mice in response to transthyretin immunoprecipitates from NPS and PES is shown. A representative image from staining of at least three animals per group is presented. All values represent means ± SD of at least 3 to 18 animals per group. *$P < 0.05$ between treatment groups indicated.

should result in normal pregnancy, with no increased blood pressure, new-onset proteinuria, and production of factors, such as sEng. As shown in Figure 6, transthyretin-depleted PES failed to induce blood pressure increase, proteinuria, and production of sEng. PES treated with isotype-matched IgG still maintained pathological characteristics compared with NPS.

Transthyretin Functions as a Binding Partner for Anti-Angiogenic Protein sEng

Because transthyretin functions as a binding partner for thyroxine, RBP-4, and β-amyloid protein,[23,28] we hypothesized that native transthyretin could function as a scavenger protein for preeclampsia-associated factors. Initially, we searched the SELDI-TOF mass spectrometry profile for additional proteins that disappeared with transthyretin depletion in NPS. Along with several proteins, the analysis suggested that a protein of approximately 66.5 kDa could be a part of the complex (Supplemental Figure S4 and Supplemental Table S3). Because sEng is 67 kDa molecular weight and its levels in preeclampsia are altered,[7] we pursued the hypothesis that transthyretin interacts with this protein. We confirmed this interaction using the Far Western technique, which depends on binding of a prey protein to bait proteins.[38] Native transthyretin was used as the bait protein and purified sFlt-1, sEng, and RBP-4 as prey proteins. RBP-4 was included as a positive

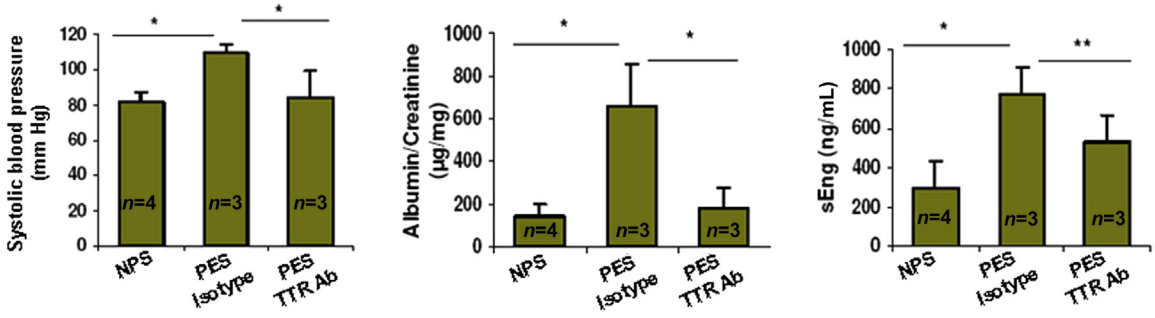

Figure 6 Transthyretin-immunodepleted preeclampsia serum (PES) fails to induce preeclampsia-like features. Transthyretin-depleted PES (100 µL) was injected on gd10 in *IL-10*$^{-/-}$ mice, and its effects on fetal size, blood pressure, proteinuria, and serum levels of sEng were compared with injection of an equal volume of normal pregnancy serum (NPS) or isotype-matched IgG-depleted PES. Immunodepletion of transthyretin in PES fails to cause significant changes in blood pressure, proteinuria, or sEng levels, suggesting that dysregulated transthyretin in PES is responsible for induction of preeclampsia pathological characteristics in *IL-10*$^{-/-}$ mice. All values represent means ± SD of at least three to four animals per group. *$P < 0.05$ between treatment groups tested.

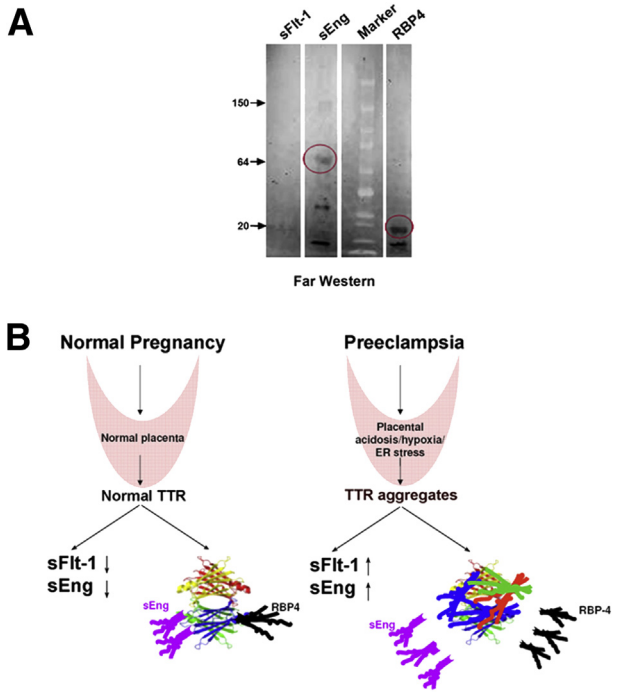

Figure 7 Transthyretin binds to soluble endoglin and its possible mode of action. **A:** Far Western blot showing the binding of transthyretin with sEng and RBP-4 (red circle) and lack of binding to sFlt-1. Purified sFlt-1, sEng, and RBP-4 were used as prey proteins, and purified human transthyretin was used as probing bait protein. A representative immunoblot from multiple independent experiments is shown. **B:** Proposed model showing the role of upstream factors, such as hypoxia, acidosis, and stress, in triggering aggregation of transthyretin that induces production of anti-angiogenic factors and impairs the binding and scavenging of preeclampsia-causing soluble factors.

control. As shown in Figure 7A, transthyretin binds to sEng and RBP4. We did not observe significant binding of transthyretin to sFlt-1 under these experimental conditions.

Discussion

In this study, we report novel findings associated with dysregulated transthyretin in preeclampsia. By using PES or transthyretin immunoprecipitated from PES, we demonstrate that administration of aberrant transthyretin to pregnant *IL-10*$^{-/-}$ mice induces a full spectrum of preeclampsia-like features. The relevance of using *IL-10*$^{-/-}$ mice lies in the fact that IL-10 is a potent anti-inflammatory and vascular cytokine.[20,46,47] IL-10 is a potent inducer of heme oxygenase-1, which has been shown to be critical for placentation and fetal development.[48,49] IL-10 and heme oxygenase-1 exert protective effects against oxidative stress and induce negative regulation of sFlt-1 and sEng.[50–52] Thus, the absence of IL-10 may predispose to inflammatory activities and poor vascularization at the maternal-fetal interface. In this regard, it is tempting to speculate that aggregated and toxic transthyretin may cause preeclampsia-like pathological characteristics in *IL-10*$^{-/-}$ mice, even at low doses.

The extraordinary observation of aberrant transthyretin in preeclampsia is further supported by immunodepletion of transthyretin from PES, which ameliorates most of the disease features. Measurement of serum transthyretin from normal pregnancy and preeclampsia by SELDI-TOF suggested that this protein was significantly reduced in PES compared with NPS. On the other hand, we observed transthyretin deposits in preeclampsia placental tissue and in the mouse placenta from animals administered with human transthyretin from PES. This prompted us to focus on the aggregation properties of endogenous transthyretin, a feature widely associated with unscheduled misfolding and aggregation in amyloid diseases and possibly in Alzheimer disease.[24–26,44] By using several complementary approaches, we provide evidence for aggregation of transthyretin protein in PES and in placental tissue from preeclampsia pregnancies. NPS and PES differed in their susceptibility to pH-dependent aggregation that was abolished after immunodepletion of transthyretin. Thus, we propose that preeclampsia is a disease of protein misfolding and aggregation. A similar scenario has been proposed for human serine protease inhibitor A1,[53] although it is not yet clear how this protease inhibitor is involved in preeclampsia. Serine protease inhibitor A1 has been shown to undergo fragmentation, misfolding, and aggregation in response to oxidative stress.[54]

Transthyretin is protective in a murine model of Alzheimer disease because of its ability to sequester β-amyloid peptides in a chaperone-like manner.[27,28] Our gain-of-function studies support a similar protective role of exogenous transthyretin in preeclampsia. The rescue of maternal symptoms in mice was associated with reduction in sFlt-1 and sEng. The role of native transthyretin in inhibiting production of anti-angiogenic factors is noteworthy because these factors are thought to be key regulators of local and systemic manifestations of preeclampsia.[21,22] On the other hand, immunoprecipitated transthyretin from PES induces production of these factors in pregnant *IL-10*$^{-/-}$ mice, implying a direct role of dysregulated protein in programming the events that lead to the onset of preeclampsia pathological characteristics. We provide evidence that dysregulated transthyretin reaches the mouse placenta, forms deposits, and induces placental apoptosis.

Our findings raise questions about the mechanisms and microenvironment that lead to transthyretin misfolding and aggregation in preeclampsia. Several factors, such as mutations in transthyretin, local change in pH at membranes, and oxidative stress, are likely to contribute to destabilization of transthyretin, causing tetramer dissociation, a rate-limiting step in transthyretin amyloidogenesis.[25,42] However, both natural transthyretin and mutant variants form aggregates and amyloid fibrils.[24,25,45] How may this happen at the maternal-fetal interface during pregnancy, even without mutations? Hypoxia is thought to be associated with the induction of endoplasmic reticulum (ER) stress and, possibly, preeclampsia pathological characteristics.[55,56] The ER is a hub for proper folding and export of peptides,

guided by ER-specific chaperones. ER stress can dysregulate the function of chaperones, resulting in export of misfolded proteins into circulation.[56] Such a scenario is manifest in the placenta of IUGR.[57] These conditions can also overlap in preeclampsia, leading to poor placentation. In this context, our published studies have shown that maternal hypoxia causes preeclampsia-like features in pregnant mice.[14] Interestingly, hypoxia has been shown to control TTR expression and uptake.[58] Thus, it is plausible that chronic hypoxia and ER stress destabilize transthyretin into a misfolded conformation and aggregation. More important, failure to adapt to a challenging intrauterine milieu may trigger conformational changes in proteins, causing aggregation and loss of function that lead to development of preeclampsia.

Our SELDI-TOF analysis and Far Western data on protein-protein interactions reveal novel transthyretin binding partners, including sEng (Supplemental Figure S4 and Supplemental Table S3). Because normal transthyretin binds to sEng, we propose that aggregated transthyretin fails to trap factors, such as sEng, which allows such proteins to contribute to the pathogenesis of this syndrome. Autophagy is known to dispose unwanted proteins and maintain homeostasis,[59] but it has been shown to be impaired in preeclampsia.[60] Its impairment is caused by endoglin.[60] Interestingly, dysregulated transthyretin present in PES induces production of sFlt-1 and sEng. It is, thus, possible that free soluble endoglin inhibits autophagy, and this effect could be imparted by aggregated transthyretin. When homotetrameric transthyretin transforms into misfolded and self-aggregated structures, the binding and scavenging potential of this protein is lost while acquiring the potential to induce production of sFlt-1, sEng, and other unknown factors (Figure 7B). Thus, preeclampsia pathological characteristic–associated aggregation of transthyretin is most likely an upstream event of production of anti-angiogenic factors.

Overall, our studies provide compelling evidence for the concept that preeclampsia is, in part, a syndrome of protein misfolding and aggregation. These observations offer a new framework for understanding the complex nature of preeclampsia pathogenesis. Intriguingly, exogenous transthyretin can reverse the features of the syndrome in a humanized mouse model of preeclampsia. These studies reveal innovative frontiers for identifying potential translational targets for therapy.

Acknowledgments

We thank the members of the Sharma laboratory for critical discussion, Phil Gruppuso for insightful comments, Sheryl-Vi Rico for transthyretin measurement by ELISA, Paula Weston for histological data, Christian Melle and Nico Escher for their support in SELDI-TOF analyses, and Justine S. Fitzgerald and Andreas Brückmann for providing patient serum samples for supplemental experiments.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.07.022*.

References

1. Roberts JM, Hubel CA: Is oxidative stress the link in the two-stage model of pre-eclampsia? Lancet 1999, 354:788–789
2. Redman CW, Sargent IL: Latest advances in understanding preeclampsia. Science 2005, 308:1592–1594
3. Dekker G, Sibai B: Primary, secondary and tertiary prevention of pre-eclampsia. Lancet 2001, 357:209–215
4. Steegers EA, Dadelszen P, Duvekot JJ, Pijnenborg R: Pre-eclampsia. Lancet 2010, 376:631–644
5. Parikh SM, Karumanchi SA: Putting pressure on pre-eclampsia. Nat Med 2008, 14:810–812
6. Meekins JW, Pijnenborg R, Hanssens M, McFadyen IR, van Asshe A: A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. Br J Obstet Gynaecol 1994, 101:669–674
7. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadani R, Karumanchi SA; CPEP Study Group: Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N Engl J Med 2006, 355:992–1005
8. Zhou CC, Zhang Y, Irani RA, Zhang H, Mi T, Popek EJ, Hicks MJ, Ramin SM, Kellems RE, Xia Y: Angiotensin receptor agonistic autoantibodies induce pre-eclampsia in pregnant mice. Nat Med 2008, 14:855–862
9. Redman CW, Sargent IL: Circulating microparticles in normal pregnancy and pre-eclampsia. Placenta 2008, 29:S73–S77
10. Zhou Y, Damsky CH, Fisher SJ: Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype: one cause of defective endovascular invasion in this syndrome? J Clin Invest 1997, 99:2152–2164
11. Hupperts B: Placental origins of preeclampsia: challenging the current hypothesis. Hypertension 2008, 5:970–975
12. Hung TH, Skepper JN, Charnock-Jones DS, Burton GJ: Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. Circ Res 2002, 90:1274–1281
13. Sharma S, Norris WE, Kalkunte S: Beyond the threshold: an etiological bridge between hypoxia and immunity in preeclampsia. J Reprod Immunol 2010, 85:112–116
14. Lai Z, Kalkunte S, Sharma S: A critical role of interleukin-10 in modulating hypoxia-induced preeclampsia-like disease in mice. Hypertension 2011, 57:505–514
15. Lim HJ, Wang H: Uterine disorders and pregnancy complications: insights from mouse models. J Clin Invest 2010, 120:1004–1015
16. Johnson L: Cancer: clinical trials unite mice and humans. Nature 2012, 483:546–548
17. Gitler AD, Lehmann R: Modeling human diseases. Science 2012, 337:269
18. Kalkunte S, Boij SR, Norris W, Friedman J, Lai Z, Kurtis J, Lim KH, Padbury JF, Matthiesen L, Sharma S: Sera from preeclampsia patients elicit symptoms of human disease in mice and provide a basis for an in vitro predictive assay. Am J Pathol 2010, 177:2387–2398
19. Hennessy A, Pilmore HL, Simmons LA, Painter DM: A deficiency of placental IL-10 in preeclampsia. J Immunol 1999, 163:3491–3495
20. Kalkunte S, Nevers T, Norris WE, Sharma S: Vascular IL-10: a protective role in preeclampsia. J Reprod Immunol 2011, 88:65–69
21. Venkatesha S, Toporsian M, Lam C, Hanai J, Yamamoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA, Stillman IE, Roberts D, D'Amore PA, Epstein FH, Sellke FW, Romero R, Sukhatme VP, Latarte M, Karumanchi SA: Soluble endoglin

contributes to the pathogenesis of preeclampsia. Nat Med 2006, 12: 642–649
22. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP, Karumanchi SA: Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 2003, 111:649–658
23. Raz A, Goodman DS: The interaction of thyroxine with human plasma prealbumin and with the prealbumin-retinol-binding protein complex. J Biol Chem 1969, 244:3230–3237
24. Dobson CM: Protein folding and misfolding. Nature 2003, 426: 884–890
25. Hammarström P, Wiseman RL, Powers ET, Kelly JW: Prevention of transthyretin amyloid disease by changing protein misfolding energetics. Science 2003, 299:713–716
26. Goldsteins G, Persson H, Anderson K, Olofsson A, Dacklin I, Edvinsson A, Saraiva MJ, Lundgren E: Exposure of cryptic epitopes on transthyretin only in amyloid and in amyloidogenic mutants. Proc Natl Acad Sci U S A 1999, 96:3108–3113
27. Buxbaum JN, Ye Z, Reixach N, Friske L, Levy C, Das P, Golde T, Masliah E, Roberts AR, Bartfai T: Transthyretin protects Alzheimer's mice from the behavioral and biochemical effects of Abeta toxicity. Proc Natl Acad Sci U S A 2008, 105:2681–2686
28. Stein TD, Anders NJ, DeCarli C, Chan SL, Mattson MP, Johnson JA: Neutralization of transthyretin reverses the neuroprotective effects of secreted amyloid precursor protein (APP) in APPSW mice resulting in tau phosphorylation and loss of hippocampal neurons: support for the amyloid hypothesis. J Neurosci 2004, 24:7707–7717
29. Altland K, Winter P, Saraiva MJ, Suhr O: Sulfite and base for the treatment of familial amyloidotic polyneuropathy: two additive approaches to stabilize the conformation of human amyloidogenic transthyretin. Neurogenetics 2004, 5:61–67
30. Henze A, Rohn S, Gericke B, Raila J, Schweigert FJ: Structural modifications of serum transthyretin in rats during protein-energy malnutrition. Rapid Commun Mass Spectrom 2008, 22:3270–3274
31. Schweigert FJ, Gericke B, Wolfram W, Kaisers U, Dudenhausen JW: Peptide and protein profiles in serum and follicular fluid of women undergoing IVF. Hum Reprod 2006, 21:2960–2968
32. Gericke B, Raila J, Deja M, Rohn S, Donaubauer B, Nagl B, Haebel S, Schweigert FJ, Kaisers U: Alteration of transthyretin microheterogeneity in serum of multiple trauma patients. Biomark Insights 2007, 2:299–306
33. Burnum KE, Tranguch S, Mi D, Daikoku T, Dey SK, Caprioli RM: Imaging mass spectrometry reveals unique protein profiles during embryo implantation. Endocrinology 2008, 149:3274–3278
34. McKinnon B, Li H, Richard K, Mortimer R: Synthesis of thyroid hormone binding proteins transthyretin and albumin by human trophoblast. J Clin Endocrinol Metab 2005, 90:6714–6720
35. Graham CH, Connelly I, MacDougall JR, Kerbel RS, Stetler-Stevenson WG, Lala PK: Establishment and characterization of first trimester human trophoblast cells with extended lifespan. Exp Cell Res 1993, 206:204–211
36. Kalkunte S, Lai Z, Tewari N, Chichester C, Romero R, Padbury J, Sharma S: In vitro and in vivo evidence for lack of endovascular remodeling by third trimester trophoblasts. Placenta 2008, 29:871–878
37. Reixach N, Foss TR, Santelli E, Pascual J, Kelly JW, Buxbaum JN: Human-murine transthyretin heterotetramers are kinetically stable and non-amyloidogenic: a lesson in the generation of transgenic models of diseases involving oligomeric proteins. J Biol Chem 2008, 283: 2098–2107
38. Wu Y, Li Q, Chen XZ: Detecting protein-protein interactions by Far western blotting. Nat Protoc 2007, 2:3278–3284
39. Guntern R, Bouras C, Hof PR, Vallet PG: An improved thioflavine S method for staining neurofibrillary tangles and senile plaques in Alzheimer's disease. Experientia 1992, 48:8–10
40. Schweigert FJ, Wirth K, Raila J: Characterization of the microheterogeneity of transthyretin in plasma and urine using SELDI-TOF-MS immunoassay. Proteome Sci 2004, 2:5
41. Vorderwülbecke S, Cleverley S, Weinberger SR, Wiesner A: Protein quantification by the SELDI-TOF-MS–based ProteinChip System. Nat Methods 2005, 2:393–395
42. Kelly JW: The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. Curr Opin Struct Biol 1998, 8:101–106
43. Lai Z, Colón W, Kelly JW: The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid. Biochemistry 1996, 35:6470–6482
44. Koo EH, Lansbury PT Jr., Kelly JW: Amyloid diseases: abnormal protein aggregation in neurodegeneration. Proc Natl Acad Sci U S A 1999, 96:9989–9990
45. Karlsson A, Olofsson A, Eneqvist T, Sauer-Eriksson AE: Cys114-linked dimers of transthyretin are compatible with amyloid formation. Biochemistry 2005, 44:13063–13070
46. Thaxton JE, Sharma S: Interleukin-10: a multifaceted agent of pregnancy. Am J Reprod Immunol 2010, 63:482–491
47. Didion SP, Kinzenbaw DA, Schrader LI, Chu Y, Faraci FM: Endogenous IL-10 inhibits angiotensin II-induced vascular dysfunction. Hypertension 2009, 54:619–624
48. Lee TS, Chau LY: Heme oxygenase-1 mediates anti-inflammatory effect of interleukin-10 in mice. Nat Med 2002, 8:240–246
49. Zenclussen AC, Lim E, Knoeller S, Knackstedt M, Hertwig K, Hagen E, Klapp BF, Arck PC: Heme oxygenases in pregnancy II: HO-2 is downregulated in human pathologic pregnancies. Am J Reprod Immunol 2003, 50:66–76
50. Huet O, Laemmel E, Fu Y, Dupic L, Aprico A, Andrews KL, Moore SL, Harrois A, Meikle PL, Vicaut E, Chin-Dusting JP, Duranteau J: Interleukin 10 antioxidant effect decreases leukocytes/endothelial interaction induced by tumor necrosis factor α. Shock 2013, 39:83–88
51. Soares MP, Bach FH: Heme oxygenase 1: from biology to therapeutic potential. Trends Immunol 2009, 15:50–58
52. Cudmore M, Ahmed S, Al-Ani B, Fujisawa T, Coxall H, Chudasama K, Devey LR, Wigmore SJ, Abbas A, Hewett PW, Ahmed A: Negative regulation of soluble Flt-1 and soluble endoglin release by heme oxygenase-1. Circulation 2007, 115:1789–1797
53. Buhimschi IA, Zhao Z, Funai EF, Harris N, Bernstein IM, Saade GR, Buhimschi CS: Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. Am J Obstet Gynecol 2008, 199:551.e1–551.e16
54. Matheson NR, Wong PS, Travis J: Enzymatic inactivation of human alpha-1-proteinase inhibitor by neutrophil myeloperoxidase. Biochem Biophys Res Commun 1979, 88:402–409
55. Feldman DE, Chauhan V, Koong AC: The unfolded protein response: a novel component of the hypoxic stress response in tumors. Mol Cancer Res 2005, 3:597–605
56. Redman CW: The endoplasmic reticulum stress of placental impoverishment. Am J Pathol 2008, 173:311–314
57. Yung HW, Calabrese S, Hynx D, Hemmings BA, Cetin I, Charnock-Jones DS, Burton GJ: Evidence of placental translation, inhibition and endoplasmic reticulum stress in the etiology of human intrauterine growth restriction. Am J Pathol 2008, 173:451–462
58. Patel J, Landers K, Li H, Mortimer RH, Richard K: Oxygen concentration regulates expression and uptake of transthyretin, a thyroxine binding protein, in JEG-3 choriocarcinoma cells. Placenta 2011, 32:128–133
59. Klionsky DJ: Good riddance to bad rubbish. Nature 2006, 441: 819–820
60. Nakashima A, Yamanaka-Tatemastu M, Fujita N, Koizumi K, Shima T, Yoshida T, Nikaido T, Okamoto A, Yoshimori T, Siato S: Impaired autophagy by soluble endoglin, under physiological hypoxia in early pregnant period, is involved in poor placentation and preeclampsia. Autophagy 2013, 9:1–14