

Online Supplement

Hypoxia-independent up-regulation of placental HIF-1 α gene expression contributes to the pathogenesis of preeclampsia

By

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Methods

Animals

Wild type (WT) 8 to 10 week-old timed pregnant C57BL/6 mice (mated with syngeneic males) were obtained from Harlan Laboratories (Indianapolis, IN). The mice were housed in the animal care facility of the University of Texas Health Science Center Houston and had access to food and water ad libitum. All protocols involving animal studies were reviewed and approved by the Institutional Animal Welfare Committee.

Introduction of human autoantibody (AT1-AA) or LIGHT into pregnant mice

PE mouse models induced by AT1-AA or LIGHT were conducted. Briefly, purified IgGs were isolated from preeclamptic (PE) or normotensive (NT) pregnant patient sera (PE-IgG, NT-IgG respectively) as previously described ¹. Pregnant mice were treated with NT-IgG or PE-IgG (0.8mg) on E13.5 and E14.5 by retro-orbital sinus injection. For the neutralization experiments, either losartan (8 mg/kg), an angiotensin II receptor antagonist, or 7-amino acid epitope peptides (AFHYESQ), were premixed with PE-IgG and coinjected with PE-IgG on E13.5 and E14.5. For LIGHT injection experiments, recombinant mouse LIGHT (2 ng; R&D Systems) or the same volume of saline was introduced into pregnant mice by retro-orbital sinus injection on E13.5 and E14.5 as

previously reported ². For neutralization experiments, either lymphotoxin β receptor (LT β R) monoclonal Antibody (100 μ g) or herpes virus entry mediator (HVEM) monoclonal antibody (100 μ g) was simultaneously coinjected with LIGHT. All mice were sacrificed on E18.5 prior to delivery, and their blood and organs were collected.

***In vivo* siRNA-induced knockdown of *Hif-1 α* in pregnant mice**

To knockdown *Hif-1 α* mRNA levels in PE-IgG or LIGHT-injected pregnant mice, a siRNA knockdown method (Altogen Biosystems) using nanoparticles to surround siRNA constructs was used as previously reported ³. Briefly, scrambled siRNA control or *HIF-1 α* siRNA (Sigma) encapsulated in nanoparticles was prepared according to instructions from the company (Altogen Biosystems) and was administered on E13.5 and E14.5 retro-orbital sinus injection together with PE-IgG or LIGHT.

The measurement of blood pressure and proteinuria

The systolic blood pressure of all mice was measured at the same time daily by tail cuff plethysmography using a carotid catheter-calibrated system (CODA, Kent Scientific).

The mice were kept warm using a warming pad (AD Instruments Co). For the measurement of proteinuria, urine was collected for analysis using metabolic cages (Nalgene). Total microalbumin and creatinine in the urine were determined by ELISA

(Exocell) and then the ratio of urinary albumin to creatinine was calculated as an index of proteinuria as previously described ¹.

Real-time RT-PCR analysis

RNA isolation and real-time RT-PCR were conducted as previously described ². Syber green was used for the analysis of all transcripts measured using the following primers:

Mouse	HIF-1 α :	forward;	5'-GAAATGGCCCAGTGAGAAAA-3'	and	reverse;	
			5'-CTTCCAVGTTGCTGACTTGA-3',	Mouse	Flt-1:	forward;
			5'-CCACCTCTCTATCCGCTGG-3'	and	reverse;	5'-
			ACCAATGTGCTAACCGTCTTATT-3',	Mouse	GAPDH:	forward;
			5'-TGACCTCAACTACATGGTCTACA-3'	and	reverse;	5'-
			CTTCCCATTCTCGGCCTTG-3',	Human	HIF-1 α :	forward;
			5'-TGCTCATCAGTTGCCACTTC-3'	and	reverse;	
			5'-TCCTCACACGCAAATAGCTG-3',	Human	Flt-1:	forward;
			5'-TTTGCCTGAAATGGTGAGTAAGG-3'	and	reverse;	
			5'-TGGTTTGCTTGAGCTGTGTTC-3',	Human	GAPDH:	forward;
			5'-TGCACCACCAACTGCTTAGC-3'	and	reverse;	
			5'-ACAGTCTTCTGGGTCGCAGTG-3'.			

Immunohistochemistry

Formalin fixed tissue blocks were cut into 4- μ m thick sections and subjected to immunohistochemistry. Briefly, endogenous peroxidase activity was quenched by 10 min of incubation in a 3% hydrogen peroxide/methanol buffer. Antigen retrieval was conducted by incubating slides in sodium citrate buffer (pH 6.0) at 89°C for 15 min. After blocking with the normal goat serum, the slides were then incubated with antibody against mouse CD31 (1:200, ab124432, Abcam) or human/mouse HIF-1 α (1:100, LS-B2823, Lifespan Biosciences) in a humidified chamber at 4°C overnight. After the primary antibody incubation, ABC staining system kit (VEACTASTAIN, VECTOR LAB) was used according to the manufacturer's suggested protocol. Antigen-antibody reactions were visualized with a dual alkaline phosphatase / fluorescence development system (VECTOR Red Substrate Kit, VECTORLAB). The slides were counterstained with Mayer's hematoxylin. The positive staining for HIF-1 α was quantified by Image J software. The intensity of red positive staining was obtained from 6 fields under X100 magnification and averaged to get mean values.

Immunoblotting

Placental explants were lysed with RIPA lysis buffer (Santa Cruz) in the presence of proteinase inhibitor cocktail (Roche Diagnostics). Lysates were resolved on SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. After blocking with Odyssey Blocking Buffer (LI-COR), the membranes were probed with antibody against human / mouse HIF-1 α (1:1000, LS-B2823, Lifespan Biosciences), and then probed with secondary antibodies labeled with IRDye fluorophores (LI-COR). The antibody/antigen complexes were scanned and detected using the ODYSSEY infrared imaging system and software (LI-COR).

Patients

Patients who were admitted to Memorial Hermann Hospital were identified by the obstetrical faculty of the University of Texas Medical School at Houston. Preeclamptic patients were diagnosed with severe disease based on the definition set by the National High Blood Pressure Education Program Working Group Report ⁴. The criteria of inclusion, including no previous history of hypertension, were reported previously ¹. Control pregnant women were selected on the basis of having an uncomplicated, normotensive (NT) pregnancy, with an expected normal-term delivery. Human subject data utilized in current study were summarized and indicated in **Table S1**. The research

protocol was approved by the Institutional Committee for the Protection of Human Subjects.

Human Placental Villous Explant Culture

Human placentas were obtained from preeclamptic or normotensive patients who delivered vaginally at term at Memorial Hermann Hospital in Houston. The explant culture system was conducted as described previously⁵. On delivery, the placentas were immediately placed on ice and submerged in phenol red-free DMEM containing 10% BSA and antibiotics. Villous explant fragments weighing 50mg were dissected from the placenta and transferred to 24-well plates at 37°C under 5% CO₂. The explants were incubated for 24 hours and then pretreated with or without HIF-1 α inhibitor (10 μ M CAY10585) (Santa Cruz) for 15 min and then treated with NT- or PE-IgG (100 μ g /ml) or recombinant human LIGHT (100 pg/mL) (R&D Systems) or dimethyloxaloylglycine (DMOG) (10 μ M), a prolyl hydroxylase (PHD) inhibitor that results in the stabilization and accumulation of HIF-1 α (Santa Cruz) for 24 hours. The explants were also treated with nanoparticle encapsulated control-or *HIF-1 α* -siRNA for 24 hours as previously described in *in vivo* knockdown method section and then were treated with NT- or PE-IgG (100 μ g /ml) or recombinant human LIGHT (100 pg/mL) for 24 hours.

Statistical analysis

All data are expressed as the mean \pm SEM. Mann-Whitney's *U* test was applied in two-group analysis in Figure 4A, 4C, and 4E. Comparison of the data obtained at different time points from multiple groups as repeated measurements in Figures 2B and 3B were analyzed by two-way repeated measures analysis of variance, followed by the Bonferroni post hoc test. Differences among the means of multiple groups in every analysis except for ones described above were compared by the one-way analysis of variance (ANOVA), followed by a Tukey's post hoc test. Categorical variables in Table S1 were analyzed by the Fisher's exact test. Statistical significance was set as $P < 0.05$. Statistical programs were run by GraphPad Prism 5 statistical software (GraphPad).

Supplementary References

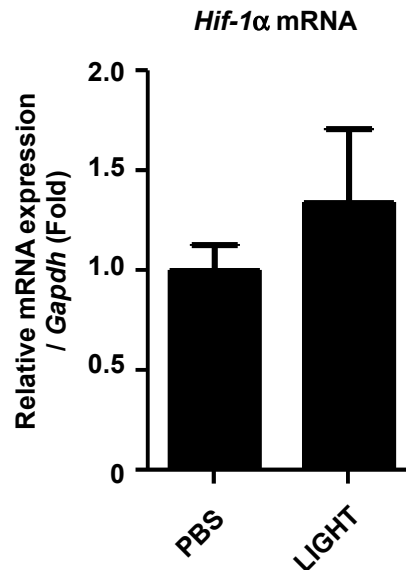
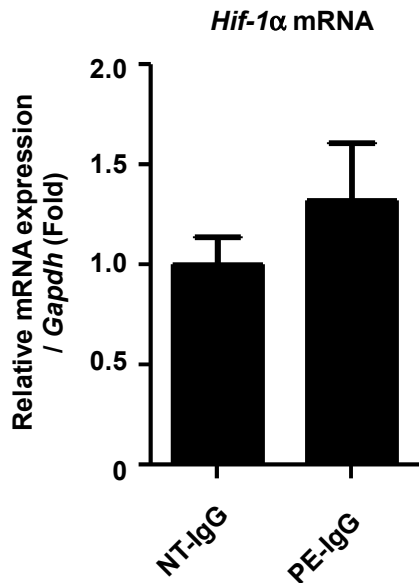
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5. Ahmed A, Rahman M, Zhang X, Acevedo CH, Nijjar S, Rushton I, Bussolati B, St John J. Induction of placental heme oxygenase-1 is protective against tnfa-induced cytotoxicity and promotes vessel relaxation. *Molecular medicine*. 2000;6:391-409.

Table S1 Clinical characteristics for human subjects

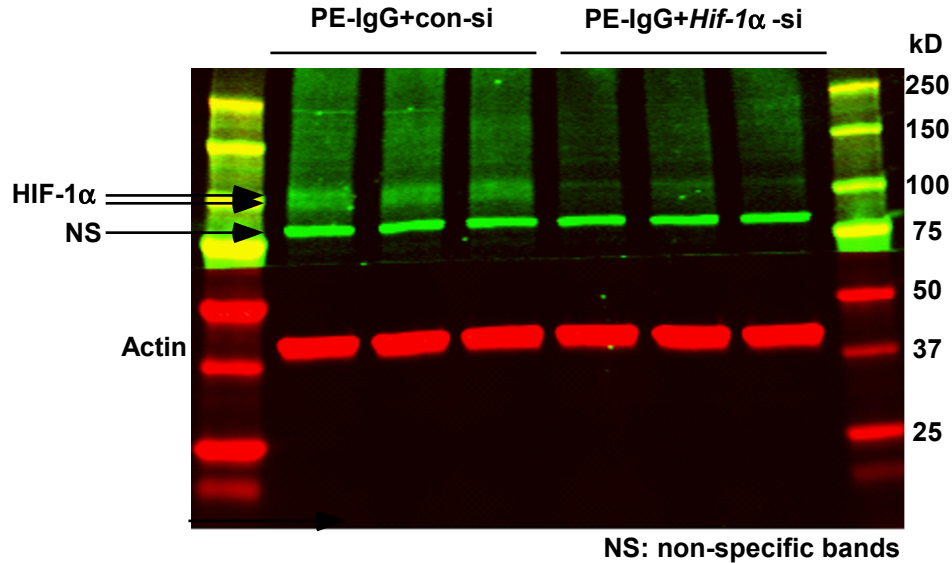
Clinical characteristics	NT (n=10)	PE (n=10)
Age (yr)	26.5±2.7	28.1±1.6
Primigravida - no. (%)	4 (40%)	5 (50%)
Race - no. (%)		
Caucasian	3 (30%)	4 (40%)
African American	4 (40%)	5 (50%)
Hispanic	1 (10%)	1 (10%)
Other or unknown	2 (20%)	0 (0%)
Body-mass index	31.8±1.8	34.3±2.2*
Systolic blood pressure (mmHg)	119.3±3.4	168.8±5.9**
Diastolic blood pressure (mmHg)	70.8±1.9	107.2±4.1**
Proteinuria (mg/24h)	N/A	1546±290
Gestational age at delivery (week)	37.9±2.1	36.3±1.9
Infant's birth weight (g)	3032±153	2321±342**
Small-for gestational age infant - no. (%)	0 (0%)	3 (30%)*

* $P<0.05$, ** $P<0.01$ vs NT



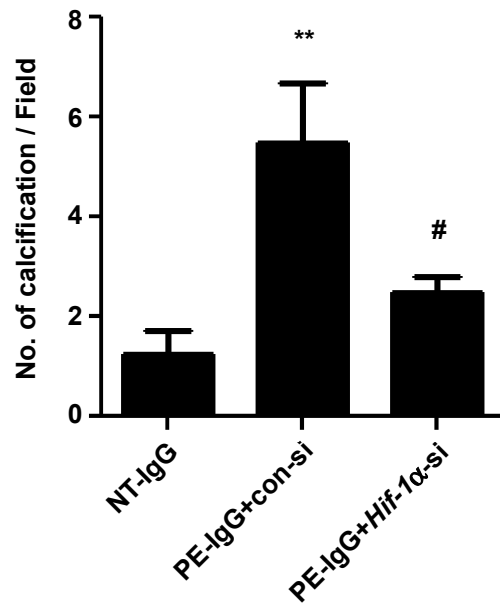
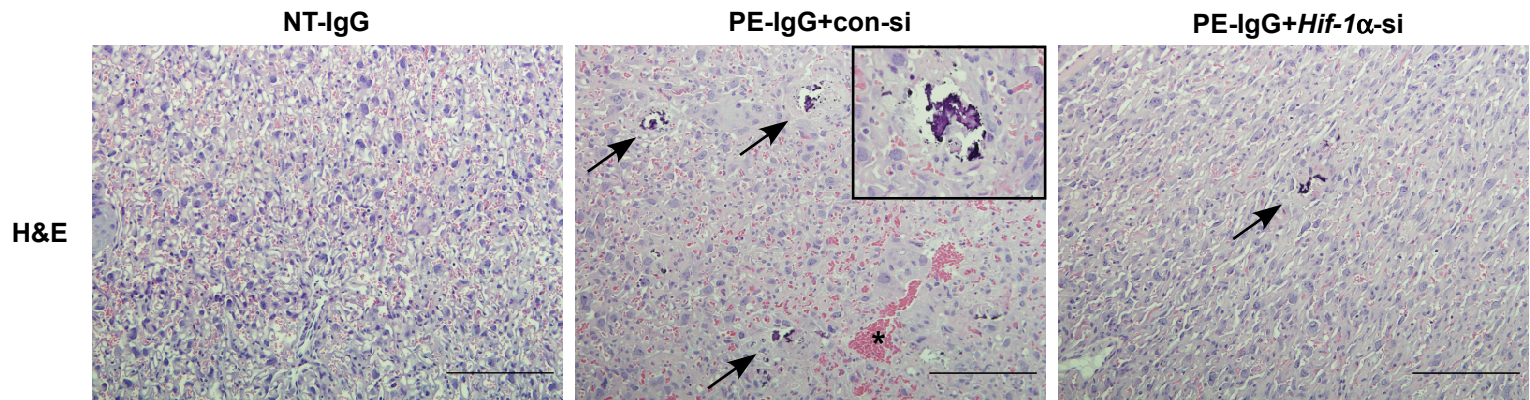
S1. Renal *Hif-1 α* mRNA expression assessed by real-time RT-PCR.

Hif-1 α mRNA expression levels in kidneys of mice injected with NT- or PE-IgG (n=4 mice per group) or LIGHT (n=3 mice per group) were determined by real-time RT-PCR. mRNA expression level was determined as a relative value to *Gapdh*, and each value was expressed as fold induction relative to placentas of NT-IgG- or PBS-treated mice. No significant difference was observed between groups.



S2. The effect of *in vivo* siRNA-induced knockdown of *Hif-1α* in pregnant mice.

Expression of HIF-1α protein in mouse placentas detected by immunoblotting. Placental HIF-1α protein expression was successfully reduced in *Hif-1α* siRNA-injected mice compared with control scrambled siRNA-injected mice.



S3. Placental histology assessed by H&E staining. PE-IgG-induced placental pathologic changes seen in the labyrinth zone of control siRNA-injected mice (calcification; arrows, the disorganization of tissue resulting in abnormal blood pooling; *) was reduced in the placenta of mice injected with *Hif-1* α siRNA. Scale bar, 200 μ m. The number of calcification per field obtained under X100 magnification is quantified. (5 fields per placenta; 4 mice per group). (** P <0.01 vs NT-IgG, # P <0.05 vs PE-IgG+con-si)