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Relationship between hypoxia and downstream pathogenic pathways in preeclampsia

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ABSTRACT

Defects in angiogenesis and mitochondrial function in the placenta contribute to the pathogenesis of preeclampsia; however upstream regulators of these pathways are not known. It has been argued that placental hypoxia secondary to abnormal spiral artery remodeling may play a causal role in the angiogenic and mitochondrial abnormalities noted in preeclampsia. The aim of this study was to evaluate the relationship between hypoxia-inducible factor-1α (HIF-1α) , a surrogate of hypoxia, and soluble fms-tyrosine kinase 1 (sFlt1), a circulating anti-angiogenic factor, and microRNA 210 (miR-210), a microRNA that regulates mitochondrial function, in human placentas from preeclamptic and non-hypertensive pregnancies. We first confirmed a 2.5-fold upregulation of HIF-1α protein in placentas from preeclampsia when compared to non-hypertensive controls. Consistent with prior studies, we also observed a 10-fold upregulated sFlt1 mRNA and 2-fold upregulated miR-210 in preeclamptic tissue. Interestingly, while sFlt1 mRNA correlated with miR-210 in preeclampsia ($R^2 = 0.77$, $p = 0.0004$), there were no significant correlations between these molecules and HIF1α expression. We conclude that non-hypoxia pathways may be involved in the abnormal angiogenic and metabolic alterations noted in preeclampsia.

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HIF-1α protein; miR-210; placenta; preeclampsia; pregnancy; sFlt1

Introduction

Preeclampsia (PE) complicates ~5% of human pregnancies, and together with its infrequent sequel, eclampsia, is a leading cause of maternal deaths directly due to pregnancy (1,2). It is diagnosed with new-onset hypertension after the 20th week of gestation associated with significant proteinuria (3) or, in the absence of proteinuria, when in association with thrombocytopenia, impaired liver function, the new development of renal insufficiency, pulmonary edema, or new-onset cerebral or visual disturbances (4). Abnormalities in placental expression of anti-angiogenic factors and mitochondrial dysfunction have been reported to play a key role in the pathogenesis of the disease (5,6). Although many abnormalities in angiogenesis have been described in preeclampsia, upregulated soluble-fms like tyrosine kinase (sFlt1), a circulating anti-angiogenic factor, has emerged as a major pathway that contributes to the maternal syndrome of preeclampsia (7). Mitochondrial dysfunction, thought to play a key role in oxidative stress observed in preeclampsia has also been evaluated as a major therapeutic target in preeclampsia (8).

Placental hypoxia has long been hypothesized as a key mechanism that leads to PE (9). Reduced placental perfusion, resulting in fewer spiral arteries both in number and modification, leads to placental hypoxia. Although hypoxia has not been formally demonstrated in PE tissues, up-regulation of hypoxia-inducible factor-1 (HIF-1), a transcription factor and a master regulator of the cellular response to low oxygen tension, has been noted in preeclamptic tissues (10,11). HIF-1α is rapidly inactivated and degraded during normoxia. But, under hypoxic conditions, stabilized HIF-1α combines with HIF-1β, a constitutively expressed protein to form the HIF-1 heterodimer, which binds to the hypoxia-responsive element of various genes, modulating their expression levels (12). Gene expression changes in the placenta have also confirmed upregulation of hypoxia related pathways during PE (13). Placental expression of hypoxia related proteins reflects the gestational oxygen

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levels, being elevated in early pregnancy and decreasing toward the term (14). Prior studies suggest that PE placentas show a 2-fold increase in HIF-1α protein levels, however whether this is a cause or effect of the disease is still being debated (15–17).

PE placentas produce excess soluble VEGF receptor, sFlt1 (7,18), and micro RNA miR-210, a hypoxia inducible microRNA, which has been implicated in mitochondrial dysfunction (19,20). Circulating sFlt-1 levels are increased prior to the onset of PE, are altered in most women with severe PE, and correlate with adverse maternal and neonatal outcomes related to PE (21–23). Cell culture studies suggest that hypoxia may promote upregulation of both sFlt1 and miR-210 (19,24). Uteroplacental ischemia in pregnant rats and baboons leads to preeclampsia-like manifestations accompanied by increases in circulating sFlt1 (25–28). However, sFlt1 and other vasoconstrictive substances elevated in humans with PE may impair blood flow to the uteroplacental unit and induce placental ischemia. A strong correlation of circulating angiogenic factors with uterine artery Doppler findings in humans is consistent with this hypothesis (29).

The aim of this study was to evaluate the relationship between HIF-1α and pathogenic biomarkers of PE, sFlt1, and miR-210, in human placentas from preeclamptic and non-hypertensive control pregnancies.

Methods

Placental collection and processing

Placentas were collected from normal and PE subjects (n = 11 in each group) in accordance with all institutional policies and with the approval of the institutional review board at the Beth Israel Deaconess Medical Center (Boston, MA, USA). The diagnosis of PE was based on the updated criteria of the American College of Obstetrician and Gynecology Task force on Hypertension in Pregnancy (4). Patients with history of diabetes, chronic hypertension, renal disease or multiple gestations were excluded. We excised placental biopsies (2 × 2 cm) without basal and chorionic plates and dabbed with a cotton gauze to remove blood and debris. These tissue samples were flash-frozen in liquid nitrogen within half an hour of placental delivery.

Protein extraction and Western blot analysis

For the total protein extraction, placental samples (~50 mg) were homogenized by sonication (Fisher Scientific Model 120 Sonic Dismembrator, microprobe, setting 70 for 45s) in 4 volumes of 1X Laemmli buffer containing phenyl-methyl sulfonyl fluoride, sodium vanadate, and f protease inhibitor cocktail (Calbiochem, Billerica, MA). The total proteins from these placentas were isolated and used in western blot analysis as described previously (15). For the detection of hypoxia inducible transcription factor 1 alpha (HIF-1α) protein, 100 µg of the total protein was subjected to protein electrophoresis on an 8% SDS-polyacrylamide gel and western blots performed as described previously (14,15). HIF-1α monoclonal antibodies (Cat. H72320, Transduction Laboratories, Lexington, KY) at a final concentration of 1.25 µg/ml in Tris-buffered saline buffer containing 0.05% Tween-20 and 5% non-fat dry milk. The mouse monoclonal β-actin antibodies (Sigma Chemical Company, St Louis, MO, Cat. A 5441) were diluted 1:1000 yielding a final concentration of 2.2 µg/ml. We performed densitometry using the UN SCAN_IT software (Silk Scientific Inc., Orem, Utah).

RNA extraction and real-time quantitative PCR

The total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform extraction technique (AGPC) with Trizol (Invitrogen, Carlsbad, California) and their concentrations were determined using a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware).

Taqman® quantitative real-time PCR (qRT-PCR) was performed for the quantitation of the total sFlt1 (all isoforms of sFlt1 and Flt1, Applied Biosystems Hs01052961_m1) and micro RNA miR-210 (Applied Biosystems has-miR-210 id # 000512), between control and preeclamptic placentas. To perform reverse transcription (RT), 5 µL RNA sample was added to 3 µL 5X RT primer and 7 µL RT reaction master mix for a total volume of 15 µL as recommended by the manufacturer (Applied Biosystems). To prepare the quantitative PCR (qPCR) reaction mix, 1.33 µL product from RT reaction, 1 µL TaqMan small RNA assay, and 10 µL TaqMan master mix (Applied Biosystems) were combined with nuclease-free water in a well on the reaction plate for a total volume of 20 µL. The real-time PCR reactions were read and analyzed using a 7900HT Sequence Detection System (Applied Biosystems). β-actin and RNU-49 were used as loading controls.

Statistical analyses

Statistical analyses were performed using the Graphad software. The Student t-test was used to analyze controls versus PE. The p value of less than 0.05 was

considered to be significant. We calculated Pearson's correlation coefficients to determine the strength of association between various analytes.

Results

Clinical characteristics of the study population

Table 1 shows the clinical characteristics of the patient population in the study. The PE group delivered earlier and did not, surprisingly, have higher blood pressures and proteinuria. The maternal ages were similar in both groups.

Biomarker levels

HIF-1α protein levels were significantly increased in PE placentas, 32679 ± 2170 when compared to controls 12904 ± 1857 ($p < 0.0001$, Figure 1A and B) as detected by Western blot analyses. This 2.5-fold increase in HIF-1alpha is consistent with previous findings that demonstrated that PE placentas have stabilized HIF protein expression (15,30).

We then estimated the transcript levels of sFlt1 and miR-210 genes in the same placental cohort using real-time PCR. Consistent with prior studies, we found that PE placentas significantly demonstrated increased sFlt1 mRNA compared to controls, 14.70 ± 3.605 vs 1.047 ± 0.1065 fold, respectively ($p = 0.0012$, Figure 2A). In accordance with the published reports (19,20), we also found increased miR-210 levels in PE placentas compared to controls, 2.737 ± 0.4714 vs 1.125 ± 0.1645, respectively ($p = 0.0042$, Figure 2B).

Correlation among the PE biomarkers

To evaluate the relationship between HIF-1α and these PE biomarkers, we performed correlation analyses between placental HIF protein expression and sFlt1 and miR-210 expression in both control and PE placental samples (Figure 3). There was no correlation between the HIF-1α and sFlt1 levels in controls and PE placentas (HIF-1α vs sFlt1; $R^2 = 0.05$ in controls and 0.003 in PE). Likewise, there was no correlation between the HIF-1α and miR-210 levels in controls and PE placentas ($R^2 = 0.02$ in control and PE). In contrast, PE biomarkers, sFlt1 and miR-210, correlated modestly in controls ($R^2 = 0.27$) and robustly in PE ($R^2 = 0.77$, $p = 0.0004$, Figure 3).

Discussion

Here, we demonstrate that while both hypoxia inducible genes, sFlt1 and miR-210, are upregulated in PE, we

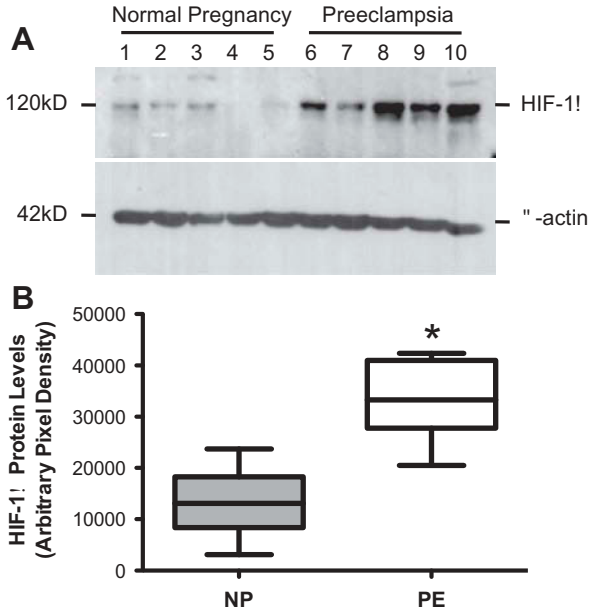

Figure 1. HIF-1α expression in control and PE placentas. A representative Western blot of HIF-1α protein expression in placentas from five controls (Lanes 1–5) and five PE (Lanes 6–10) is presented in Figure 1. Quantitation of HIF-1α expression ($n = 11$ in each group) is shown in the bottom panel. * represents $p = 0.0001$, PE vs controls.

Table 1. Clinical characteristics of subjects.

	Controls ($n = 11$)	Preeclampsia ($n = 11$)
Mothers age (years)	34.36 ± 1.5	31.6 ± 1.63
Gestational age (weeks)	38.77 ± 0.3	33.2 ± 1.03*
Race	Caucasian 73%, African American 18.0%, Asian 9.0%	Caucasian 64%, African American 9%, Asian 18%, mixed 9%
Systolic BP mmHg (at delivery)	118 ± 2.73	172.5 ± 4*
Diastolic BP (mmHg) (at delivery)	70.4 ± 1.45	104.7 ± 8.9*
Proteinuria	N/A	100%
Fetal growth restriction	0/11	2/11
C-section	11/11	7/11

* represents $p < 0.05$

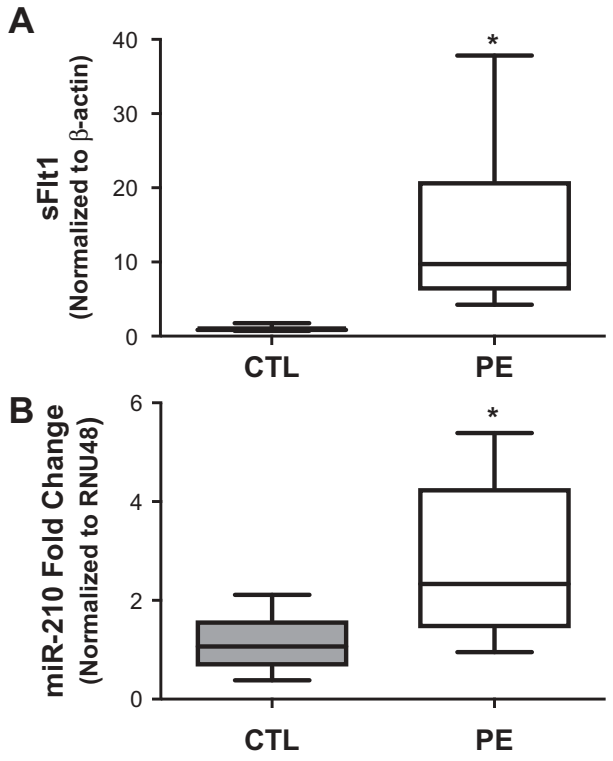

Figure 2. sFlt1 and miR-210 expression in control and PE placentas. (A) Total sFlt1 mRNA levels were estimated by real-time PCR and the values were normalized to β-actin and summary results from controls (n = 11) and PE (n = 11) are depicted. * represents p = 0.0012, PE vs controls. (B) miR-210 levels were estimated by real-time PCR and values were normalized to RNU gene and summary results from controls (n = 11) and PE (n = 11) are depicted. * represents p = 0.0042, PE vs controls.

found no correlation for these analytes with HIF1α expression in the placenta. HIF-1α is a well-established surrogate of hypoxia and has been consistently reported to be elevated in humans with PE. Several *in vitro* studies and *in vivo* studies in rodents have suggested that placental hypoxia and increased HIF-1α proteins may be sufficient to induce sFlt1 and miR-210 upregulation (19,24,26,28). In contrast, some have argued that defects in placental trophoblast differentiation may be the primary abnormality and that placental hypoxia may not be critical for the development of PE (31). Taken together with our studies, we conclude that while hypoxia may induce sFlt1 and miR-210 expressions, it is unlikely to be a major driver of these angiogenic and metabolic abnormalities noted in humans with PE.

It has been argued that the hypoxic environment of the placenta causes overexpression of HIF-1α proteins by about 2-fold. HIF-1α, a regulator of the cellular response to low oxygen tensions, is essential for the maintenance of cellular homeostasis. Several groups established evidence and the role of HIF-1α in pregnancy and their possible involvement in the pathogenesis of PE (14,32–34). However, PE is also characterized by abnormal placental perfusion, suggesting that the placental up-regulation of HIF-1α may be secondary to impaired flow abnormalities noted in these patients. Similarly, an association between miR-210 and HIF-1α has been described as a form of positive feedback. In this model, the increased expression of miR-210 in hypoxia may promote HIF-1α stability. This is facilitated by blocking of the glycerol-3-phosphate dehydrogenase 1-like enzyme (GPD1L) by miR-210. Accordingly, miR-210 may stabilize HIF-1α (35). Zhang et al. found high levels of miR-210 in placentas of patients with PE (20), and reported that it interferes with trophoblast invasion. Xu et al. have also shown that several miRNAs up-regulated in PE placentas are produced by various subtypes of trophoblasts including villous cytotrophoblast cells, syncytiotrophoblasts, column cytotrophoblast cells, and interstitial trophoblast cells invading the decidual stroma (36). This altered miRNA expression along with the reduced mitochondrial respiration (19) suggests the possibility of a functional role for miRNA-210 in the pathogenesis of PE (37). In our results, miR-210 correlated weakly with the expression of HIF-1α suggesting a lack of the direct relationship between these two molecules, at least in placental tissues. In contrast, miR-210 was strongly correlated with sFlt1 expression during PE, suggesting that both these pathways may have a common upstream regulator.

Our study has some limitations. HIF-1α expression rapidly changes following exposure to normoxia. To minimize the variability of the HIF-1α expression, we snap froze placental tissues in liquid nitrogen within 30 minutes of delivery, however, we cannot rule out the possibility that changes in oxygen concentrations *ex vivo* may have affected HIF expression. It was reassuring to us that PE tissues consistently demonstrated HIF upregulation and therefore believe that our studies are valid. Our study was also limited by sample size. PE was believed to be a very heterogeneous disorder and therefore we cannot exclude the possibility that HIF-1α overexpression may play a role in certain sub-types of PE.

The etiology of the abnormal placentation in PE is still being debated. Various pathways have been proposed to have key roles in inducing placental disease, including deficient heme oxygenase expression, impaired corin expression, autoantibodies against the angiotensin receptor, oxidative stress, inflammation, altered natural killer cell signaling, and deficient catechol-*O*-methyl transferase (5). Interestingly, most of these pathways were shown to increase placental production of the antiangiogenic factors *in vitro* or in animal studies. However, the etiology of the abnormal angiogenic and metabolic dysfunction in

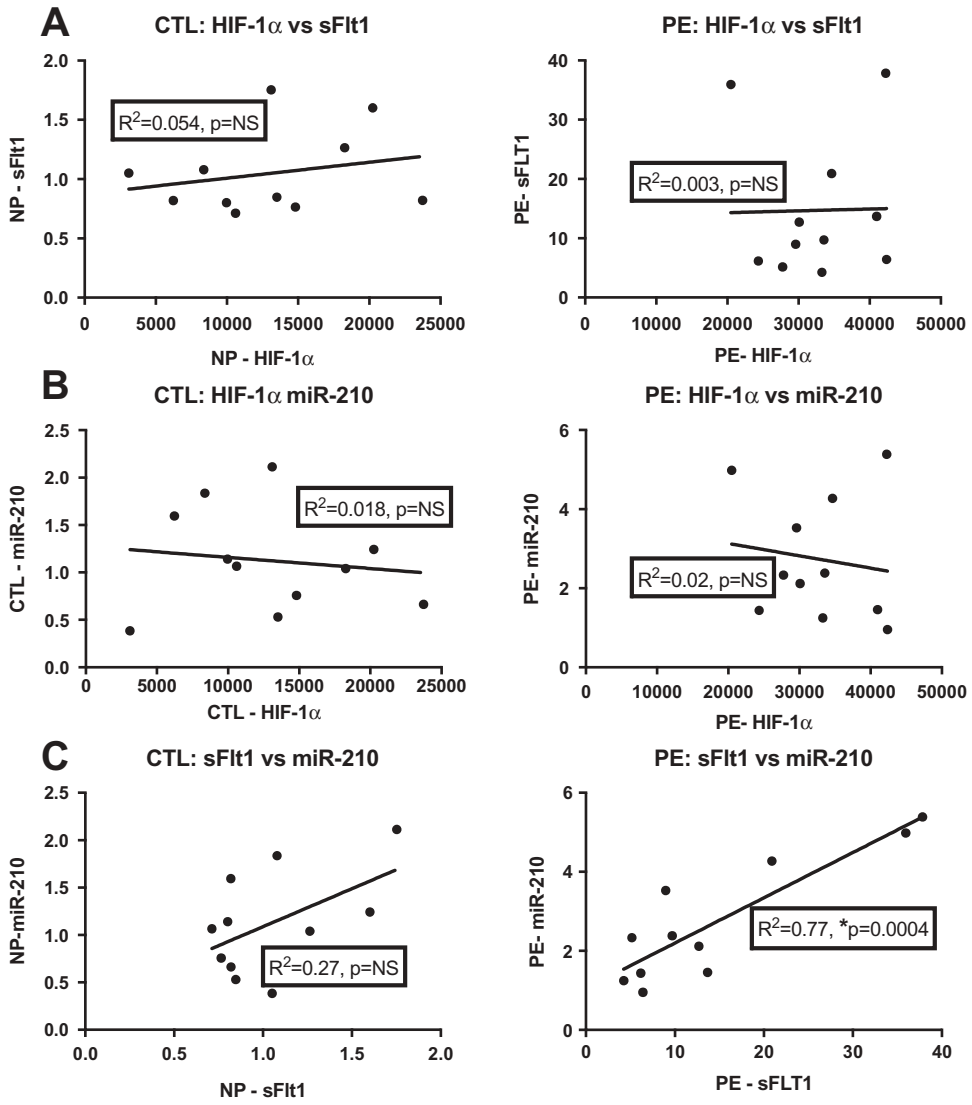

Figure 3. Correlation between HIF-1α and PE biomarkers. (A) Correlation plot for HIF1α levels vs sFlt1 levels in controls (left) and PE (right) are depicted. (B) Correlation plot for HIF1α levels vs miR-210 levels in controls (left) and PE (right), ©. Correlation plot for sFlt1 levels vs miR-210 levels in controls (left) and PE (right) are depicted.

humans with PE is still being debated. Further studies are needed to evaluate the role of these non-hypoxic pathways in humans to determine key drivers of placentation abnormalities that lead to the maternal syndrome of PE.

Disclosures

Dr. Karumanchi is a co-inventor of patents related to pre-eclampsia biomarkers that are held by Beth Israel Deaconess Medical Center. S.A.K. has financial interest in Aggamin LLC and reports serving as a consultant to Siemens Diagnostics, Roche and Thermofisher.

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