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Protective proteins and telomere length in placentas from patients with pre-eclampsia in the last trimester of gestation

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Abstract

Introduction—Visfatin/nicotinamide phosphoribosyltransferase (Nampt), an enzyme involved in energy metabolism and sirtuins, SIRT1 and SIRT3, which are NAD-dependent deacetylases, are critical for cellular function. All three either regulate or are regulated by intracellular NAD⁺ levels and therefore available cellular energy, important for placental cell survival and successful pregnancy. This study investigates whether these protective proteins are involved in the placental pathophysiology of pre-eclampsia (PE) and if they are associated with 8-oxo-deoxyguanosine (8OHdG), a marker of oxidative damage or with placental telomere length.

Methods—Maternal blood and placental samples were collected from 31 patients with PE and 30 controls between 31 and 40 weeks gestation. Quantitative immunohistochemistry was performed on placental specimens for visfatin/Nampt, SIRT1, SIRT3, and nuclear 8OHdG. Plasma visfatin was measured by ELISA and telomere length by Southern blot analysis of telomere restriction fragments.

Results—Visfatin/Nampt and SIRT1 in syncytiotrophoblast decreased in PE compared to controls ($p < 0.0001$, $p = 0.004$ respectively). SIRT3 decreased in PE most significantly at preterm ($p = 0.002$). 8OHdG was only significantly lower in preterm controls compared to term controls ($p = 0.01$) and correlated with SIRT1 in all samples ($r = 0.27$). Telomere length was not different in PE and controls.

Discussion—Decreased visfatin/Nampt, SIRT1 and SIRT3 in syncytiotrophoblast in PE suggests a lack of placental reserve in metabolic energy efficiency, increased inflammation, and lower resistance to environmental stressors. However, there was little effect on nuclear function, or evidence of genomic DNA damage, which would lead to cellular senescence and death.

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Keywords

Visfatin; Sirtuins; 8-Oxo-deoxyguanosine; Telomeres; Pre-eclampsia; Hypertension

1. Introduction

Preeclampsia (PE) is a condition of pregnancy classically characterized by new-onset hypertension and proteinuria, affecting 3–6% of pregnancies [1]. The clinical severity and gestational age of onset varies widely, with early-onset PE (28–34 weeks) mostly due to deficient placentation and late-onset PE (34 weeks-term) to maternal systemic vascular inflammation [2]. The early form is the most severe and involves derangement and injury of multiple organ systems. Current data suggests that in normal pregnancy, as the placenta ages with advancing gestation, the syncytiotrophoblast is under increasing stress, which is even greater in PE [3]. However, little is known about how syncytiotrophoblast health is maintained throughout gestation [4]. Although PE is associated with a greater level of oxidative stress, it is unknown how much energy the placenta expends in its protection [4]. We hypothesized here that the placenta may utilize some of the same mechanisms that the entire organism employs in the regulation of biological aging. Thus, we investigated some key protective proteins in the placenta and attempted to link them to some biomarkers of nuclear stress [5–7].

Three longevity proteins have been studied: nicotinamide phosphoribosyltransferase (Nampt, pre-B-cell colony enhancing factor or visfatin) [8] and sirtuins, SIRT1 and SIRT3 [9]. All either regulate, or are regulated by intra-cellular NAD⁺ levels and therefore available cellular energy. Accordingly, this regulates cell survival, critical for placental maintenance and successful pregnancy. Rongvaux and colleagues [10] identified visfatin/Nampt as a key NAD⁺ biosynthetic enzyme, which catalyzes the rate-limiting step in the NAD salvage pathway. However, its acetylation levels determine whether it remains intracellular or is secreted to act as a hormone [11]. When secreted, it is an important inflammatory modulator, which can both induce, and be induced by other proinflammatory cytokines [12]. Hypoxia upregulates the *Nampt* gene in vitro [12] and this in turn upregulates vascular endothelial growth factor (VEGF) and matrix metalloproteinases [13]. Nampt is produced by the human syncytiotrophoblast, with lower levels found in the placental bed in PE [14,15]. However, levels in maternal serum in PE are controversial, showing it either decreased [16], unchanged [17] or increased [18,19].

The seven mammalian sirtuins (SIRT1–7) serve as key controls for cell metabolism, stress-response and cellular proliferation. They are NAD-dependent protein deacetylases and/or ADP-ribosyltransferases [20,21]. They are important sensors of metabolic activity and NAD⁺ depletion and thus critical for cell survival [22]. SIRT1 is the major deacetylase with multiple down-stream targets and is involved in angiogenesis, inflammation, apoptosis, DNA repair, stress resistance, lipid metabolism and gluconeogenesis [23,24]. SIRT1 is present in placental syncytiotrophoblast and cytotrophoblast, where it is anti-inflammatory [25]. Nampt regulates SIRT1 levels and increases its activity [26]. However, SIRT3 has not been studied in placenta, but is located primarily in mitochondria and is cell-protective by

decreasing production of reactive oxygen species (ROS), increasing cellular respiration and preventing hypoxia-induced apoptosis [22,27]. Nampt also increases SIRT3 activity [28]. Therefore, these three proteins are all intimately related in function and protect the cells from excessive stress and senescence. Our aims were to show whether they might be involved in the placenta in PE, and whether they are associated with 8-oxo-deoxyguanosine (8OHdG) in the nucleus, as a biochemical marker of oxidative damage [6]. In addition, as chronic oxidative stress negatively influences DNA damage and telomere length, this was also measured [7].

2. Materials and methods

2.1. Patient selection and sample collection

This study was approved by Hawaii Pacific Health and Western Institutional Review Board. Study participants were admitted for induction of labor, scheduled cesarean delivery or spontaneous labor. Exclusion criteria included maternal connective tissue/autoimmune disease, renal disease, pre-gestational diabetes mellitus, active infection, chronic corticosteroid use, illicit drug or tobacco use, anemia (hemoglobin < 10 mg/dl), obstructive sleep apnea, multiple gestation, uterine malformations, fetal chromosomal/congenital anomalies. Each tissue was examined by a placental pathologist for histologic evidence of placental inflammation or chorioamnionitis and discarded if positive. Patients with PE were diagnosed using the criteria defined in the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy [1]. Gestational age was classified as preterm (< 36 weeks 6 days) or term (≥ 37 weeks 0 days). Preterm controls had spontaneous preterm labor and/or preterm premature rupture of membranes. SGA was diagnosed in preterm neonates using gender-specific Fenton growth curves [29]. Prior to delivery, maternal blood samples were collected and plasma stored at -80 °C. Immediately following delivery, two full-thickness placental samples near the cord insertion site excluding basal plate, were either fixed in neutral-buffered formaldehyde for 72 h and embedded in paraffin, or frozen and kept at -80 °C until used.

2.2. Maternal plasma analyses

Plasma samples were previously used for sFlt-1 measurement [30]. Visfatin/Nampt in plasma was measured by ELISA (Phoenix Pharmaceuticals, Burlingame, CA). Samples were assayed in duplicate, with intra-assay coefficient of variation of 10% and inter-assay variation of <15% and personnel blinded to clinical information.

2.3. Quantitative immunohistochemistry

Serial sections (5 µm) were heated at 95 °C in sodium citrate buffer (10 mM, pH 6.0) for 30 min for Nampt and SIRT3 and in 10 mM Tris/1 mM EDTA (pH 9.0) for 10 min for SIRT1 and 8OHdG. The Vectastain Elite kit (Vector Labs, Burlingame, CA) used the manufacturer's protocol. Non-specific binding was blocked with 2.5% normal serum for 30 min before incubation with primary antibodies: Nampt rabbit monoclonal (ABCAM, Cambridge, MA; ab109210), rabbit polyclonal antibodies to SIRT1 (Sigma-Aldrich, St. Louis, MO; s5322), SIRT3 (ABCAM; ab137698), or mouse monoclonal to 8OHdG

(ABCA, ab48508). Negative controls were species specific non-immune IgG with 3,3'-diaminobenzidine (DAB) substrate solution for visualization.

Multispectral imaging was performed with an Olympus BX51 microscope (Olympus America Inc, Mellville, NY) and a CRI Nuance spectral analyzer (Perkin Elmer, Waltham, MA) to obtain brightfield cubes between 420 and 700 nm wavelength at 20 nm intervals. The Inform Tissue Finder software (version 2.0.2, Perkin Elmer) unmixed spectral components and performed tissue segmentation for quantification of staining intensity, expressed as average signal intensity per pixel in mean optical density units (ODU) from 5 five different fields per patient. The average signal intensity was quantified at $\times 400$. Nuclear staining of 8OHdG was segmented by multispectral imaging with DAB positive (8OHdG) nuclei expressed as percentage of total nuclei in five different fields. Laboratory personnel were blinded to clinical information.

2.4. Telomere length analysis

A randomly selected subgroup of samples with PE but without SGA ($n = 18$) and gestational-age matched controls ($n = 16$) were used. Genomic DNA was extracted from frozen placental tissue (100 μ g) with phenol/chloroform and digested with HinfI and RsaI restriction enzymes at 37°C. Southern blotting was performed with a radiolabeled telomere-specific 24 base-pair probe. Mean telomere length was determined by software Quantity One (Bio-Rad, Hercules, CA) and base-pair lengths calibrated to radiolabeled standards.

2.5. Statistical analysis

Demographic and clinical information were summarized (means $\pm$ SD) for continuous variables, frequencies and percentages for categorical variables. Chi-square test compared percentages between controls and PE and Fisher's exact test used if any expected frequency was <1 or 20% of expected frequencies were <5 . Two sample *t*-test and Wilcoxon Rank Sum test for non-parametric analysis were used when normality was not met. Least square means for comparisons between two groups, adjusted by gestational age and slopes compared based on generalized linear models with Pearson correlation coefficient. Data analysis used SAS statistical software version 9.3 (SAS Institute Inc., Cary, NC). Two sided *p*-values 0.05 were considered statistically significant.

3. Results

3.1. Patient demographics

Patient demographics are shown in Table 1. Patients with PE ($n = 31$) consisted of 18 preterm (58%) and 13 term (42%). Of these, 23% had HELLP syndrome. Controls ($n = 30$) were 15 preterm and 15 term. There were no significant differences in gestational ages or chronic hypertension in women with PE and controls. Consistent with demographics of PE, gravidity, parity, mean blood pressures, SGA and mean birthweights were all significantly different in women with PE compared to controls at term. In preterm PE these patients had significantly higher blood pressures, but gravidity, parity, birthweight and SGA were not different from controls. However, gravidity and parity were significantly different between term PE and term controls ($p = 0.03$ and 0.01 respectively). Term controls were less likely to

have labored compared to women with PE, although there was no difference in the preterm groups. In term controls, neonates had significantly higher birthweights than those with term PE, but there was no difference in birthweights in the preterm groups. Because preterm (early-onset) and term (late-onset) PE, incidence of HELLP syndrome as well as labor/no-labor could have affected our results, each set of results obtained were analyzed separately for these factors. If any significant differences were found, the data are shown, otherwise two main groups, those with PE and those without (controls) are presented.

3.2. Visfatin/Nampt in placenta and maternal plasma

In the placenta, immunolocalization of visfatin/Nampt confirmed prominent staining in the syncytiotrophoblast and residual cytotrophoblast [15], with lighter staining in PE (Fig. 1A) compared to controls (Fig. 1B) and no staining in the negative control (Fig. 1C). There were no differences in staining location or intensity at preterm/term, in women with/without HELLP syndrome, or labor/no labor. Quantitation showed significantly decreased visfatin/Nampt ($p < 0.0001$) in all PE compared to all controls (Fig. 1D). However, maternal plasma visfatin/Nampt was not significantly different in PE (Fig. 1E). Adjusting for gestational age (31–40 weeks), there were no significant differences in either placental or plasma levels in all controls (Fig. 1F). However, in the placenta, visfatin/Nampt was significantly lower in PE ($p = 0.0002$) compared to controls (Fig. 1G), although the slopes were not significantly different.

3.3. SIRT1 localization in placenta

SIRT1 prominently stained the syncytiotrophoblast and remaining cytotrophoblast, agreeing with Lappas et al. [25]. In PE, staining was lighter (Fig. 2A) compared to controls (Fig. 2B) with no staining in the negative control (Fig. 2C). There were no differences in its localization at preterm/term or in HELLP syndrome. Quantitation in all subjects showed significantly decreased SIRT1 in PE ($p = 0.004$) compared to controls (Fig. 2D). Adjusted for gestational age (31–40 weeks), SIRT1 was significantly lower in PE ($p = 0.006$) with the slopes significantly different ($p = 0.038$) (Fig. 2E).

3.4. SIRT3 localization in placenta

SIRT3 prominently stained the syncytiotrophoblast and remaining cytotrophoblast. There was lighter staining in preterm PE (Fig. 3A) compared to preterm controls (Fig. 3B) and no staining in the negative controls (Fig. 3C). This difference was not evident in the term samples. There were no differences in patients with HELLP syndrome. Quantitation in all samples showed significantly decreased SIRT3 in all PE compared to controls ($p = 0.049$), but this was highly significant at preterm ($p = 0.002$) (Fig. 3D). Adjusted for gestational age (31–40 weeks), there was no significant difference in PE. However, the slopes for PE and controls were significantly different ($p = 0.022$) (Fig. 3E).

3.5. 8OHdG localization in placenta

As expected, 8OHdG localized to the nuclei of the villous stroma (Fig. 4A), shown in the insert at double the magnification (Fig. 4B). With the Inform software, the same insert showed segmentation of all nuclei (green) (Fig. 4C) and their further segmentation into

either DAB positive (red) or DAB negative (blue) nuclei (Fig. 4D). The negative control showed no staining (Fig. 4E). Quantitation showed large variability and no difference in all PE compared to controls and no differences in either preterm or term PE with their respective controls (Fig. 4F). However, 8OHdG levels were significantly lower in all preterm samples compared to those at term ($p = 0.03$) (not shown) and especially so when the controls were compared at preterm and term ($p = 0.0105$) (Fig. 4F). When adjusted for gestational age, 8OHdG showed no difference in PE and controls, but the slopes were markedly different ($p = 0.002$) (Fig. 4G). Placental 8OHdG levels significantly correlated with SIRT1 levels in all samples ($r = 0.27$ ($p = 0.039$)) whereas there were no significant correlations with either visfatin/Nampt or SIRT3.

3.6. Telomere length analysis in PE

There was no significant difference in telomere length in PE (Fig. 5A). Adjusting for gestational age, the slopes were not significantly different, although there was a slight decline in telomere length towards term in PE (Fig. 5B). Telomere length significantly correlated ($r = 0.32$) with placental SIRT3 levels.

4. Discussion

We hypothesized that PE would cause increased production of placental visfatin/Nampt, SIRT1 and SIRT3 as cell-protective proteins, in an attempt to counteract some of the detrimental effects of this disease. However, we show here that all declined in PE compared to controls. Thus, it appears that the fundamental mitochondrial energy generating system of the syncytiotrophoblast was severely damaged in PE and provided no compensatory protection. On the other hand, maternal plasma visfatin, nuclear 8OHdG and placental telomere length showed no changes in PE, suggesting that although the placenta is inherently frail, the nucleus is still capable of protecting many fundamental cellular functions.

We confirmed placental localization of visfatin/Nampt and SIRT1 [14,15,25] and show their concurrent decline in PE. However, maternal blood levels of visfatin/Nampt were unchanged, agreeing with others [17]. Although our patient cohort included some with both PE and SGA, the numbers of patients with SGA neonates were too small to allow separate analysis. SIRT1 and visfatin/Nampt comprise a novel feedback loop, which produces a circadian oscillation of NAD⁺ [31,32]. In addition, SIRT1 causes deacetylation of Nampt, allowing its secretion and action as a hormone [11]. Thus, a placental decline of SIRT1 in PE would prevent Nampt deacetylation, reducing its secretion and levels in maternal blood, resulting in the maintenance of the intracellular pool of Nampt for intracellular functions. As an enzyme, Nampt activates SIRT1 [26], a relationship recently suggested in syncytiotrophoblast and important in maternal obesity [33]. Nampt also salvages NAD⁺, required for SIRT1 activity. SIRT1 is described as a gauge for cellular energy levels, protecting against physiological damage, thereby resulting in increased cell survival [9]. The NAD⁺ requirement of SIRT1 links cellular energy levels to epigenetic changes for organism adaptation to environmental conditions. Thus, Nampt and SIRT1 comprise an important partnership and cells with very low levels of Nampt are considered to be critically frail,

because they rely upon extra-cellular sources of NAD intermediates to maintain sufficient levels of NAD⁺ for function [31,32]. The lower levels of placental Nampt and SIRT1 in PE probably reflect the true frailty of the trophoblast. However, our results require confirmation since we only use immunocytochemistry for protein quantitation in the placenta. In addition, concurrent measurements of NAD⁺ activity and some key intermediates involved in this pathway would also provide valuable confirmation.

SIRT3 is primarily mitochondrial, where it deacetylates and activates mitochondrial enzymes involved in fatty acid beta-oxidation, amino acid metabolism, electron transport and antioxidant defenses [34,35]. Mitochondria have their own NAD⁺ generating system, driven by SIRT3 [28] and NAD⁺ salvage pathway, driven by Nampt [36]. Nampt increases SIRT3 activity and maintains mitochondrial levels of NAD⁺ under conditions of genotoxic stress, suggesting their contribution to cell survival [28]. We show SIRT3 in the syncytio/cytotrophoblast and highly significant differences in the slopes with gestational age in PE and controls, suggesting the greatest difference occurred in early gestation. Morphological and proteomic studies suggest mitochondrial involvement in PE [37,38]. Indeed, defects in mitochondria might be a possible cause of PE [39]. The mitochondrial transport chain is the primary source of reactive oxygen species (ROS) [40] and its energy metabolism increases with SIRT3 activation, resulting in inhibition of apoptosis [35]. One of the most abundant DNA mutations caused by ROS is 8OHdG, considered a biomarker of oxidative DNA damage [40]. We show a correlation between SIRT1 and 8OHdG levels, reflected in their nuclear co-location. Although there were no differences in 8OHdG in PE, its levels in controls significantly increased at term compared to preterm. Previous data also showed no changes in PE, however levels increased in those with PE and SGA [41]. Accumulation of 8OHdG and mitochondrial DNA damage are exponential and inversely proportional to lifespan in the heart and brain [42]. Further studies are required to elucidate the exact roles of mitochondrial dysfunction in PE and its relationship with nuclear function.

Genomic DNA fragmentation analysis, considered the “gold standard” for measurement of placental telomere length showed no change in PE. A subset of patients was used, only those who were non-smokers and without SGA because both are associated with telomere shortening [43,44]. Our results agree with other work showing no change in placental telomere length in PE [45]. However, the marked correlation between telomere length and SIRT3 suggests a role for SIRT3 in the control of telomere length, possibly by placental telomerase activity. This has not been measured here and clearly this needs to be integrated in further studies. On the other hand, the significant increase in 8OHdG only in controls from preterm to term, not evident in PE, suggests differences in some nuclear protective systems throughout normal gestation, as well as in PE, which need to be further explored.

In summary, we have shown a reduction in some cell-protective proteins in the trophoblast in PE, with marginal effects on nuclear function. Thus, in spite of mitochondrial damage in PE, the nuclei appear to still be capable of protecting the trophoblast from cellular senescence and death.

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Abbreviations

PE	preeclampsia
HELLP	hemolysis, elevated liver enzymes, and low platelets
ROS	reactive oxygen species
sFlt-1	soluble vascular endothelial growth factor receptor-1
SGA	small for gestational age
Nampt	nicotinamide phosphoribosyltransferase
SIRT1	sirtuin 1
SIRT3	sirtuin 3
8OHdG	8-oxo-deoxyguanosine

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Fig. 1.

Visfatin/Nampt in placenta and maternal plasma. Representative examples of placental immunostaining at term, A) PE, B) control, C) negative IgG control. Visfatin/Nampt localized to the syncytiotrophoblast (ST) and remaining cytotrophoblast (CY) (arrows). D) Quantitation showed a significant decrease ($p < 0.0001$) in placenta in PE ($n = 31$) compared to controls ($n = 30$). E) Maternal plasma levels were not significantly different. F) As a function of gestational age (31–40 weeks), there was no significant difference in placental (solid line, closed triangles) or maternal plasma (dotted line, open circles). G) Adjusted for gestational age, placental levels were significantly lower ($p = 0.0002$) in PE (solid line, closed triangles) compared to controls (dotted line, open circles). The slopes were not significantly different. Original magnification 400 $\times$.

Fig. 2. Placental SIRT1 in PE. Representative examples of placental immunostaining at term, A) PE, B) control, C) negative control. SIRT1 localized to the syncytiotrophoblast (ST) and remaining cytotrophoblast (CY) (arrows). D) Quantitation showed SIRT1 significantly decreased ($p < 0.004$) in PE ($n = 31$) compared to controls ($n = 30$). E) Adjusted for gestational age (31–40 weeks), SIRT1 was significantly lower in PE (solid line, closed triangles) compared to controls (dotted line, open circles) ($p = 0.006$). The slopes were significantly different ($p = 0.038$). Original magnification 400 $\times$.

Fig. 3.

Placental SIRT3 in PE. Representative examples of placental immunostaining at preterm, A) PE, B) control, C) negative control. SIRT3 localized to the syncytiotrophoblast (ST) and cytotrophoblast (CY) (arrows). D) Quantitation showed SIRT3 significantly decreased ($p = 0.002$) in preterm PE ($n = 18$) compared to preterm controls ($n = 15$). E) Adjusted for gestational age (31–40 weeks), PE (solid line, closed triangles) was not significantly different from controls (dotted line, open circles). However, the slopes were significantly different ($p = 0.022$). Original magnification 400 $\times$.

Fig. 4.

Placental 8OHdG in PE. Representative example of a term placenta (control) immunostained for 8OHdG, demonstrating nuclear quantitation with Inform software. A)

Immunolocalization using DAB (brown) showing nuclei within the villous stroma positively stained, original magnification 400× and insert from (A) at twice the original magnification in (B). The same image (C) showing segmentation of all nuclei (green). D) The same image segmented into either DAB positive (red) or DAB negative (blue) nuclei. E) Negative control.

F) Quantitation expressed as 8OHdG positivity as percentage of total nuclei showed no significant differences in PE and controls due to wide variability. However, controls at preterm ($n = 15$) were significantly lower ($p = 0.0105$) than controls at term ($n = 15$). G) When adjusted for gestational age (31–40 weeks), PE (closed triangles, solid line) compared to controls (open circles, dotted line) were not significantly different. However, the slopes were significantly different ($p = 0.002$).

Fig. 5.

Placental telomere length in PE. A subset of samples with PE and no SGA ($n = 18$) were compared to controls ($n = 16$), A) showed no significant difference. B) Adjusted for gestational age (31–40 weeks), PE (closed triangles, solid line) and controls (open circles, dotted line) showed slopes were not significantly different, although there was a trend for a decline towards term in PE.

Table 1

Patient demographics.

Variables ^a	Preterm		Term		<i>p</i> -value
	PE n = 18	Controls n = 15	PE n = 13	Controls n = 15	
Mean Age, years	28.2 ± 7.7	29.1 ± 5.8	27.7 ± 5.8	30.1 ± 6.6	0.74
Gravidity	2.1 ± 1.4	3.5 ± 2.7	2.0 ± 1.5	3.5 ± 1.8	0.03
Parity	0.7 ± 0.9	1.6 ± 1.5	0.6 ± 1.4	1.7 ± 1.2	0.01
Mean Systolic BP, mmHg	166.2 ± 17.6	119.1 ± 9.1	160.2 ± 17.8	112.8 ± 8.6	0.0001
Mean Diastolic BP, mmHg	96.1 ± 10.9	71.9 ± 9.3	97.8 ± 8.4	65.9 ± 7.9	0.0002
Labor, n (%)	14 (77.8)	7 (46.7)	12 (92.3)	5 (33.3)	0.002
Chronic Hypertension, n (%)	3 (16.7)	0	0 (0)	0 (0)	N/A
Small for gestational age neonate, n (%)	1 (5.6)	3 (20.0)	8 (61.5)	0 (0)	0.0004
Mean birthweight, g	2243.7 ± 595	2440.6 ± 630.5	2733.4 ± 413.8	3295.8 ± 289.7	0.004

^aMean ± SD are denoted, except when indicated.