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The expression of sirtuin1 in normal and preeclamptic villous explants under hypoxia

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Summary

Purpose of investigation: To investigate the expression of sirtuin1 and hypoxia-inducible factor-1α in normal and preeclamptic placenta under hypoxia. *Materials and Methods*: Twelve women with severe preeclampsia and ten normotensive women were enrolled in the study. Villous explants from the placenta were collected following cesarean delivery. Quantitative reverse transcription polymerase chain reaction, Western blot analysis, and immunohistochemical staining were performed to evaluate mRNA expression, as well as to quantify and identify the tissue localization of SIRT1 and HIF-1α in each placenta. *Results*: SIRT1 mRNA expression was lower in preeclamptic villous explants than in normal explants, while HIF-1α mRNA expression was higher in the preeclamptic placenta than in the normal placenta. Expression of SIRT1 mRNA decreased under hypoxia, although expression of HIF-1α mRNA increased after 6 and 24 hours of hypoxia. *Conclusion*: Down-regulation of SIRT1 might be associated with stabilization of HIF-1α following prolonged hypoxia in preeclamptic placenta.

Key words: Sirtuin1, Hypoxia-inducible factors-1, alpha Subunit, Pre-Eclampsia, Placenta.

Introduction

Preeclampsia (PE) affects approximately 6–8% of all pregnancies worldwide and is one of the leading causes of maternal and perinatal morbidity and mortality [1]. Preeclampsia is characterized by the onset of hypertension and proteinuria after 20 weeks of gestation [2]. Advanced-stage clinical symptoms include seizures, renal failure, fetal growth retardation (FGR), and/or HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome [3]. Without proper treatment, it can develop into eclampsia, which is a life-threatening seizure occurring during pregnancy [2,3]. Current treatment for this disorder is limited to symptomatic therapy, and the only known cure is rapid delivery of the placenta [2].

The syndrome is thought to originate from insufficient trophoblast invasion and abnormal spiral artery remodeling, leading to placental ischemia, which initiates a hypoxia-induced stress response and endothelial dysfunction [4]. When uteroplacental hypoxia occurs, maternal blood is normoxic, but the flow into the intervillous space is heterogeneous and locally compromised. Consequently, placental villi are exposed to a higher hypoxic oxygen supply, and this leads to the clinical symptoms of preeclampsia [5]. Cellular responses to changes in oxygen tension during normal development or pathological processes are regulated by the hypoxia-inducible transcription factor (HIF) [6]. HIF-1α is a major transducer of hypoxia signaling in the placenta, and is highly expressed in the hypoxic environment of the placenta during early gestation, playing an important role in embryonic development, placental vascularization, and differentiation of trophoblasts [7,8]. HIF-1α, through regulation of soluble fms-like tyrosine kinase 1 and soluble endoglin, has been shown to be abnormally up-regulated in preeclamptic women and may play a key role in the development of preeclampsia [9,10].

Silent mating type information regulation 2 homolog (Sirtuin 1, SIRT1) is the most studied sirtuin family member, mainly due to its ability to promote longevity in *Caenorhabditis elegans*, *Drosophila*, and mammals [11-13]. The deacetylase activity of SIRT1 plays an important function by targeting histones and results in tighter chromatin structure and transcriptional repression [14]. Importantly, SIRT1 controls the stability and activity of a broad range of transcription factors including HIF-1α [15]. It is reasonable to hypothesize that alterations in SIRT1 expression may be involved in preeclampsia via changes in HIF-1α expression; our aim was to investigate the expression of SIRT1 and HIF-1α in the placenta of normal and preeclamptic women under early and prolonged hypoxia.

Materials and Methods

Sample collection

Placentas from 12 patients with severe PE and 10 control placentas from normal pregnancies were collected at the time of

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their cesarean section at the Severance Hospital, Yonsei University Health System. To standardize collection methods, the same investigator collected all samples, and only the central portions of the placentas were collected after placental delivery. Control subjects were normotensive pregnant women admitted for elective cesarean section. Collection and processing of human placentas were approved by the hospital's institutional review board, and informed consent was obtained from each patient. All women in the PE group had severe PE, diagnosed in accordance with the American College of Obstetricians and Gynecologists guidelines [16], as the presence of hypertension and proteinuria beyond 20 weeks of gestation. Hypertension was defined as blood pressure >160/110 mmHg on two occasions at least 6 hours apart; proteinuria was defined as >5 g protein in a 24-hour urine collection, or a score of >3+ on dipstick testing of two random urine samples collected at least 4 hours apart. Women with preterm rupture of membranes, multiple pregnancies, fetal structural or genetic anomalies, maternal chronic hypertension, HELLP (hemolysis, elevated liver function and low platelets) syndrome, cardiovascular disease, renal disease, hepatic disease, diabetes, or other infectious or autoimmune diseases were excluded from this study.

Placenta villous explant culture

Several cotyledons from third trimester placentas were excised at random and rinsed extensively in sterile saline to remove blood. Decidua and large vessels were removed from the villous placenta by blunt dissection. The villous tissue was finely dissected into 5-mg pieces and washed repeatedly before culture in sterile phosphate-buffered saline (PBS) and antibiotics (penicillin and streptomycin) to remove maternal blood. About three pieces of explant tissue were placed into 12-well plates containing 3 mL of DMEM/F12 (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (penicillin, streptomycin). All explants were incubated at 37°C for 24 hours under standard tissue culture conditions of 20% O2/5% CO2-balance room air (normoxia) in a cell culture incubator. For hypoxia treatment, explants (n = 5) were cultured at 37°C, 5% CO2 in an atmosphere of 20% or 1% O2 for 6 and 24 hours in a hypoxic chamber (Thermo Scientific, Waltham, MA, USA) with an oxygen probe for continuous monitoring. At the end of the incubation period, explants were removed, excess medium was removed with sterile cotton gauze, and samples were flash frozen in liquid nitrogen and stored at -70°C. The medium was pooled and then stored at -70°C in aliquots.

Extraction of total RNA and reverse transcription

Total RNA was extracted from placental tissue according to the manufacturer's protocol (Intron Biotechnology, Seongnam, Republic of Korea); 1 mg of total RNA was used in reverse transcription using Superscript III reverse transcriptase (Invitrogen).

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

SIRT1 and HIF-1α mRNAs and expression of the internal standard [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] were quantified by real-time polymerase chain reaction (PCR). PCR was performed using the following primers SIRT1-forward 5'-TGCGGGAATCCAAAGGATAA-3' and reverse-5'-CAGGCAAGATGCTGTTGCA-3'; HIF-1a-forward 5'-ACGTTCCTTCGATCAGTTGT-3' and reverse-5'-TTTGAGGACTTGCGCTTTCA-3'; and GAPDH forward-5'-TCGACAGTCAGCCGCATCTTCTTT-3' and reverse-5'-ACCAAATCCGTTGACTCCGACCTT-3'. Expression of SIRT1 and HIF-1a were normalized to GAPDH expression.

Western blot analysis

Proteins were extracted from freeze-dried, dissected, and homogenized placental samples using lysis buffer (Cell Signalling Technology, Beverly, MA, USA). Lysates were loaded on a 6% mini-SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) for 1 hour after electrophoresis. Blocking was performed with 5% non-fat dry milk and 0.1% Tween 20 in Tris-buffered saline (TBST) for 2 hours at room temperature. Membranes were incubated with primary polyclonal SIRT1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), monoclonal HIF-1α antibody (BD Bioscience, Franklin lakes, NJ, USA), or monoclonal GAPDH antibody (Millipore) overnight at 4°C with gentle shaking. After incubation, the membranes were washed three times in TBST for 10 minutes, and then exposed to anti-mouse immunoglobulin G horseradish-peroxidase-conjugated antibody (Jackson ImmunoResearch Inc., West Grove, PA, USA) for 1 hour at room temperature. The membranes were treated with enhanced chemiluminescence reagents I and II (Amersham Life Science, Arlington Heights, IL, USA), and exposed to hyper-X-ray film (AGFA, Mortsel, Belgium). GAPDH was used as an internal control to compare data from different films.

Densitometry

Membranes were visualized for densitometric scanning using a densitometer (IMAGE READER LAS-1000 lite, Fuji Photo Film Co. Ltd., Tokyo, Japan). Densitometric analysis was performed with digital analysis software (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Immunohistochemical staining

Paraffin embedded tissues were sectioned into 5-μm thick slices. Sections were deparaffinized and rehydrated using xylene and alcohol. The sections were pretreated for 10 minutes in a microwave oven for antigen retrieval and incubated at room temperature for 30 minutes. After rinsing, sections were incubated in 2% H2O2 in PBS (pH 7.4) for 10 minutes to inhibit endogenous peroxidase activity. Sections were then incubated with 5% bovine serum albumin for 1 hour to block nonspecific binding.

Tissue sections were then exposed to primary antibodies: 1:100 SIRT1 (Santa Cruz Biotechnology, Inc.) or 1:400 HIF-1α polyclonal antibody (Thermo Scientific) for 60 minutes at room temperature. All sections were sequentially treated with biotinylated anti-rabbit immunoglobulin for 30 minutes, followed by peroxidase-labeled streptavidin for 30 minutes and diaminobenzidine in the presence of hydrogen peroxide. Controls were incubated with PBS in place of primary antibody; no positive staining was observed in any case. The reaction was visualized using a solution containing 0.0125% diaminobenzidine and 0.005% hydrogen peroxide. After rinses, sections were counterstained with hematoxylin and mounted with mounting medium. Staining was scored as follows: 1, no staining; 2, weak or focal staining; 3, moderate staining in most cells; and 4, strong staining in most cells. Photographs were taken with a 2048×1536 pixel digital CCD camera (DP70, Olympus, Tokyo, Japan) for immunohistochemical images. Images were analyzed and each cell was semi-quantitatively scored for the expression of SIRT1 and HIF-1α as follows: 0, no staining; 1, weak intensity; 2, moderate intensity; and 3, high intensity.

Statistical analyses

Data were expressed as mean±standard error of the mean. The clinical parameters, real time-PCR and densitometric data analyses were compared by Mann-Whitney U-test using SPSS software (version 20.0.1, SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

Table 1. — *Clinical characteristics of the study groups.*

	Control (n=5)	Preeclampsia (n=6)	P-value
Age (years)	34.7±3.7	35.0±3.9	0.91
BMI (kg/m^2) *	22.5±5.4	22.2±5.2	0.94
BP (mmHg) †			
Systolic	113.6±9.9	177.4±41.8	<0.05
Diastolic	72.5±12.7	108.8±27.8	<0.05
GA‡ at delivery (weeks)	38.0±0.5	34.0±3.6	0.06
Birth weight (g)	3194.5±371.5	1994.0±791.7	<0.05

*BMI; body mass index, †BP; blood pressure, ‡GA; gestational age.

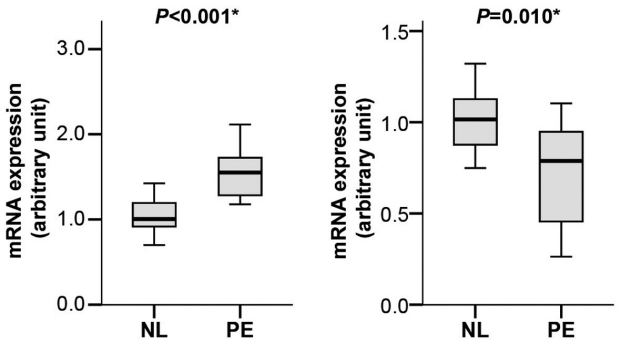

Figure 1. — Quantitative reverse transcription polymerase chain reaction (RT-PCR) showing that HIF-1α mRNA expression (Left) is higher and SIRT1 mRNA expression (Right) is lower in the preeclamptic (PE) placenta than in the normal (NL) placenta ($P < 0.005$).

Results

Demographic and clinical data from the studied subjects are shown in Table 1. There was no significant difference in maternal age between the normal and PE groups. In the PE group, systolic and diastolic blood pressures were significantly higher, and gestational age at delivery and birth weight were lower.

HIF-1α and SIRT1 mRNA were detected in normal and preeclamptic placental tissues by quantitative RT-PCR. HIF-1α mRNA expression was found to be significantly higher in the preeclamptic placenta than in the normal placenta ($P < 0.001$), while SIRT1 mRNA expression was found to be significantly lower in the preeclamptic placenta than in the normal placenta ($P < 0.005$) (Figure 1).

Immunohistochemical staining was performed for tissue localization of HIF-1α and SIRT1 in each placental sample. HIF-1α was expressed in the syncytiotrophoblasts and endothelial cells of chorionic villi vessels of the placenta. HIF-1α immunoreactivity was greater in the syncytiotrophoblasts of the preeclamptic placenta specimens compared to those of the normal placenta, but was only weakly expressed in the endothelial cells of chorionic villi vessels in both groups (Figure 2). SIRT1 was expressed in syncytiotrophoblasts and endothelial cells of chorionic villi vessels of the placenta. However, SIRT1 staining intensity was significantly lower in the syncytiotrophoblasts of the preeclamptic placenta specimens compared to those of the normal placenta, and was also found to be of lower intensity in the endothelial cells of chorionic villi vessels of the preeclamptic placenta compared to the normal placenta (Figure 3).

HIF-1α protein was expressed to a greater extent in the preeclamptic placenta specimens compared to specimens from the normal placenta, while HIF-1α protein expression increased greatly 6 hours and 24 hours after exposure to hypoxia than in samples in absence of hypoxia. SIRT1 protein showed a lower expression both in the preeclamptic

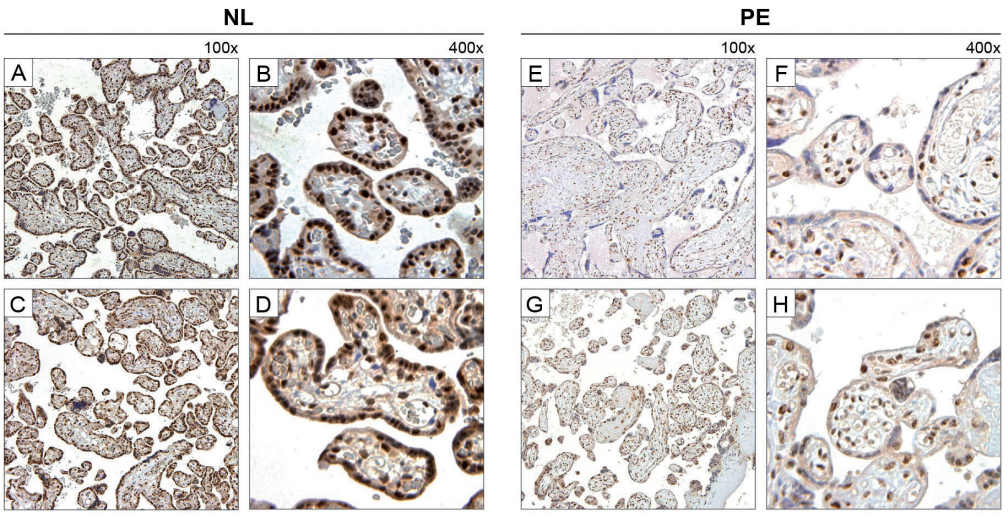

Figure 2. — Immunohistochemical staining of SIRT1 in normal and preeclamptic placenta. The intensity of immunohistochemical staining for SIRT1 is significantly lower in the syncytiotrophoblasts and endothelial cells of chorionic villi vessels of preeclamptic (PE) placenta than in the normal (NL) placenta. A, B, C and D, normal placenta; E, F, G and H, preeclamptic placenta.

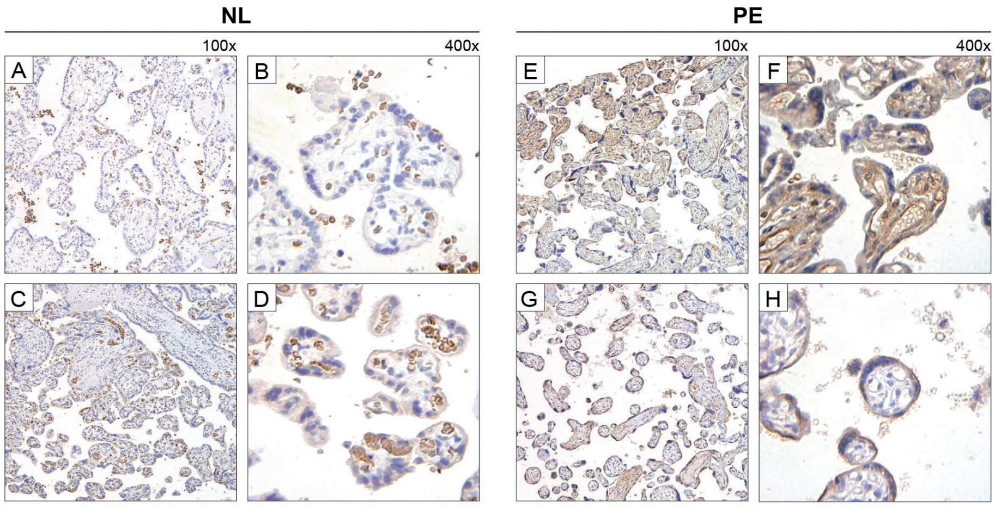

Figure 3. — Immunohistochemical staining of HIF-1α in normal and preeclamptic placenta. The intensity of immunohistochemical staining for HIF-1α is significantly higher in the syncytiotrophoblasts of preeclamptic (PE) placenta than in the normal (NL) placenta, while the intensity of HIF-1α expression in endothelial cells of chorionic villi vessels is equally weak in both normal and preeclamptic placenta. A, B, C and D, normal placenta; E, F, G and H, preeclamptic placenta.

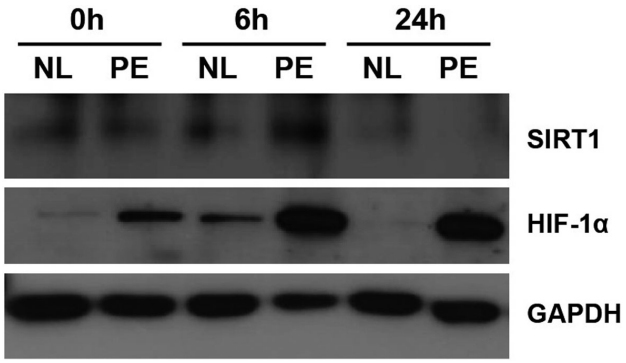

Figure 4. — Expression of HIF-1α mRNA and SIRT1 mRNA under hypoxia. Western blots showing that HIF-1α protein expression is stronger in preeclamptic (PE) placenta samples than in the normal (NL) placenta. HIF-1α expression increases after exposure to hypoxic conditions. SIRT1 protein expression is lower in both normal and preeclamptic placenta after a 24-hour exposure to hypoxia.

placenta and in the normal placenta following prolonged hypoxia (24-hours), while SIRT1 protein was expressed both in normal and preeclamptic placenta samples after 6 hours of hypoxia to the same extent as non-hypoxic placentas (Figure 4).

Discussion

In this present study, we investigated SIRT1 and HIF-1α expression in trophoblasts of the preeclamptic placenta. We showed that preeclampsia was coupled with a low level of SIRT1 and high expression of HIF-1α, suggesting that an activated hypoxic response is correlated with a hypoxic dysfunction of the trophoblast.

We first compared the expression of SIRT1 and HIF-1α in preeclamptic and normal placentas. The expression of SIRT1 was lower and HIF-1α was higher in the preeclamptic placenta, particularly in the syncytiotrophoblast. HIF-1α is the key mediator of oxygen homeostasis under hypoxic conditions and modulates the expression of various genes regulating placental development and maturation [7,17-19]. HIF-1α protein increased under hypoxia in syncytiotrophoblasts, cytotrophoblasts, and in the placental vasculature [20]. Interestingly, recent evidence suggests that HIFs are activated in normoxic conditions by several stimuli, including the renin angiotensin system, growth factors, immunogenic cytokines [17,18], and SIRT1. Thus, SIRT1 may also play a role in the human placenta; however, to date, there is little data available regarding the expression and regulation of SIRT1 proteins in the placenta.

SIRT1 modulates gene expression in target tissues by regulating transcriptional coregulators or by directly interacting with transcription factors. SIRT1 expression was localized in the syncytiotrophoblast layer and in the cytotrophoblasts of the placenta, amnion epithelium, trophoblast layer of the chorion, and decidual cells. SIRT1 expression levels have been reported to decrease in human gestational tissues with spontaneous labor onset, indicating that SIRT1 may be involved in regulating pregnancy and parturition [21].

Endothelial dysfunction has been implicated in the pathophysiology of preeclampsia. SIRT1 inhibition induces premature senescence of endothelial cells, which is involved in endothelial dysfunction, decreased eNOS expression and NOS activity in human umbilical vein endothelial cells (HUVEC) [22]. Moreover, SIRT1 overexpression prevented senescence and enhanced stress resistance in HUVEC [23]. This suggests that SIRT1 may protect from endothelial dysfunction and thereby contribute to maintenance of vascular homeostasis.

Recent reports indicate SIRT1 can also regulate HIF-1α activity; however, the interaction between SIRT1 and HIF-1α is still unclear. SIRT1 is regulated by the intracellular NAD+/NADH ratio and hypoxia is known to reduce this ratio [15,24-26]. Two recent reports have shown different results. Lim et al. have reported that SIRT1 negatively regulated HIF-1α activity by decreasing NAD+ levels, therefore inhibition of SIRT1 may provoke an exaggerated HIF-1α-mediated response, especially with prolonged hypoxia [15]. However, Laemmle et al. have reported that inhibition of SIRT1 decreases HIF-1α-mediated transcriptional activity, suggesting that SIRT1 is necessary for HIF-1α protein stability [26]. Instead in the trophoblast, hypoxia up-regulated SIRT1 expression, which then enhanced the expression of N-Myc down-regulated gene1 (NDRG1) [27] found to be elevated in complicated pregnancies such as preeclampsia [28,29].

To confirm the differences in expression profiles of SIRT1 and HIF1α between normotensive women and preeclamptic women, culture of villous explants from the normotensive and preeclamptic groups were performed. The expression of SIRT1 decreased in the prolonged hypoxic condition both in normal and preeclamptic placenta, while the expression of SIRT1 remained unchanged in both normal and preeclamptic placentas after 6 hours of exposure to hypoxic conditions. Moreover, this change was greater in the preeclamptic placenta compared to the normal placenta. Expression of HIF-1α was greater in preeclamptic placenta than in normal placenta in early and prolonged hypoxic conditions. HIF-1α expression increased in response to hypoxia and then decreased following prolonged hypoxia in the normal placenta, while highly expressed HIF-1α remained elevated in the preeclamptic placenta. Moreover, levels of SIRT1 also changed following early hypoxia and prolonged hypoxia exposure in preeclamptic explants. HIF-1α is expressed and degraded constantly in normoxic condition. Once the tissue is exposed in hypoxic condition, degradation of HIF-1α is inhibited and HIF-1α induce adaptation of the tissue under hypoxia [8]. In normotensive placenta, degradation of HIF-1α could be inhibited under early hypoxia while degradation of HIF-1α was stabilized under late hypoxia. In preeclamptic placenta, degradation of HIF-1α could be constantly repressed under hypoxia. This results suggest that pattern of expression of SIRT1 in preeclamptic placenta under hypoxia is different from that in normotensive placenta. This differences could arise from the different process in placentation under preeclampsia and normal pregnancy. Besides, the result of increased expression of both SIRT1 and HIF-1α after six hours of exposure to hypoxia is opposite the hypothesis that HIF-1α is repressed by SIRT1. Overall, our results suggested that the mechanism of SIRT1 in regulating HIF-1α function could be different in early and prolonged hypoxia in preeclamptic and normal placenta.

The limitations in this study include the lack of experimental data confirming the signaling cascades involved in the regulation by SIRT1. However, Lim et al reported that SIRT1 could repress HIF-1α by deacetylation in colon cancer [15]. Since primary trophoblasts do not divide in vitro, transfection efficiency is low. Therefore, experimental protocols involving cell transfection, including siRNA or co-immunoprecipitation were not feasible. Further experimental studies activators or inhibitors of SIRT1 activity on villous explants are needed. In addition, in this study, chorionic villous explants from term placenta after delivery were obtained and expression of SIRT1 was confirmed under hypoxia by using QF-PCR, immunohistochemistry and western blot. It could reflect secondary changes after progression of preeclampsia, not differences between early change of preeclamptic placenta and normal placenta.

Nonetheless, this is the first study to show decreased expression of SIRT1 and increased expression of HIF-1α in the placentas of severe preeclamptic patients. In conclusion, there are significant alterations of SIRT1 expression in cells and tissues derived from preeclamptic placenta compared to placentas in normal pregnancies. This finding might contribute to a better understanding of the pathophysiology of PE and to HIF-1α regulation in preeclamptic placentas. Further insight into the control of HIF-1α regulation under non-hypoxic conditions may provide additional and definitive evidence for its role in the complexities of placentology. We revealed that changes of expression in SIRT1 and HIF-1α in preeclamptic and normotensive placenta under hypoxia in this study. Further research should be followed to reveal correlation between SIRT1 and HIF-1α in preeclampsia, and the role of SIRT1 and HIF-1α in pathogenesis of preeclampsia.

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