Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Evaluation of protein levels of autophagy markers (Beclin 1 and SQSTM1/p62) and phosphorylation of cyclin E in the placenta of women with preeclampsia

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Received September 21, 2017; **Accepted** December 18, 2017; **Published** December 30, 2017

Doi: http://dx.doi.org/10.14715/cmb/2017.63.12.12

Abstract: Preeclampsia is a severe multisystem disorder, and its pathophysiology is still not completely understood. Autophagy, a recycling process that maintains cellular homoeostasis during differentiation and development, is controversial regarding increased or decreased autophagic activity in preeclampsia. The aim of this study was to determine whether autophagy is increased in the placentas of women with preeclampsia by examining the protein levels of autophagy markers (Beclin 1 and SQSTM1/p62) and phosphorylation of cyclin E. For this purpose, placentas from preeclampsia (n=10) and control (n=10) pregnancies were included in this study. The protein expression of autophagy-related markers Beclin1, SQSTM1/p62 and phosphorylation status of cyclin E were detected by Western blot. Our data showed that the protein levels of both Beclin 1 and SQSTM1/p62 were significantly increased, while the phosphorylation level of cyclin E was significantly decreased in placentas with preeclampsia compared to those derived from controls. The results of this study suggest that the autophagic activity is perpetually increased in preeclampsia and cyclin E protein stabilisation might be involved in the induction of autophagy.

Key words: Preeclampsia; Placenta; Beclin 1; SQSTM1/p62; Cyclin E.

Introduction

Preeclampsia is a severe pregnancy disorder characterised by hypertension and proteinuria and is tightly related to maternal morbidity and mortality. Although it affects approximately 3-10% of entire pregnancies worldwide (1,2), the underlying mechanism of preeclampsia is still not completely understood. The abnormal placenta or the placental dysfunction is considered to be the leading cause of preeclampsia as the removal of the placenta seems to remain the only current cure.

The placenta is the site of complicated maternal and fetal interactions such as gas and nutrient exchange and is a branching villous structure. It is known that each villus consists of endothelial cells in a mesenchymal connective tissue and trophoblasts. This mesenchymal connective tissue is surrounded by cytotrophoblast and syncytiotrophoblast cells (3,4). Syncytiotrophoblast cells enclose the entire surface of the placental villus and create an impermeable barrier between maternal blood and fetal tissues, while the extravillous trophoblasts differentiate from cytotrophoblast cells, migrate and invade into the decidua and then remodel the maternal spiral arteries (4). As the pathogenesis of preeclampsia is established at the time of trophoblast invasion and remodelling of the spiral arteries during the first trimester of pregnancy, it is implicated that the migration and invasion of trophoblasts are essential for the development of the placenta.

Autophagy a process of self-degradation of cellular constituents plays a major role in maintaining cellular homoeostasis (5). Autophagy is activated by intracellular stress, such as starvation, hypoxia, pathogen infection or reactive oxygen species and implied in the participation of other cellular processes including cellular differentiation, tissue remodelling, growth control, ageing and cellular immunity (6). When it is activated an autophagosome, a double-membrane cytosolic vesicle, is formed and autophagosome sequesters cytoplasm and delivers it to the lysosomes for degradation (7).

Initiation of autophagy and formation of autophagosome is regulated by autophagy-specific genes (Atg), including Beclin-1 (Vps30), the human homolog of yeast Atg6 / vacuolar sorting protein 30 (8). Beclin 1 contains three main domains: a BH3 domain (Bcl-2 homology domain, amino acids 114- 123), a central coiled-coiled domain (CCD, amino acids 144- 269) and an evolutionarily conserved domain (ECD, amino acids 244–337) (9). During autophagosome formation, Beclin 1 forms a complex by interacting with a class III phosphoinositide 3-kinase (PI3K) and Vps34, while the interaction between Beclin 1 and Bcl-2 or Bcl-XL leads to the inhibition of autophagy (9).

p62/sequestosome-1 (SQSTM1) is a cellular adaptor protein with several structural binding motifs and functions in the formation of multimeric signalling complexes (10). SQSTM1 is itself degraded by autophagy and involved in autophagic degradation of ubiquitinated protein aggregates in lysosomes by directly interacting with microtubule-associated protein 1A/1B-light chain

3 (LC3) (11). Conversion of cytosolic LC3I, also known as Atg8, to the membrane-bound LC3-II form results in its accumulation to autophagosomal membranes (12). As SQSTM1 is a multi-domain protein it is involved in the activation of NFκB signalling pathway, regulation of apoptosis via activation of polyubiquitinated caspase 8 and regulation of oxidative stress signalling by binding Keap and therefore blocking Nrf2 degradation (13). The role of autophagy has been identified in the human placentas (14). Biomarkers of autophagy including Beclin 1 were observed in cytotrophoblast, syncytiotrophoblast, and extravillous trophoblast, as well as maternal decidual stroma cells in normal gestation (15,16). Higher LC3II expression an indicator of increased autophagy was detected in villous cytotrophoblast and syncytiotrophoblasts in cesarian section placentas compared to placentas from spontaneous vaginal delivery (17). Autophagy found to be induced by hypoxia enhances extravillous trophoblast invasion in vitro (18).

Cyclin E1 is one of the regulators of cell cycle progression and forms a complex with Cyclin-dependent kinase-2 (Cdk2). This complex coordinates the G1-S transition by phosphorylation/ inactivation of pRb, which releases E2F-1 and promotes S-phase entry (19). Therefore, Cyclin E1-Cdk2 activity is highest in G1–S cells and lowest in quiescent cells. Cyclin E2, a second member of the Cyclin E family, shares 47% overall amino acid homology with cyclin E1 (20). The importance of Cyclin E family for the placenta was shown in double knockout cyclin E1-/- E2-/- mice embryos. Although cyclin E1-/- E2-/- embryos did not represent an apparent impairment in cell proliferation, these embryos die due to defects in endoreplication of the trophoblast giant cells, important for the development of the placenta, placental attachment and provision of nutrients to the developing embryo (20). For the reason that the placenta is exposed to hypoxia, reactive oxygen species, cytokines, low glucose concentrations and pathogens, the activation of autophagy is currently being considered as an important cytoprotective mechanism of the cells to overcome microenvironmental challenges. Despite the fact that the role of autophagy in the pathogenesis of preeclampsia is still controversial, it seems to be required for appropriate growth of the embryo and placenta. The objective of the current study was to determine whether autophagy is increased in preeclamptic placentas by examining the protein expression levels of autophagy markers and phosphorylation status of cell cycle regulator cyclin E.

Materials and Methods

Ethical approval performed in accordance with the Declaration of Helsinki was obtained from Dicle University Faculty of Medicine. Informed written consent for placental tissues was collected from all participants. Placentas from preeclampsia (n=10) and control (n=10) pregnancies were included in this study. Women with preeclampsia were chosen based on an elevated systolic and diastolic blood pressure (>140 mmHg, >90 mmHg) accompanied by proteinuria (300 mg/ 24 h) on urine analysis. Preeclampsia patients complicated with infection, chronic hypertension and any other chronic diseases were excluded from this study. To match the gestational ages of preeclamptic placentas, control placentas were derived from pregnant women who had spontaneous preterm delivery induced by uterine distension were received immediately after cesarean deliveries. These pregnant women who had spontaneous preterm delivery were selected by having a non-proteinuric pregnancy with no medical or obstetric complications. Demographic and clinical features of the groups were given in Table 1.

Sample preparation

Placental tissue samples were cut out from the maternal side near around the umbilical cord. These samples were immediately frozen using liquid nitrogen. The flash-frozen placental tissues were stored at -86 ºC until Western blot analysis. Western blot analyses were performed as described previously (21). Polyclonal rabbit anti-Beclin 1, polyclonal rabbit anti-p62/SQSTM1, polyclonal rabbit anti-pCyclinE1 (Thr 395) purchased from Santa-Cruz Biotechnology and anti-β-actin performed as a loading control (Abcam) were used in this study. The snap-frozen placenta samples were ground to a fine powder in a chilled mortar in the presence of liquid nitrogen. Immediately after grinding, the placenta powder was lysed on ice in a RIPA buffer (Sigma-Aldrich) consisting of protease and phosphatase inhibitor cocktail (Thermo Scientific). The amount of total cellular protein was measured using a BCA protein assay kit according to the manufacturer's instructions (Pierce, Thermo Scientific). Aliquots (20 µg of protein per lane) of total cellular protein were separated using SDS-polyacrylamide gel electrophoresis (10% gels) and blotted onto polyvinyl difluoride (PVDF) membrane (Bio-Rad). To prevent nonspecific binding, each membrane was blocked with 5% non-fat dry milk in PBS-T (0.05% Tween-20) for one hour at room temperature and then

Table 1. Demographic and clinical characteristics of the study groups.

Characteristics	Preeclampsia (n=10)	Control (n=10)	*p
Maternal age (years)	30.2±5.7	29.3±3.8	>0.05
Gestational age (week)	35.3±2.25	36.1±1.2	>0.05
BMI (kg/ m^2)	25.13±4.53	23.9±2.85	>0.05
Systolic blood pressure (mmHg)	164.5±12	117.8±4.4	<0.001
Diastolic blood pressure (mmHg)	104.5±17	75.3±4.7	<0.001
Proteinuria (g/24 h)	0.44±0.15	_	_
Method of delivery	Caesarean section	Caesarean section	_

Data are shown as mean ± SD. *p values <0.05 are represented in boldface.

incubated with primary antibodies for two hours at room temperature. Followed by Extensive washing with PBS-T, each membrane was further incubated anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP-goat-anti-rabbit). Immunoreactive bands were visualised using an enhanced chemiluminescence reagent (Bio-Rad), according to the manufacturer's protocol. The images were taken using ChemiDocTM MP (Bio-Rad).

Statistical analysis

Student's t-test was used to determine the significance of the results using the Sigmaplot 12 software package (Systat). A p-value of $< 0{\cdot}05$ was considered statistically significant.

Results

Protein expression levels of Beclin 1 and SQSTM1/p62

Expression status of Beclin 1 has been routinely used to monitor the autophagic activity. In the present study, the levels of Beclin 1 were examined in placentas from control and preeclampsia patients and found that the protein levels of Beclin-1 were significantly higher in preeclamptic placentas compared to those derived from control women (Figure 1). SQSTM1/p62 is an autophagic adaptor and links ubiquitinated substrates to the autophagy pathway. To further confirm increased activation of autophagy in preeclampsia, protein expression of SQSTM1/p62 was investigated. A decrease in SQSTM1/p62 level has been reported to be associated with the activation of autophagy (11). In our study, SQSTM1/p62 expression in addition to the presence of Beclin 1 expression was found to be increased in preeclamptic placentas compared to those derived from the control group (Figure 2).

Phosphorylation status of cyclin E in the preeclampsia group and control group

To compare the phosphorylation level of cyclin E between the preeclampsia and control group, phosphorylation of threonine 395 (T395) residue was examined in preeclamptic and control placentas by Western blot. It was found that the phosphorylation of cyclin E at T395 residue, been linked to the proteasome-mediated degradation of full-length cyclin E (22), was found to be significantly decreased in preeclamptic placentas compared to those derived from control women (Figure 3).

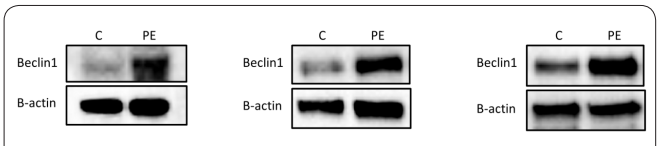

Figure 1. Increased Beclin 1 protein expression in preeclamptic placentas. Representative Western blot analysis of Beclin 1 expression in placenta samples from patients with preeclampsia (PE) and different controls (C). β-actin was used as a loading control. The image shown represents three representative examples of 10 separate experiments.

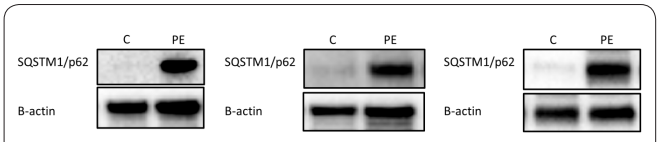

Figure 2. Protein levels of SQSTM1/p62 were elevated in preeclampsia. Representative Western blots of SQSTM1/p62 in preeclamptic (P) and control (C) placentas. The lowest panels represent loading control (β-actin). The image shown represents three representative examples of 10 separate experiments.

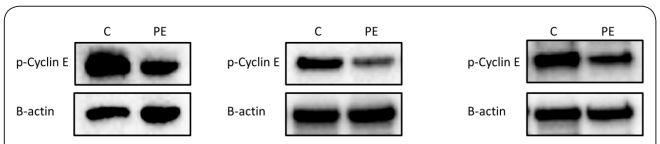

Figure 3. Phosphorylation of cyclin E at threonine 395 was decreased in preeclamptic placentas. Representative Western blots for p-cyclin E in preeclamptic (P) and control (C) placentas. β-actin was used to ensure equal loading. The image shown represents three representative examples of 10 separate experiments.

Discussion

Preeclampsia which underlying mechanism is still not fully understood is characterised by hypertension and proteinuria and one of the leading causes of maternal morbidity and mortality worldwide (1). Abnormal development and dysfunction of the placenta due to changes in the proliferation, differentiation, cell death, and invasion of trophoblasts were implicated in this severe disorder. Autophagy, responsible for degradation of misfolded proteins or damaged organelles, is considered as an important cytoprotective mechanism for human placentation and has recently been studied in preeclampsia. Several reports showed an altered and inconsistent autophagy activity in placentas from patients suffering from preeclampsia (24-29). Furthermore, a model in which cAMP signalling via ERK-mediated induction of cyclin E leads to enhanced perinuclear recruitment of Beclin 1 and formation of autophagosomes was introduced in mesenchymal stem cells (23). These reports prompted us to evaluate the protein expression levels of autophagy markers, Beclin 1 and SQSTM1/p62, and phosphorylation status of cyclin E in preeclamptic placenta tissues.

It appears that there have been no previous reports regarding on evaluation of autophagy-related proteins Beclin1, p62 and cyclin E in preeclamptic placentas. In our study, an increase in protein levels of both Beclin 1 and SQSTM1/p62 was detected in placental tissues from preeclampsia patients compared to those derived from control women. Furthermore, the phosphorylation of cyclin E at threonine 395 (T395) reported to be essential for its degradation through the ubiquitin/proteasome pathway (22) was significantly decreased in preeclamptic placentas which in turn may lead to an increase in cyclin E protein stabilisation.

In our study, we detected a significant increase in protein levels of both Beclin 1 and SQSTM1/p62 in preeclamptic placentas. Data from Oh et al. showed an elevated protein expression of LC3-II, an indicator of autophagosome formation in mammalian cells and reflects autophagic activity, in the placentas from severe preeclampsia compared with the placentas from normal pregnancies (24). However, in the same study unlike the LC3-II protein, the expression of Beclin 1 protein was

not changed in the same samples (24). Nevertheless, Gao and colleagues revealed that the expression of LC3 and Beclin 1 was significantly increased in placentas from pregnancies complicated by early-onset preeclampsia (25). Furthermore, Akaishi et al. also showed a significant increase in the expression of LC3-II concomitant with a significant decrease in the expression SQSTM1/p62 in placentas of women with preeclampsia irrespective of the presence or absence of fetal growth restriction, indicating activated autophagy in preeclampsia (26). Although the decreased SQSTM1/p62 expression is generally expected after activation of autophagy (26), we observed significantly higher protein expression of SQSTM1/p62 in preeclamptic placentas compared to control placentas. Explanations for this discrepancy are not clear; however, our results suggest that elevated protein levels of SQSTM1/p62 reflect a constant autophagy activity in preeclampsia.

In contrary to our and other's (24-26) results Hung et al. reported higher placental levels of autophagy-related protein LC3B-II in women with intrauterine growth restriction but not in women with preeclampsia (27). Similarly, Zhang et al. showed a decreased level of autophagy proteins, Beclin 1 and LC3II/LC3I, in preeclamptic placentas (27). In line with results of Zhang and colleagues (28), Nakashima proposed a protective role for autophagy in both trophoblasts and extravillous trophoblasts to prevent poor placentation in the early stage of pregnancy (29).

Cyclin E regulates cell cycle progression through S-phase entry by in conjunction with its catalytic partner cyclin-dependent kinase 2 (CDK2) and is critical for DNA replication, and centrosome duplication (19,20). A previous study showed a regular expression of Cyclin E at moderate levels in all stages of normal placental development (30). In the present study, we have provided evidence that the phosphorylation of cyclin E at T395 is decreased in the preeclampsia placentas. As phosphorylation of cyclin E at threonine 395 has been linked to the proteasome-mediated degradation of full-length cyclin E (31), decreased phosphorylation of this protein might be related to an increase in total cyclin E which may be involved in autophagy activation. Gao et al. revealed that cyclin E expression was downregulated after p53 upregulation in human umbilical cord vein endothelial cells (HUVECs) from preeclampsia pregnancies (32), demonstrating that cell cycle-dependent role of cyclin E is required for endothelial cell growth and proliferation.

It is worth mentioning that the limitations of this study are the collection of the tissue samples from only one site in which may not be a good cross-sectional representation of a whole placenta and the number of the study group. Therefore, further studies in a large number of preeclampsia patients will be necessary to confirm our findings.

As a conclusion, the results of this study showed that autophagy is prominently increased in preeclamptic placentas. Furthermore, decreased phosphorylation of cyclin E in placentas with preeclampsia might be related to an increase in total cyclin E protein which may be involved in the induction of autophagy.

Acknowledgements

The authors declared that this study had received no financial support.

Declaration of Interest

The author(s) declared no potential conflicts of interest concerning the research, authorship, and publication of this article.

Author's Contribution

Authors' contributions: D.A-Y designed the study. D.A-Y and S.I-K. performed the experiments, participated in the analysis of the data and drafted the manuscript. E.A provided the placental tissue samples. E.D participated in the analysis of the data. All authors took part in the interpretation of data, revision of the manuscript, and in the final approval of the version to be published.

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