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Autophagy protects against oxidized low density lipoprotein-mediated inflammation associated with preeclampsia

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ABSTRACT

Introduction: Inflammatory responses play an important role in the pathogenesis of preeclampsia. Recently, the anti-inflammatory role played by autophagy has drawn increasing attention. Our aim was to investigate variations in autophagy in preeclampsia and protection against oxidized low-density lipoprotein (oxLDL)-mediated inflammation by autophagy.

Methods: We used immunohistochemistry, immunofluorescence, quantitative real-time PCR, and western blotting to analyze the expression of autophagy proteins (beclin-1 and LC3II/LC3I) in preeclampsia placentas and in JEG-3 cells treated with oxLDL and rapamycin.

Results: We found a decreased level of autophagy proteins in preeclampsia placentas, and oxLDL did not induce autophagy in JEG-3 cells. Furthermore, when cells were pretreated with rapamycin, autophagy was activated and expression of inflammatory factors (tumor necrosis factor-α and interleukin-6) induced by oxLDL was downregulated.

Conclusion: We conclude that impaired autophagy in preeclampsia has potential to decrease trophoblast protection from oxidative and inflammatory stress, thereby contributing to the pathogenesis of preeclampsia.

1. Introduction

Preeclampsia, a serious hypertensive disorder of pregnancy, is a leading cause of maternal death and a major contributor to maternal and perinatal morbidity. However, the triggering factors and underlying mechanisms responsible for the pathogenesis of preeclampsia are elusive. The leading hypotheses rely strongly on disturbed placental functions. Studies have supported a two-stage model: stage one is inadequate placental perfusion, and stage two is the maternal syndrome resulting from inadequate placental perfusion. The consequences of inadequate perfusion are intermittent hypoxia and the generation of oxidative stress, which lead to the release of antiangiogenic proteins and inflammation [1,2].

In addition to apoptosis, autophagy is another programmed cell death pathway. It is a reparative and life-sustaining process by which cytoplasmic components are sequestered in double-membrane vesicles and degraded on fusion with lysosomes under stress to maintain cellular homeostasis [3]. Autophagy plays important roles in immunity and inflammation. The beneficial and detrimental effects of immunity and inflammation can be balanced by autophagy, which may protect against infections as well as autoimmune and inflammatory diseases [4]. Recently, autophagy has become a highly researched field in obstetrics, and this process may be essential for preimplantation development beyond the four- and eight-cell stages, and for blastocyst survival [5], extravillous trophoblast functions, invasion, and vascular remodeling [6]. Preeclampsia displays many characteristics, including hypoxia and inflammatory responses, and these are associated with autophagy. However, divergent views on how autophagy varies in pregnancy appear in studies of preeclampsia. A study has suggested excessive activation of autophagy in the placenta of mothers with hypertensive disorders compared with normotensive pregnancies, indicating the involvement of excessive autophagy in the development of the disease [7]. Goldman-Woh et al. used published microarray datasets to analyze differential expression of autophagy pathway genes, and no statistically significant difference in autophagy-associated gene expression was found in preeclamptic placenta samples compared with normal samples [8]. Nakashima et al. reported that impaired autophagy in extravillous trophoblast cells contributes to the pathophysiology of preeclampsia [9].

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Oxidized low density lipoprotein (oxLDL) is the oxidative modification of native LDL in numerous disease states resulting from oxidative stress and vascular endothelial injury. OxLDL binding to its receptor, lectin-like oxidized low density lipoprotein receptor 1, causes endothelial dysfunction and could play a significant role in the pathobiology of atherosclerosis, diabetes, hypertension, and preeclampsia [10]. Previous studies have shown that the cytotoxicity induced by oxLDL differs in diverse cell types. A study of human umbilical vein endothelial cells (HUVECs) suggested that oxLDL increases the autophagic level in a concentration-dependent manner [11]. OxLDL inhibits autophagy in macrophages and smooth muscle vascular cells [12,13]. However, there are only a few studies on the effects of oxLDL in the autophagy of human trophoblasts. Therefore, we aimed to investigate autophagy variations in preeclampsia, the influence of oxLDL on autophagy in the JEG-3 cell line, and whether autophagy can protect against oxLDL-mediated inflammation.

2. Materials and methods

2.1. Placenta and patient information

Placental tissues were collected after caesarean delivery from normotensive pregnancies (NP, n = 20) and preeclampsia pregnancies (PE, n = 26). Samples were collected from each placenta and then pooled to obtain one sample per placenta, after which they were placed on ice, transported to the laboratory, processed within 30 min, rinsed in ice-cold phosphate buffered saline (PBS), and then immediately frozen at −80 °C until analysis. This study was approved by the Committee for Ethical Review of Research at Qingdao University, China.

Preeclampsia is defined as a sustained systolic blood pressure of ≥140 mmHg, or a sustained diastolic blood pressure of ≥90 mmHg on two separate readings and 24-h urine protein collection with ≥300 mg in the specimen. Patients with complicated HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome were excluded to avoid clinical phenotypic differences. Normotensive pregnancy is defined as a pregnancy with normal blood pressure (<140/90 mmHg) and no proteinuria [2] (See Table 1).

2.2. Immunohistochemistry

Autophagy-related protein beclin-1 forms an early complex that promotes the synthesis and growth of pre-autophagosomal membranes. LC3 is synthesized as proLC3 and integrated into the membranes of autophagosomes. Thus, beclin-1 and LC3 are considered as markers for detecting autophagy [14,15].

Immunohistochemistry was performed using 4 μm-thick sections of samples embedded in paraffin. The sections were incubated for 1 h at 64 °C, deparaffinized in xylene, and rehydrated in ethanol and water. The sections underwent antigen retrieval in citrate buffer (pH 6.0) followed by blocking peroxidase activity (Block; Dako, Tokyo, Japan), and then incubated with the appropriate primary antibody diluted in PBS for 1 h at room temperature. Detection was performed using the Envision horseradish peroxidase-conjugated secondary antibody and 3,3-diaminobenzidine color development system (DAKO) for consistent development times between samples. Rabbit polyclonal antibodies against beclin-1 (dilution 1:600; Abcam, USA) and LC3 (dilution 1:2200, Abcam) were used as the primary antibodies. Normal rabbit serum was substituted for primary antibodies with the negative control. Positive sections from the manufacturers were used as positive controls.

Table 1
Characteristics of subjects from whom the placentas were obtained.

Characteristic	NP	PE
n	20	26
Maternal age (y)	32.0 ± 5.0	33.2 ± 4.1
BMI (kg/m^2)	24.1 ± 3.5	25.6 ± 5.0
Gestational age at delivery (wk)	36.2 ± 2.3	35.1 ± 3.2
Systolic BP (mmHg)	120.0 ± 2.2	166.3 ± 5.8
Diastolic BP (mmHg)	74.1 ± 3.2	98.17 ± 5.5
Proteinuria (mg/24 h)	21 ± 12	4201 ± 300.0

BP, blood pressure; BMI, body mass index.

2.3. Cell culture

The human choriocarcinoma JEG-3 cell line was obtained from the Shanghai Institute for Biological Sciences (Chinese Academy of Sciences). All experiments were performed in complete culture medium to avoid induction of autophagy via the serum starvation pathway. The cell line was maintained in α-minimum essential medium (41500034; GIBCO, Shanghai, China) containing 10% fetal bovine serum and an antibiotic mixture at 37 °C in a humidified atmosphere with 5% CO_2.

Cells were treated with various concentrations of oxLDL (25, 50, 100, and 150 mg/l; Yiyuan Biotech, China) for various times (6, 12, 24 and, 48 h), or were pretreated with rapamycin (100 nM; Qcbio Science, China) for 1 h.

2.4. Immunofluorescence

To assess the presence and location of the LC3 protein, JEG-3 cells were seeded into 24-well plates and cultured under various conditions. Then, the cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton for 5 min, and blocked in 5% bovine serum albumin for 30 min. The cells were then incubated with a rabbit anti-LC3 antibody (diluted 1:500; Abcam ab48394) at 4 °C overnight. After washing with PBS, the cells were incubated with CY3-conjugated goat anti-rabbit IgG (diluted 1:500; Jackson, USA) at 37 °C for 30 min. The cells were counterstained with 4′,6-diamidino-2-phenylindole (diluted 1:1000; Boster, Wuhai, China) at room temperature for 5 min. Images were captured using a confocal microscope (ZEISS, Germany).

2.5. Quantitative real-time PCR for mRNA expression analysis

Total RNA from placental tissues and cell lysates was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA (500 ng) was used to synthesize cDNA using a PrimeScript RT reagent Kit (Takara, Japan). The cDNA was subsequently amplified with SG Fast qPCR Master Mix (High Rox, 2 × ; BBI, Canada) using a StepOnePlus instrument (ABI, USA). The primers used are listed in Table 2. PCR conditions were as follows: 30 s at 95 C, followed by 40 cycles of 7 s at 95 °C, 10 s at 57 °C, and 15 s at 72 °C. Relative mRNA expression levels were determined by normalizing the expression of each gene to the β-actin gene using the $2^{-\Delta\Delta Ct}$ method [16] relative to the expression in the control group. Normal JEG-3 cells without oxLDL or rapamycin were used as the control group.

2.6. Western blotting

Total proteins were extracted in RIPA lysis buffer (Beyotime, Zhejiang, China). After centrifugation, the supernatants were collected to determine the protein concentrations. Equal protein