Placenta 60 (2017) 9–20

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Mammalian target of rapamycin signaling is a mechanistic link between increased endoplasmic reticulum stress and autophagy in the placentas of pregnancies complicated by growth restriction

Tai-Ho Hung [a, b, *, 1], T'sang-T'ang Hsieh [a], Chung-Pu Wu [c, d], Meng-Jen Li [a], Yi-Lin Yeh [a], Szu-Fu Chen [e, 1]

[a] Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Taipei, Taiwan
[b] Department of Chinese Medicine, College of Medicine, Chang Gung University, Taoyuan, Taiwan
[c] Graduate Institute of Biomedical Sciences, Department of Physiology and Pharmacology and Molecular Medicine Research Center, College of Medicine, Chang Gung University, Taoyuan, Taiwan
[d] Department of Neurosurgery, Chang Gung Memorial Hospital, Taoyuan, Taiwan
[e] Department of Physical Medicine and Rehabilitation, Cheng Hsin General Hospital, Taipei, Taiwan

ARTICLE INFO

Article history:
Received 12 May 2017
Received in revised form
1 October 2017
Accepted 6 October 2017

Keywords:
Autophagy
Endoplasmic reticulum stress
Intrauterine growth restriction
Mammalian target of rapamycin
Placenta

ABSTRACT

Introduction: Increased endoplasmic reticulum (ER) stress and autophagy have been noted in the placentas of pregnancies complicated by idiopathic intrauterine growth restriction (IUGR); however, the cause of these phenomena remains unclear. We surmised that oxygen-glucose deprivation (OGD) may increase ER stress and autophagy and that mammalian target of rapamycin (mTOR) signaling is involved in regulating placental ER stress and autophagy in pregnancies complicated by IUGR.
Methods: We obtained placentas from women with normal term pregnancies and pregnancies complicated by IUGR to compare ER stress, mTOR signaling, and levels of autophagy-related proteins between the two groups and used primary cytotrophoblast cells treated with or without salubrinal (an ER stress inhibitor), MHY1485 (an mTOR activator), or rapamycin (an mTOR inhibitor) to investigate the effects of OGD on ER stress, mTOR activity, and autophagy levels *in vitro*.
Results: Women with pregnancies complicated by IUGR displayed higher placental ER stress and autophagy levels but lower mTOR activity than women with normal pregnancies. Furthermore, OGD increased ER stress, regulated in development and DNA damage responses-1 (REDD1), phosphorylated tuberous sclerosis complex 2 (TSC2), and autophagy levels and decreased mTOR activity compared to the standard culture condition; however, the salubrinal treatment attenuated these changes. Moreover, the administration of MHY1485 or rapamycin to OGD-treated cells decreased or increased autophagy levels, respectively.
Discussion: Based on our results, mTOR is a mechanistic link between OGD-induced ER stress and autophagy in cytotrophoblast cells; thus, mTOR plays an essential role in the pathogenesis of pregnancies complicated by IUGR.

1. Introduction

Idiopathic intrauterine growth restriction (IUGR), defined as suboptimal growth that prevents the fetus from achieving its genetically determined size, is one of the major causes of perinatal morbidity and mortality [1]. The causes of IUGR are not fully understood; however, the most widely recognized factor that predisposes pregnancies to this particular complication is deficient extravillous cytotrophoblast invasion of the endometrium during the first trimester of pregnancy, which leads to incomplete transformation of the myometrial segments of the maternal spiral arteries [2]. The persistence of the contractile state in these vessels causes decreases or fluctuations in the perfusion of the intervillous space, resulting in profound changes in prevailing

* Corresponding author. Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital at Taipei, 199 Dun-hua North Road, Taipei 105, Taiwan.
E-mail address: thh20@cgmh.org.tw (T.-H. Hung).
[1] These authors contributed equally to this work as corresponding authors.

https://doi.org/10.1016/j.placenta.2017.10.001
0143-4004/

tissue oxygen and glucose concentrations and nutrient supply [3]. These changes may induce mitochondrial and rough endoplasmic reticulum (ER) stress, leading to trophoblast dysfunction and suboptimal placental performance [4]. Indeed, mitochondrial and ER stress and apoptosis levels are increased in the syncytiotrophoblast and underlying cytotrophoblast layers of placentas in pregnancies complicated by IUGR compared to those in placentas in normal pregnancies [5–7].

Autophagy is a catabolic process involving the invagination and degradation of cytoplasmic components, such as misfolded proteins and damaged organelles, through a lysosomal pathway and the recycling of the constituent elements of these components or organelles to synthesize macromolecules and generate ATP [8]. In the human placenta, the ultrastructures of autophagic vacuoles were clearly observed in the trophoblast layers and autophagy-related proteins, such as beclin-1, microtubule-associated protein light chain 3B (LC3B), and damage-regulated autophagy modulator (DRAM), are consistently transcribed and expressed throughout gestation, indicating that autophagy is important during placental development [9]. However, increases in autophagy-related changes have been noted in the placentas from women with pregnancies complicated by IUGR compared to placentas from women with normal pregnancies [10,11]. At present, the relationship between ER stress and autophagy in the placentas of pregnancies complicated by IUGR remains unclear.

One key component that regulates the balance between cell growth and autophagy in response to cellular physiological conditions and environmental stress is mammalian target of rapamycin (mTOR) [12]. Both amino acid transporter and mTOR activity levels are reduced in the placentas of pregnancies complicated by IUGR compared to those in the placentas of normal pregnancies [5,13,14]. Furthermore, the exposure of cytotrophoblast cells to rapamycin (an mTOR inhibitor) plus bafilomycin (an autophagosome inhibitor) resulted in higher LC3B-II levels in these cells compared to cells exposed to bafilomycin alone, indicating that reductions in mTOR activity are associated with increases in autophagic flux [15]. Based on these results, mTOR may play a role in regulating placental autophagy in pregnancies complicated by IUGR.

We hypothesized that there are differences in ER stress, autophagy, and mTOR activity levels between the placentas of pregnancies complicated by IUGR and those of normal pregnancies. We also surmised that oxygen-glucose deprivation (OGD) causes increases in ER stress and autophagy in the placentas of pregnancies complicated by IUGR and that mTOR signaling is a mechanistic link between placental ER stress and autophagy under OGD conditions. Therefore, the objectives of this study were (1) to compare ER stress, mTOR activity, and autophagy levels between placentas from normal pregnancies and placentas from pregnancies complicated by IUGR and (2) to investigate the effects of *in vitro* OGD on ER stress, mTOR activity, and autophagy levels in cultured cytotrophoblast cells.

2. Materials and methods

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital, Taiwan (102–5860B). All placental samples were collected after the subjects enrolled herein provided written informed consent for the use of the samples. Unless otherwise indicated, the reagents used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Collection of placental tissues from normal pregnancies and pregnancies complicated by IUGR

We obtained placentas from women with singleton term pregnancies who underwent elective cesarean deliveries prior to the onset of labor to compare ER stress, mTOR signaling activity, and autophagy-related protein levels between women with normal pregnancies and appropriate for gestational age fetuses (fetuses with birth weights between the 10th and 90th percentiles for their gestational ages, n = 15) and women with pregnancies complicated by IUGR (n = 15), which was diagnosed in cases in which the birth weight of the fetus was below the 5th percentile when corrected for gestational age and fetal gender. None of the women had any medical diseases, such as overt diabetes, preeclampsia or renal or autoimmune diseases. The characteristics of the women who participated in this study and their pregnancies are summarized in Table 1.

We randomly collected villous tissue samples from five distinct sites on the maternal side of the placenta after it was delivered. Each site was midway between the cord insertion site and placenta periphery and midway between the chorionic and basal plates. The villous samples were quickly washed in ice-cold phosphate-buffered saline to clear the maternal blood and then frozen in liquid nitrogen before being stored at −70 °C for further processing. All villous samples were collected and processed within 10 min after delivery.

2.2. Isolation and culture of cytotrophoblast cells from normal term placentas

We isolated cytotrophoblast cells from 33 normal term placentas, as previously described [16]. The purified cells were plated in 6-well plates at a minimum density of 4×10^5 cells/cm^2 and cultured in RPMI 1640 medium (catalog no. 11875; Invitrogen, Life Technologies, Grand Island, NY, USA) containing 2 mg/ml D-glucose, 5% fetal bovine serum, antibiotics, and antimycotics in a humidified atmosphere with 5% CO_2 and balanced air. After an overnight rest, the cells were rinsed twice with pre-warmed medium to remove non-attached cells and then used in individual experiments. Cell viability was determined by assessing the degree of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromides) reduction. Characters of the isolated cytotrophoblast cells were verified by immunofluorescent staining for cytokeratin 7 and measurements of the secretion of human chorionic gonadotropin (hCG) into the medium (Supplementary data and Supplementary Fig. 1).

2.3. OGD

We cultured cytotrophoblasts in RPMI 1640 media without D-glucose (catalog no. 11879; Invitrogen) in 2% O_2 with 5% CO_2/balanced N_2 (OGD group) or media with 2 mg/ml D-glucose with 5% CO_2/balanced air (standard conditions group), as previously described [9], to study the effects of reduced oxygen and glucose concentrations on ER stress, mTOR activity, and levels of autophagy-related proteins. After the cells had been incubated for 24 h, their lysates were collected and stored at −70 °C for further processing.

To determine the role of ER stress in OGD-induced autophagy, we cultured cytotrophoblast cells under standard or OGD conditions and treated them with or without 50 μM salubrinal (an ER stress inhibitor) for 24 h. Salubrinal was dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 50 mM. In addition, we cultured cytotrophoblast cells under standard or OGD conditions and treated them with or without 2 μM MHY1485 (CAS no. 326914-06-1, Merck Ltd., Taipei, Taiwan), an mTOR activator, or 100 nM rapamycin (an mTOR inhibitor) for 24 h to investigate the interaction between mTOR and autophagy under these conditions. MHY1485 and rapamycin were dissolved in DMSO as 1000× stock