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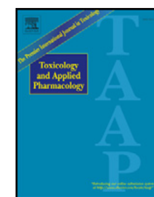

Prolonged endoplasmic reticulum stress alters placental morphology and causes low birth weight☆

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ABSTRACT

The role of endoplasmic reticulum (ER) stress in pregnancy remains largely unknown. Pregnant mice were subcutaneously administered tunicamycin (Tun), an ER stressor, as a single dose [0, 50, and 100 μg Tun/kg/body weight (BW)] on gestation days (GDs) 8.5, 12.5, and 15.5. A high incidence (75%) of preterm delivery was observed only in the group treated with Tun 100 μg/kg BW at GD 15.5, indicating that pregnant mice during late gestation are more susceptible to ER stress on preterm delivery. We further examined whether prolonged *in utero* exposure to ER stress affects fetal development. Pregnant mice were subcutaneously administered a dose of 0, 20, 40, and 60 μg Tun/kg from GD 12.5 to 16.5. Tun treatment decreased the placental and fetal weights in a dose-dependent manner. Histological evaluation showed the formation of a cluster of spongiotrophoblast cells in the labyrinth zone of the placenta of Tun-treated mice. The glycogen content of the fetal liver and placenta from Tun-treated mice was lower than that from control mice. Tun treatment decreased mRNA expression of Slc2a1/glucose transporter 1 (GLUT1), which is a major transporter for glucose, but increased placental mRNA levels of Slc2a3/GLUT3. Moreover, maternal exposure to Tun resulted in a decrease in vascular endothelial growth factor receptor-1 (VEGFR-1), VEGFR-2, and placental growth factor. These results suggest that excessive and exogenous ER stress may induce functional abnormalities in the placenta, at least in part, with altered GLUT and vascular-related gene expression, resulting in low infant birth weight.

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Introduction

The prevalence rate of low birth weight in Japanese infants showed an approximately twofold increase in 2009 compared with that in 1980 (OECD, 2011). It has been reported that low birth weight is associated with an increased risk of mortality as well as the development of cardiovascular disease, hypertension, and type 2 diabetes in later life (Alexander et al., 2003; Barker and Osmond, 1986; Godfrey and Barker, 2001). Thus, it is a concern that increasing low birth weight in Japan may impose a social burden in the future; however, the underlying mechanisms involved have not been fully elucidated.

The endoplasmic reticulum (ER) is an organelle that controls calcium homeostasis, protein synthesis, and trafficking (Gorlach et al., 2006; Ron and Walter, 2007). Recently, Iwawaki et al. reported that the function of a molecule involved in ER stress, inositol-requiring protein 1α, in the placenta is essential for placental development and embryonic viability and that ER stress and inositol-requiring enzyme 1α (IRE1α) may be involved in other physiological phenomena (Iwawaki et al., 2009). However, the accumulation of misfolded proteins and the disruption of calcium homeostasis in the ER lead to ER stress, which contributes to life-style diseases such as insulin resistance through the inhibition of insulin receptor signaling and cardiovascular diseases in obese mice (Ozcan et al., 2004). Major cellular responses against ER stress are classified as the attenuation of protein synthesis and upregulation of genes of encoding chaperones that facilitate the protein folding process in the ER. Both responses reduce the accumulation and aggregation of misfolded proteins (Kaufman, 1999). These processes are known as the unfolded protein response (UPR). The major transducers of UPR are the double-stranded RNA-activated protein kinase-like ER kinase (PERK), IRE1α, and activating transcription factor 6 (ATF6). Tunicamycin (Tun) is a chemical ER stress inducer that blocks N-glycosylation and leads to the accumulation of misfolded proteins in the ER.

A genetic deficiency of UPR transducers has been found to result in prenatal mortality and developmental abnormality. PERK knockout mice show postnatal growth retardation and permanent neonatal diabetes (Harding et al., 2001; Zhang et al., 2002, 2006). ATF6 knockout

Abbreviations: ATF6, activating transcription factor 6; BiP, binding immunoglobulin protein; BW, body weight; CHOP, CCAAT-enhancer-binding protein homologous protein; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FGR, fetal growth restriction; GD, gestation day; GLUT, glucose transporter; GRP78, glucose-regulated protein 78; HIF-1α, hypoxia inducible factor 1, alpha subunit; HE, hematoxylin and eosin; IRE1α, inositol-requiring enzyme 1α; IUGR, intrauterine growth restriction; PAS, periodic acid–Schiff; PERK, protein kinase RNA-like ER kinase; PGF, placental growth factor; RT-PCR, reverse transcription-polymerase chain reaction; SEM, standard error of the mean; TBS, Tris buffer saline; TBU, Tokushima Bunri University; Tun, tunicamycin; UPR, unfolded protein response; VEGFA, vascular endothelial growth factor A; VEGFR, vascular endothelial growth factor receptor.

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mice show embryonic lethality (Wu et al., 2007; Yamamoto et al., 2007). The inositol-requiring enzyme pathway is essential for embryonic viability and development (Bertolotti and Ron, 2001; Reimold et al., 2000; Tirasophon et al., 1998); however, the effect of overwhelming ER stress during pregnancy on fetal growth and development is still unclear.

The placenta plays a critical role in the normal growth and development of the fetus, facilitating nutrition and gas exchange as well as waste product disposal. Placental insufficiency was considered to be an important etiology of intrauterine fetal growth retardation and to be attributable to a reduction in placental blood flow (Bower et al., 1993; Matijevic and Johnston, 1999). The rodent mature placenta is composed of three major layers, which are thought to play a role in the formation of blood vessels and membrane-bound nutritional cells: the labyrinth layer, the spongiotrophoblast layer (also known as the junctional zone), and the maternal decidua (Watson and Cross, 2005). More recently, it has been reported that placentas subject to maternal heavy metal exposure and preeclampsia were found to be in a state of ER stress (Lian et al., 2011; Wang et al., 2012). These researches suggest that exposure to ER stress may result in increased fetal growth retardation, teratogeny, and preterm delivery, although the critical window and underlying molecular mechanism(s) are unclear. In fact, exposure to heavy metals results in various effects, including ER stress, oxidative stress, teratogenicity, and apoptosis (Wang et al., 2012). Among the various effects, we hypothesized that maternal transient and prolonged excessive exposure to ER stress are involved in fetal growth retardation and preterm delivery.

In the present study, we investigated the effects of maternal exposure to the ER stressor Tun on fetal growth and the potential underlying mechanisms such as the transport system of nutrients, including glucose, from the dam to the fetus *via* the placenta and the possible involvement of its perturbation in the placenta and/or fetus.

Materials and methods

Reagents. Tun was obtained from Sigma-Aldrich (St. Louis, MO, USA), and was more than 99% pure. Dimethyl sulfoxide (DMSO) used to dissolve Tun was from Wako Pure Chemical Industries (Osaka, Japan). RNAiso, TaKaRa Ex Taq polymerase, and SYBR® Premix DimerEraser™ (Perfect Real Time) were purchased from TaKaRa Bio Inc. (Otsu, Japan). High-Capacity cDNA Reverse Transcription Kits were obtained from Applied Biosystems (Foster City, CA, USA). Glycogen Assay Kits were purchased from BioVision Research Products (Mountain View, CA, USA). Mouse anti-BiP/GRP78 and anti-CHOP/GADD153 were obtained from Cell Signaling (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. The primers for reverse transcription-polymerase chain reaction (RT-PCR) analysis were purchased from Operon Biotechnology (Tokyo, Japan). Unless otherwise noted, all reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and Tun treatment. All animal experiments were approved by the Animal Ethics Committee of Tokushima Bunri University (TBU) and performed in accordance with the Guidelines for Animal Experiments at TBU. Eight-week-old male and female Slc:ICR mice were obtained from Japan SLC, Inc. (Shizuoka, Japan) and were acclimated to laboratory conditions for a week before the initiation of experimental procedures. The animals were maintained in a controlled environment with temperature at 22 ± 1 °C, humidity at 45 ± 5%, and a 12/12-h light/dark cycle. They received stock food (NMF; Oriental Yeast Co., Tokyo, Japan) and filtered water *ad libitum*. The female mice were mated overnight with males at a 3:1 proportion in single cages. The next day was considered gestation day 0 (GD 0). Pregnant mice were individually housed in clear plastic cages with paper-chip bedding (Paper Clean; SLC Japan, Inc.).

Tun was dissolved in saline containing 3.0% DMSO. The vehicle used was prepared with the same concentration of DMSO and saline. In the single administration experiment, pregnant mice were subcutaneously injected with Tun [0, 50, and 100 μg/kg body weight (BW)] on GD 8.5, 12.5, and 15.5. In the repeated administration experiment, pregnant mice were subcutaneously injected with Tun (0, 20, 40, and 60 μg/kg BW) from GD 12.5 to GD 16.5. Prior to terminal euthanasia on GD 17.5 (between 11:00 and 13:00), the mice were anesthetized with pentobarbital (50 mg/kg BW) intraperitoneally and blood samples were collected from the retro-orbital sinus in a heparinized tube. The fetus and placenta were removed from the uterus and weighted. The maternal and fetal liver and placenta samples were immediately frozen in liquid nitrogen and stored at −80 °C for RNA and protein extraction.

Histopathological examination. Some placental tissues were fixed in Mildform® 15NM (Wako) and embedded in paraffin for histopathological analysis. Horizontal sections (3-μm thickness) of the placenta were cut by microtome (Yamato Koki Industrial Co., Tokyo, Japan), mounted on glass slides, deparaffinized in xylene, rehydrated in a graded ethanol series, and stained with hematoxylin and eosin (HE) for light microscopy or processed for periodic acid–Schiff (PAS) staining. Digital images of the placental sections were obtained using an Olympus digital camera (Tokyo, Japan) attached to a compound microscope.

Immunohistochemistry examination. For the immunohistochemical study, 4-μm-thick horizontal sections of placenta were prepared on MAS-coated slide glass (Matsunami Glass, Osaka, Japan). Placentas on GD 17.5 were randomly selected from the control and 60 μg Tun/kg group. The tissue sections were deparaffinized in xylene, dehydrated in a graded series of ethanol, and washed with Tris-buffered saline (TBS, pH 7.6). For antigen retrieval, the sections were treated in Target Retrieval Solution (Dako Corporation Carpentaria, CA, USA) for 15 min at 121 °C by autoclaving. The sections were blocked with Protein Block Serum-Free (Dako) for 1 h to prevent non-specific binding and then incubated overnight with the primary antibodies (1:200) for HIF-1α (Novus Biologicals, Cambridge, UK) and VEGFA (Chemicon International, Temecula, CA, USA). The biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) were used at a dilution of 1:200. The slides were further processed using a Vectastain ABC Kit according to the manufacturer's instructions (Vector Laboratories). The sections were then washed in TBS, and immunoreactivity was visualized with diaminobenzidine. After washing in TBS, the sections were counterstained with hematoxylin, dehydrated, and mounted with Entellan new (Merck, Darmstadt, Germany).

Semiquantitative RT-PCR. Semiquantitative RT-PCR analysis was performed as described previously (Kawakami et al., 2012). Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). Samples of cDNA were synthesized with 2 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's instructions. The primer sets and PCR conditions used in this study are summarized in Table 1. The iCycler System (Bio-Rad, Hercules, CA, USA) was used for the amplification of target genes in the placenta. The PCR parameters were as follows: 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s for vascular endothelial growth factor receptor-1 (VEGFR-1), VEGFR-2, and placental growth factor (PGF), respectively. The PCR products were separated by 2% agarose gel electrophoresis at 100 V for 35 min and stained with ethidium bromide. The amplified PCR products were quantified by densitometry using an ATTO Densitograph (ATTO Corporation, Tokyo, Japan). The relative amount of GAPDH mRNA as the internal control was also determined for normalization for various RT-PCR efficiencies.

The protocols for real-time RT-PCR quantification were described previously (Kawakami et al., 2009). All the primer sets used are shown in Table 1. For real-time RT-PCR, target genes were amplified with the SYBR® Premix DimerEraser® (Perfect Real Time) System using a LightCycler (Roche, Manheim, Germany). The PCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for

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