

## Endoplasmic reticulum stress induced by oxidative stress in decidual cells: a possible mechanism of early pregnancy loss

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Received: 15 November 2011 / Accepted: 9 June 2012 / Published online: 26 June 2012  
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**Abstract** Early pregnancy loss (EPL) is one of the most common complications of human reproduction. Combined with our previous proteomic studies on villous and decidual tissues of EPL, we found that alterations of the proteins involved in oxidative stress (OS), unfolded protein response (UPR) and proteolysis presented a complex and dynamic interaction at the maternal-fetal interface. In the present study, we developed a cell model of OS using normal decidual cells to examine cell viability and expression levels of proteins related to endoplasmic reticulum stress (ER stress) and UPR. We found that glucose regulated protein 78 (GRP 78) and ubiquitinated proteins

were significantly up-regulated in hydrogen peroxide ( $H_2O_2$ ) treated decidual cells in a dose-dependent manner. Excessive OS could influence proper function of UPR by decreasing VCP in decidual cells, thereby leading to cell damage as well as inhibition of cell growth and activation of apoptosis. Furthermore, when pretreated with MG 132, a pharmacological inhibition of the proteasome, the  $H_2O_2$  treated decidual cells became less viable and could not up-regulate the expression level of GRP 78 to resolve the protein-folding defects, which indicating that malfunction of UPR in decidual cells might aggravate the inhibitory effect of OS in decidual cells. The present results reveal that abnormal protein profiles associated with OS induced ER stress and malfunction of UPR might be involved in the development of EPL, and OS and ER stress are potential targets for pregnant care and prognosis in normal pregnancy and its disorders.

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**Keywords** Early pregnancy loss · Decidual cells ·  
Oxidative · Endoplasmic reticulum · Stress ·  
Ubiquitin–proteasome response

### Abbreviations

EPL	Early pregnancy loss
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
GRP 78	Glucose regulated protein 78
$H_2O_2$	Hydrogen peroxide
OS	Oxidative stress
ROS	Reactive oxygen species
UPR	Unfolded protein response
VCP	Valosin-containing protein
(ATF6)	Activating transcription factor-6
(XBP-1)	X-box binding protein-1
UPS	Ubiquitin–proteasome system

## Introduction

Early pregnancy loss (EPL) is the most common complication of human reproduction. As many as 50 % of conceptions end in EPL at or around implantation, with another 20 % ending between implantation and completion of the first trimester, carrying significant personal, social and financial consequences. Causative factors associated with EPL include chromosomal abnormalities, uterine anatomic anomalies, infectious diseases, endocrinopathies, and immunological disorders [1, 2].

Both *in vivo* and *in vitro* data suggest that oxidative stress (OS) is a common pathological background for different etiologies of EPL [2–4]. OS is defined as an imbalance between intracellular and extracellular reactive oxygen species (ROS), antioxidant, and protection systems. Elevated ROS cause DNA and protein damage, lipid peroxidation with primary effects on cell membrane structures and function [5], and, influence endoplasmic reticulum (ER) function by disturbing calcium homeostasis or protein processing/transport [6].

Endoplasmic reticulum stress (ER stress) occurs when newly-synthesized, unfolded or misfolded proteins accumulate in ER when its function has been disturbed by differing pathological conditions. Accumulation of unfolded or misfolded proteins activates an adaptive signaling cascade known as the ER stress response or unfolded protein response (UPR). This activates the PERK, IRE1/X-box binding protein-1, that in turn, activates transcription factor-6 (ATF6) signaling pathways as protective measures, resulting in general translational attenuation, up-regulation of chaperones and folding enzymes, and, enhanced ER-associated degradation of misfolded proteins (ERAD) [7, 8]. Depending on the severity of ER stress, the UPR may result in cell death through the activation of apoptotic pathways mediated specifically by the ER [9].

In our previous study, we found that ER chaperone, glucose-regulated protein 78 (GRP 78) is down-regulated in decidual cells from EPL [10]. GRP 78 is induced by physiological stress that disturbs ER function and homeostasis, protecting against tissue or organ damage under pathological conditions [11]. In non-stressed cells, GRP 78 binds to ER transmembrane sensor proteins PERK, IRE1, and ATF6, maintaining them in inactive forms. When unfolded proteins or misfolded proteins separate from GRP 78, these pathways activate, sending signals to the nucleus to trigger the UPR pathway [12].

During the UPR, the final unfolded or misfolded proteins are extracted and retro-translocated to the proteasome in the cytoplasm where they are degraded by ERAD [13]. Valosin-containing protein (VCP) also decreases in decidual cells during EPL [10]. VCP is a sensor that detects accumulation of misfolded proteins in cells and aids in

extraction of these ubiquitinated proteins (Ub) from ER, facilitating their delivery to the proteasome [14, 15].

In our previous study, we found that levels of GRP 78 and VCP, associated with ER stress and ERAD, changed significantly in decidual cells during EPL. This study seeks to determine whether, and how, OS induced ER stress and UPR in decidual cells, and, its relationships with OS, ER stress and UPR during the development of EPL.

## Materials and methods

### Sample collection

From November 2007 to February 2008, ten pregnant women with a diagnosis of EPL attending the Department of Obstetrics and Gynecology, Women's Hospital, Zhejiang, China, were recruited for this study. These pregnant women had vaginal bleeding and/or lower abdominal pain for the first time in the prior 48 h. Diagnosis of EPL was based on clinical history, physical examination, and transvaginal ultrasound scan. A gestational sac without fetal heart rate confirmed the diagnosis of EPL. Criteria for inclusion were a gestational age of eight weeks (based on the first day of the last menstrual period) and no history of recurrent spontaneous abortions, chromosomal abnormalities, endocrine diseases, anatomical abnormalities of genital tract, infections, immunologic diseases, trauma, internal diseases, etc. Twenty, age-matched women with normal pregnancies undergoing terminations of pregnancy for psychological reasons at the same gestational age were designated as the control group. Decidual tissues were taken through the cervix during dilatation and aspiration according to formal clinical procedures. Informed consent was obtained from each woman for the use of decidual tissue. The Ethical Review Committee of Women's Hospital, Zhejiang University School of Medicine approved the study.

### Isolation and culture of DCs

Isolation of primary human decidual cells was carried out with the use of Percoll gradient as described previously with modifications [10, 16]. Sterile human decidual tissues were taken during dilatation and curettage after informed consent from pregnant women with normal pregnancy or EPL at eight weeks gestation. Briefly, we placed decidual tissues in a conical flask containing collagenase (type I, 0.2 %; Sigma, USA) and deoxyribonuclease I (DNase, 2.5 mg/mL; Sigma) in PBS and incubated at 37 °C for 80 min. The final digest was filtered through 100 µm pore filters (Millipore Corp.) and centrifuged. Liberated cells were fractionated on a 5/40/60 % discontinuous, Percoll gradient and centrifuged. Cells lying above the 40 % layer

were recovered by aspiration and centrifuged. The cells were resuspended in 1 mL DMEM containing 10 % fetal bovine serum (FBS), then numbered and seeded onto 96-well plates and 10 cm culture dishes before incubation at 37 °C with 5 % CO<sub>2</sub>. At more than 90 % confluence, the cultures were exposed to various treatments. For all conditions, viability assays were performed in duplicate. The purity of the decidual cell preparation was >96 %, according to specific staining by human placental lactogen (HPL) (US Biological).

#### Immunocytochemistry

We cultured decidual cells at  $9 \times 10^5$ /well in a 6-well culture plate (Corning) which had been set with a cover glass. At 90 % cell confluence, free FBS medium replaced the culture medium. Decidual cells were treated with H<sub>2</sub>O<sub>2</sub> (500 μM) for 12 h. Then we removed culture medium from the plate, added −20 °C methanol and put it at −20 °C overnight. Decidual cells were washed with PBS containing 0.1 % Triton X-100 (Sigma) before being incubated with primary antibodies against Ub, PR, VCP and GRP78 for 2 h at room temperature.

#### Immunofluorescence confocal microscopy

We cultured decidual cells as per the previous section. After three washes in PBS, the cells were incubated with fluorochrome-conjugated secondary antibody for 1 h at RT in the dark. Cells were incubated with DAPI after three washes in PBS. We acquired optical sections using the Zeiss LSM510 confocal microscope (Carl Zeiss, Germany).

#### Cell viability assay

We assessed decidual cell growth using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay [17]. Cells were cultured in a 96-well culture plate at a density of  $3 \times 10^4$  decidual cells per well, serum starved for 12 h, and then treated with H<sub>2</sub>O<sub>2</sub> (500 μmol/L) and/or MG-132 (5 μmol/L) (Sigma) for 12–48 h. Thereafter, the cells were incubated with 0.5 mg/mL of MTT labeling reagent (Sigma) for 4 h. Then, we added DMSO solubilization solution to wells and the culture plates were vibrated for 10 min. The wavelength to measure absorbance of formazan product is 490 nm, with a reference wavelength of 630 nm. Cell viability assay was performed in triplicate.

#### Western blotting analysis

We performed Western blotting analysis of protein expression as previously described [4]. Antibodies used in the Western blot analysis included anti-Ubiquitin (Ub)

(Sigma), anti-VCP (Affinity BioReagents), anti-GRP 78 (US Biological), anti-caspase-4 (Abcam), and anti-β-Integrin (Cell Signaling Technology). A monoclonal anti-β-actin antibody (Santa Cruz Biotechnology) was used as a control for protein loading. Three separate experiments were run independently for Western blotting analysis.

#### Statistical analysis

Data from immunofluorescence confocal microscopy, cell viability and Western blot analysis were expressed as mean ± SD. Student's *t* test was used for the statistical analysis of data from Western blot. Other statistical analyses were performed using the non-parametric Mann–Whitney *U* test to compare data in different groups. The value of *P* < 0.05 was considered statistically significant.

## Results

### ER stress and UPR markers in EPL decidua

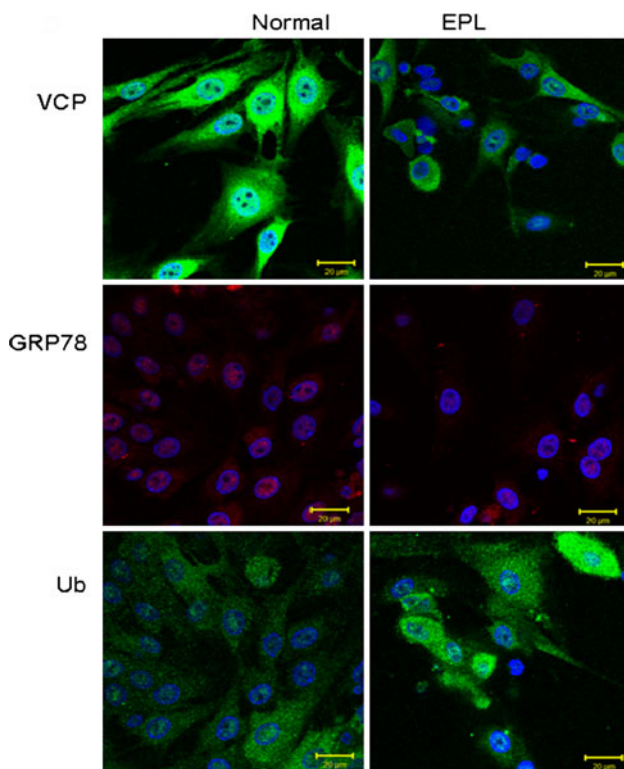
Firstly, we determined the distribution of Ub, GRP 78 and VCP in decidual cells in EPL and control groups by using immunofluorescence confocal microscopy. As shown in Fig. 1, EPL decidual cells demonstrate stronger staining of Ub in cytoplasm and nuclei when compared to that of controls. There are lighter stainings of VCP and GRP 78 in the cytoplasm of decidual cells from EPL group when compared to those of controls.

### Immunocytochemical staining of Ub, VCP and GRP 78

To determine whether OS might induce UPR in decidual cells, we examined the distribution of Ub, GRP 78, and VCP in H<sub>2</sub>O<sub>2</sub>-treated, decidual cells using an immunocytochemical staining method. The staining of Ub in the cytoplasm and nuclei of decidual cells and the staining of GRP 78 in the cytoplasm of decidual cells treated with H<sub>2</sub>O<sub>2</sub> (500 μmol/L) (Fig. 2a1, c1) were more abundant than those of controls (Fig. 2a2, c2). Yet cytoplasmic immunocytochemical staining of VCP after H<sub>2</sub>O<sub>2</sub> treatment is weaker than those of controls (Fig. 2c1, c2). Immunocytochemical staining of HPL, used as the decidual cell-specific marker, is shown for comparison with the negative controls (Fig. 2d1, d2). More than 95 % cells stained positive. Decidual cells immunostained with PBS were used as negative controls.

### Viability of decidual cells after treatment with H<sub>2</sub>O<sub>2</sub> and/or MG132 by MTT assay

Elevated ROS at the fetal-maternal interface is the common pathophysiological feature associated with different etiologies

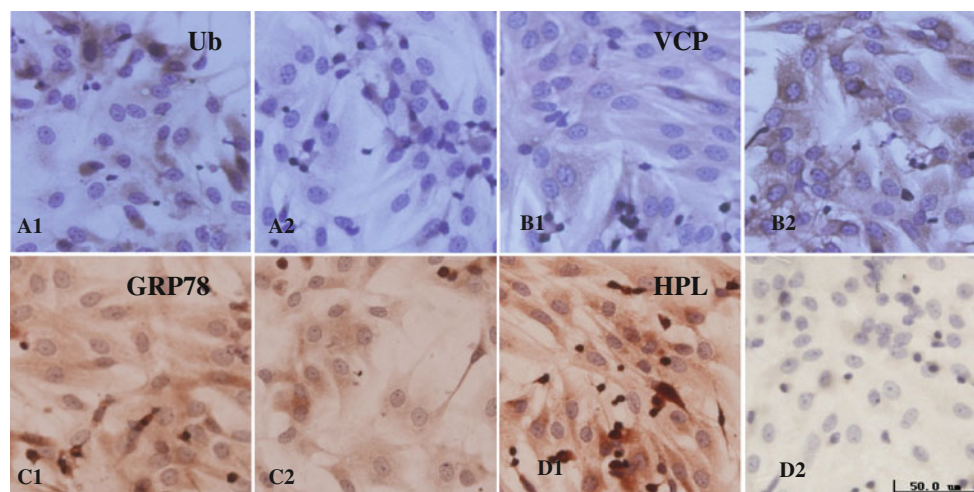


**Fig. 1** Identification the distribution of Ub, GRP 78 and VCP by using immunofluorescence confocal microscopy. EPL decidual cells showed stronger stainings of Ub in the cytoplasm and nuclei when compared with those of control cells. Compared with the control, lighter stainings of VCP and GRP 78 were detected in the cytoplasm of decidual cells from EPL group. The nuclei were stained with DAPI (Blue), Ub (Green), GRP 78 (Red), VCP (Green). Bar = 20  $\mu$ m. (Color figure online)

of EPL [2–4]. In the present study, we examined the effect of  $H_2O_2$  at different concentrations on the viability of decidual cells. As shown in Fig. 3,  $H_2O_2$  at 50–2,000  $\mu$ mol/L reduced the cellular viability of decidual cells in a dose-dependent fashion. However treatment with  $H_2O_2$  did not have obvious time-dependent effects on the viability of decidual cells. Since Ub are an indicator of inhibited UPR function, we inhibited UPR function experimentally by pharmacological inhibition of the proteasome, MG-132 [14]. In the present study, treatment of MG-132 at 5  $\mu$ mol/L significantly decreased the viability of decidual cells. MG-132 at 5  $\mu$ mol/L plus  $H_2O_2$  at 500  $\mu$ mol/L had stronger inhibitory effects on cell viability than those of  $H_2O_2$  or MG-132 alone at three different time-points (12/24/48 h). Furthermore, we found that decidual cells had significantly lower viability after treatment with  $H_2O_2$  plus MG-132 at the time-points of 24 or 48 h when compared to 12 h.

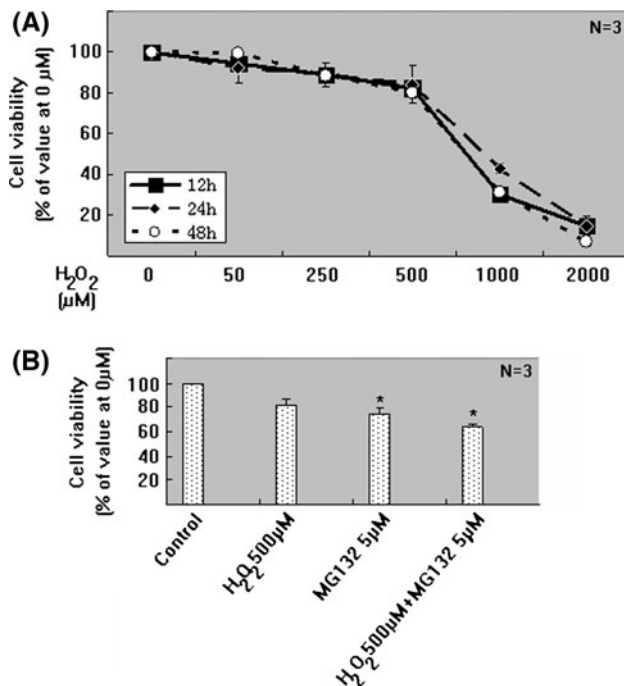
#### Ub and GRP 78 proteins expression in cultured decidual cells after treatment with MG 132 and/or $H_2O_2$

GRP 78 may play a cytoprotective role against stress in cells [11]. In 10 cm culture dishes, we treated the cells with MG-132 at 2.5 or 5  $\mu$ mol/L alone,  $H_2O_2$  at 500/1,000/2,000  $\mu$ mol/L alone, and MG-132 at 2.5  $\mu$ mol/L plus  $H_2O_2$  at 500/1,000/2,000  $\mu$ mol/L for 12 h in serum-free DMEM. After extraction of cellular proteins, we performed immunoblotting on Ub and GRP 78. Treatment with MG-132, or  $H_2O_2$  alone, dose-dependently increased Ub and GRP 78



**Fig. 2** Analysis of the expression of Ub, GRP 78, and VCP proteins by immunocytochemical staining. **a1, c1** The staining of ubiquitin in cytoplasm and nuclei of decidual cells and the staining of GRP 78 in cytoplasm of decidual cells treated with  $H_2O_2$  (500  $\mu$ mol/L) **a2, c2** were more abundant than those of control cells. **b1, b2** Yet cytoplasmic immunocytochemical staining of VCP after  $H_2O_2$

treatment was more weakened than those of control cells. **d1, d2** Immunocytochemical staining of HPL used as the decidual cell-specific marker was shown for comparison with the negative control. And we found more than 95 % cells were positive. Decidual cells immunostaining with PBS was used as the negative control



**Fig. 3** Effect of different treatments with H<sub>2</sub>O<sub>2</sub>, and/or MG-132 at various concentrations or time-points on the cell viability of decidual cells. **a** Cell viabilities of decidual cells treated with H<sub>2</sub>O<sub>2</sub> at 50–2,000 µmol/L and time-points of 12/24/48 h were measured by MTT. **b** Cell viabilities of decidual cells treated with H<sub>2</sub>O<sub>2</sub> at 500 µmol/L and/or MG-132 at 5 µmol/L for 12 h were measured. OD values of EPL decidual cells were converted to cell survival percentage as compared to control cells. Cell viability assay was performed in triplicate

expression levels in decidual cells. Treatment of decidual cells with MG-132 at 2.5 µmol/L plus H<sub>2</sub>O<sub>2</sub> at 500/1,000/2,000 µmol/L significantly decreased both ubiquitin-ated proteins and GRP 78 expression levels in decidual cells compared to treatment with MG-132 or H<sub>2</sub>O<sub>2</sub> alone, (Fig. 4).

In 10 cm culture dishes, we treated the cells with H<sub>2</sub>O<sub>2</sub> at 1000 µmol/L or MG-132 at 5 µmol/L for 12 h in serum-free DMEM. After extraction of cellular proteins, we performed immunoblotting on VCP, β-integrin, SOD and caspase-4 in turn. In the present study, we found that levels of β-integrin, recognized as a biomarker of decidual function during early pregnancy, were significantly down-regulated in decidual cells treated with H<sub>2</sub>O<sub>2</sub> at 1,000 µmol/L or MG-132 at 5 µmol/L when compared to controls. Expression levels of VCP were significantly down-regulated when treated with 1000 µmol/L H<sub>2</sub>O<sub>2</sub> or 5 µmol/L MG-132. In addition, we also detected the cleaved fragments of caspase-4. However, we were unable to observe significant changes of expression level of SOD in decidual cells when treated with H<sub>2</sub>O<sub>2</sub> at 1000 µmol/L or MG-132 at 5 µmol/L (Fig. 5).

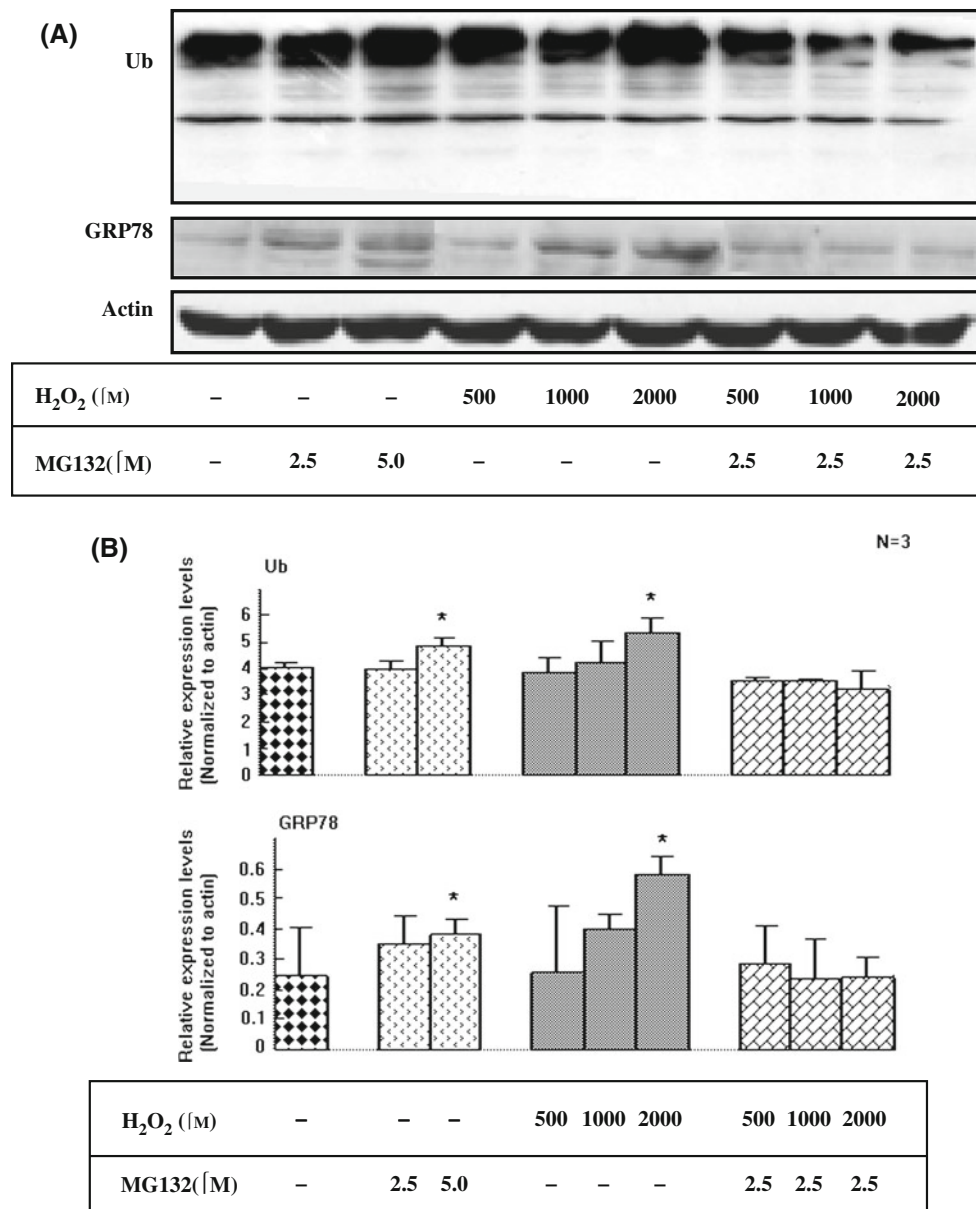
## Discussion

Recent evidence suggests that OS at the maternal-fetal interface may influence the maintenance of a viable pregnancy [2–4, 18]. It has been reported that most cases of EPL present with a thinner and fragmented trophoblastic shell, and reduced cytotrophoblast invasion of the tips of the spiral arteries [19]. This results in premature onset of the maternal circulation throughout the placenta. The excessive entry of maternal blood into the intervillous space has a direct mechanical effect on the villous tissue, and an indirect OS effect that contributes to cellular dysfunction and/or damage.

In our previous study, we demonstrated that GRP 78, VCP and two members of UPR associated with ER stress and ERAD, changed significantly in decidual cells of EPL [10]. Our proteomic study on villous tissues revealed that proteins, including three principal antioxidant enzymes-copper/zinc-superoxide dismutase, peroxiredoxin 3 and thioredoxin-like 1 protein-and members of UPR including ubiquitin-conjugating enzyme, E2N and proteasome beta-subunit are differentially expressed in villous tissues of EPL [4]. Since ROS can trigger ER stress response, we developed a cell model of OS using normal decidual cells to study the potential relationships between OS, ER stress and UPR during the development of EPL.

In this study, we found that ROS accumulation in decidual cells could trigger ER stress and UPR [20]. In mammals, GRP 78, an ER-localized chaperone, is identified as a monitor of ER stress. GRP 78 is required to transport nascent membrane and secretory proteins into the ER lumen and to fold unfolded or misfolded proteins [21, 22]. In this study, we found that the treatment of H<sub>2</sub>O<sub>2</sub> to normal decidual cells caused obvious accumulating of Ub, as well as up-regulating the levels of GRP 78 in a dose-dependent manner. These Ub might activate stress-signaling pathways to rescue the cells and are extracted and retro-translocated to the cytoplasm where they are tagged by ubiquitin and degraded by proteasome during the process of ERAD [13, 23]. Over-expressed GRP 78 in these stressed decidual cells can facilitate the restoration of proper protein folding within the ER, and attenuate UPR [24], thus alleviating ER stress. Taken together, we suggest that normal decidual cells under mild OS are probably in compensation through proper UPR. However, we found excessive OS could influence ER and ERAD function in decidual cells, thereby leading to an increase in abnormal proteins, inhibition of cell growth, and then impairment of decidual function.

UPR in decidual cells might mediate the development of EPL. The present study provided us that both in normal and EPL decidual cells, OS could cause significant accumulation of Ub, which is a characteristic of inhibited UPR

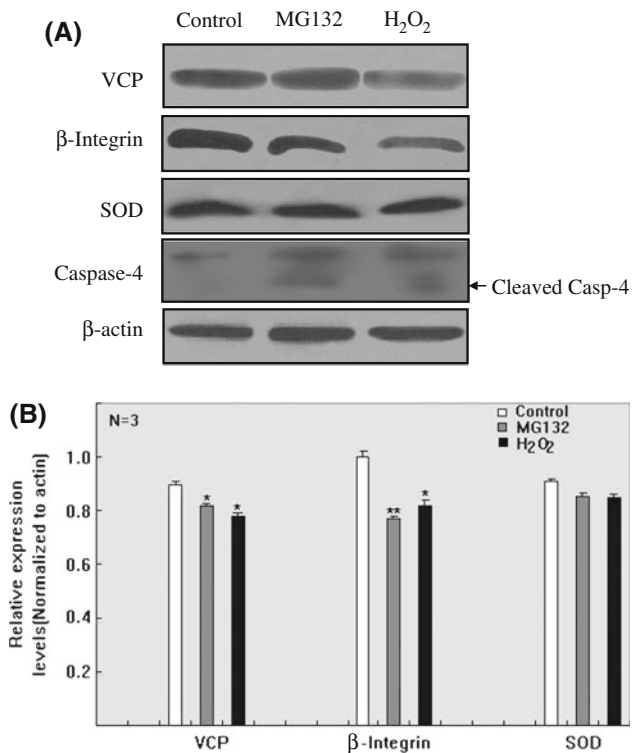


**Fig. 4** Western blot analysis of Ub and GRP 78 in cultured decidal cells after treatments with  $H_2O_2$  and/or MG-132 at various concentrations. Compared with the control group, MG-132 at 2.5 and 5  $\mu mol/L$  and  $H_2O_2$  at 500–2,000  $\mu mol/L$  does-dependently increased expression levels of Ub and GRP78. The treatments of decidal cells

with 2.5  $\mu mol/L$  MG-132 plus 500, 1000, or 2000  $\mu mol/L$   $H_2O_2$  did not show apparent effect on the abundance of Ub and GRP 78. **a** The representative autoradiograph. **b** Summary data were normalized and analyzed by using  $\beta$ -actin as internal reference. Data were presented as mean  $\pm$  SD,  $n = 3$ . \* $P < 0.05$  compared with control

function. VCP, previously reported to mediate degradation of UPR substrates [14], was found to be down-regulated in EPL and  $H_2O_2$  treated decidal cells. These results are consistent with the proteomic data in which two members of UPR were altered significantly. Further, we treated decidal cells with MG-132, a 26S proteasome inhibitor, thus disturbing the function of UPR. MG-132 made levels of Ub and GRP 78 increase but decreased level of VCP. It is probable that inhibition of UPR increased Ub [25], thus the accumulated proteins induced ER stress. During ER

stress, cells first initiated compensation mechanism including over-expression of GRP 78. Moreover, several lines of evidence implicate that in addition to its established function in proteolysis at the end step of the pathway, the 26S proteasome also participates in earlier steps, such as dislocation of ERAD substrates. The proteasome is an alternative candidate to provide the driving force for dislocation of ERAD substrates [26]. However, when pre-treated with MG-132, the  $H_2O_2$  treated decidal cells became less viable and cannot up-regulate expression of



**Fig. 5** Western blot analysis of VCP, SOD,  $\beta$ -integrin and cleaved fragments of caspase-4 in decidual cells treated with  $H_2O_2$  and MG132. Both levels of VCP and  $\beta$ -integrin were significantly decreased in  $H_2O_2$  at 1,000  $\mu\text{mol/L}$  or MG-132 at 5  $\mu\text{mol/L}$  treated decidual cells. The expression level of SOD was not changed in decidual cells treated with  $H_2O_2$  at 1,000  $\mu\text{mol/L}$  or MG-132 at 5  $\mu\text{mol/L}$ . In addition, we also detected the cleaved fragments of caspase-4 in  $H_2O_2$  or MG-132 treated decidual cells. **a** The representative autoradiograph. **b** Summary data were normalized and analyzed by using  $\beta$ -actin as internal reference and control as external reference. Data were presented as mean  $\pm$  SD,  $n = 3$ . \* $P < 0.05$  compared with control

GRP 78 to resolve the protein-folding defects. If protein-folding defect can not be resolved by severe or persistent OS, chronic activation of ER stress signaling occurs. As a result, we propose that malfunction of UPR in decidual cells is likely another important feature during the pathology of EPL, and it may aggravate the inhibitory effect of OS in decidual cells. Because of complexity of UPR, the exact mechanism of UPR involving in EPL is required to be further studied.

In the following experiment, we also evaluated the feature of ER stress-induced apoptosis in  $H_2O_2$  treated decidual cells. Usually, caspase activation is judged by the appearance of cleaved fragments of caspases. In our study, we found that cleaved fragments of caspase-4, which is specifically activated in ER stress-induced apoptosis, were found in  $H_2O_2$  treated decidual cells but not in control cells, confirming that ER stress-related caspases were activated in these stressed cells [7, 27]. Excessive OS may also lead to impairment of decidual function and activation

of apoptosis. However, the exactly functional significance of ER stress-induced apoptosis in decidual cells will be determined in our future work.

In summary, by developing a cell model of OS with normal decidual cells, we demonstrated that OS can induce UPR and ER stress in normal decidual cells, and, that excessive OS can influence proper compensatory mechanisms including UPR and ERAD in decidual cells and lead to inhibit cell growth and activate apoptosis. Thus, abnormal protein profiles associated with OS induced ER stress and malfunction of UPR might be involved in the development of EPL.

**Acknowledgments** This work was supported by the National Basic Research Program of China (2006CB504004 and 2006CB944006), the National Natural Science Foundation of China (30670789 and 30801243), the Key Research Program of Zhejiang Province, China (2006C13078) and the Fundamental Research Funds for the Central Universities of China.

**Conflict of interest** The authors have no conflicts to declare in relation to the material reported in this manuscript.

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