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Resveratrol mitigates trophoblast and endothelial dysfunction partly via activation of nuclear factor erythroid 2-related factor-2

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ABSTRACT

Introduction: Maternal endothelial dysfunction underlying preeclampsia arises from excessive placental release of anti-angiogenic factors, such as soluble fms-like tyrosine kinase-1 (sFlt1), soluble endoglin (sEng) and activin A. Resveratrol, an activator of the nuclear factor erythroid 2-related factor-2 (Nrf2) transcription factor, mediates the gene expression of antioxidant and vasoprotective factors that may counter the endothelial damage imposed by these anti-angiogenic factors. The objective of this study was to assess whether resveratrol could reduce placental oxidative stress and production of anti-angiogenic factors *in vitro* and/or improve *in vitro* markers of endothelial dysfunction via Nrf2 activation.
Method: We used *in vitro* term placental explants to assess the effects of resveratrol on placental oxidative stress and production of sFlt1, sEng and activin A. Using human umbilical vein endothelial cells we investigated the effects of resveratrol on markers of *in vitro* endothelial dysfunction, including the expression of intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), E-selectin and endothelin-1, and endothelial permeability. To confirm that resveratrol mediated its effects via Nrf2, we examined the impact of resveratrol on the same *in vitro* markers of endothelial and placental dysfunction following Nrf2 knockdown.
Results: Resveratrol significantly decreased placental oxidative stress and the production of sFlt1 and activin A. Resveratrol significantly mitigated tumor necrosis factor−α stimulated endothelial expression of ICAM1, VCAM1, E-selectin and endothelin-1 and prevented an increase in endothelial monolayer permeability. Nrf2 knockdown abolished some of the protective effects of resveratrol on endothelial cells, but not in primary trophoblast cells.
Conclusion: Features of placental and endothelial dysfunction characteristic of preeclampsia are improved by resveratrol *in vitro*, partially via the modulation of Nrf2.

1. Introduction

Preeclampsia remains a leading cause of maternal and perinatal morbidity and mortality worldwide [1]. While the use of antihypertensives to control maternal blood pressure and allow pregnancy prolongation has greatly improved clinical outcomes it is widely accepted that a more targeted therapy, focused on more than simply controlling hypertension, is needed [2–5]. Two insights have heralded opportunities to develop such a therapy. First the recognition that systemic maternal endothelial dysfunction underlies many of the maternal features of preeclampsia turned attention to exploring mechanisms of endothelial injury [6,7]. This then led to the observation that the endothelial dysfunction is likely secondary, at least in part, to excessive placental release of several anti-angiogenic factors, such as tumor necrosis factor−α (TNF-α), soluble fms-like tyrosine kinase-1 (sFlt1), soluble endoglin (sEng)

Abbreviations: Nrf2, Nuclear factor erythroid 2-related factor-2.

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and activin A [8–11]. These factors induce endothelial injury by up regulation of pro-oxidant enzymes, such as NADPH oxidase (NOX), and the induction of oxidative stress [8,12,13]. Together, these insights have afforded the possibility that established preeclampsia may be amenable to more effective management by either targeting the anti-angiogenic factors or their downstream endothelial effects, including NOX activation and oxidative stress.

In that regard, activation of the nuclear factor erythroid 2-related factor-2 (Nrf2) transcription factor offers much promise. Nrf2 mediates the response to cellular stresses by activating the gene transcription of a variety of cytoprotective enzymes, including the phase II detoxification and anti-oxidant enzymes, glutathione S-transferases, NADPH quinone oxidoreductase and heme oxygenase-1 (HO-1) [14]. To date, studies on placental expression of Nrf2 in women with preeclampsia have revealed conflicting results. Some investigators have reported increased placental Nrf2 expression in preeclampsia while others have reported a decrease [15–17]. Nonetheless, the targeted activation of Nrf2 in preeclampsia could be beneficial. Activation of a repertoire of endogenous anti-oxidant enzymes by Nrf2 protects against the oxidative stress that results from ischemia-reperfusion injury. Theoretically, this should be useful in resolving the underlying placental injury in preeclampsia [18–20]. Indeed, activation of HO-1 has been shown to ameliorate placental ischemia in a rodent model of reduced placental perfusion, which in turn reduced the production of sFlt1 and sEng [21–23]. In addition, Nrf2 activation has been shown to rescue endothelial function in mice fed a high fat diet and in a rat model of chronic renal disease [24,25]. These beneficial effects of Nrf2 activation on the endothelium arose mainly to the downstream anti-oxidant and anti-inflammatory gene targets [24,25].

Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a polyphenolic compound found in a variety of plant foods including grapes, peanuts and berries [26]. It is an Nrf2 activator with both anti-oxidant and anti-inflammatory effects [25,27]. Not surprisingly, resveratrol has been shown to improve endothelial function and prevent end-organ injury in diverse models of ischemia-reperfusion injury [28–30]. We wished to explore whether resveratrol might afford anti-oxidant protection to either trophoblast and/or endothelial cells with a future view to using it as an adjuvant therapy in preeclampsia.

2. Materials and methods

All human samples were collected with informed written consent and following approval from the Monash Health Human Research Ethics Committee (Monash Health Human Research Ethics Committee Reference Number: 13357B).

2.1. Placental tissue collection, explant culture and primary trophoblast isolation

Human placental samples were collected at the time of elective cesarean section from women with a healthy term singleton pregnancy.

For explant culture, several cotyledons were excised randomly and washed in Hank's balanced salt solution (HBSS) to remove blood. Placental villous tissue was dissected, weighed and on average 30–40 mg of tissue was placed in each well of a 24-well plate and cultured in Medium 199 supplemented with 1% antibiotics and L-Glutamine (all from Life Technologies, Carlsbad, CA), containing the different treatment conditions. A group of placental explants were treated with 2.3 mmol/L xanthine/15mU/mL xanthine oxidase (X/XO; Sigma-Aldrich, St. Louis, MO) to induce oxidative stress, in the presence or absence of 50 μmol/L, 100 μmol/L or 200 μmol/L resveratrol (Sigma-Aldrich). Placental explants treated with media only were used as negative controls. Explants were cultured in 5% O_2 at 37 °C for 48 h. A separate group of placental explants were exposed to 1% O_2 in the presence or absence of resveratrol at 37 °C for 24 h. Placental explants exposed to 5% O_2 were used as controls. Each treatment was performed in duplicate.

In order to isolate primary trophoblast cells, placental villi were scraped from washed cotyledons. Approximately 25g of villi were subjected to 3 cycles of digestion with digestion buffer (DMEM low glucose, 1% antibiotics, 0.25% trypsin, 0.25% grade II dispase, 0.2 mg/ml DNase I) in a 37 °C shaking water bath for 15 min. Cell suspensions were separated by Percoll gradient centrifugation. Primary trophoblast cells were collected and cultured in DMEM with 10% fetal bovine serum (FBS) and 1% antibiotics and incubated at 8% O_2 overnight. Following small interfering RNA treatment (details below) one group of primary trophoblast cells were exposed to X/XO while another group was exposed to 1% O_2 as with or without 100 μmol/L resveratrol for 24 h. Untreated cells at 8% O_2 were used as controls for both groups.

2.2. Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords collected from healthy term pregnancies at elective caesarean section, as previously described [31]. All HUVEC cultures were plated at a density of 12,000 cells per 100 mm^2 in Medium 199 supplemented with 20% FBS, 1% antibiotics and L-Glutamine (all from Life Technologies), and incubated at 37 °C in 5% CO_2. HUVECs were then treated with recombinant 1–100 ng/ml TNFα (R&D systems, Minneapolis, MN) in the presence or absence of 50 μmol/L, 100 μmol/L or 200 μmol/L resveratrol for 6 h prior to measurement of Nrf2 nuclear translocation and expression of the endothelial activation markers intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VACM1) and E-selectin. Endothelin-1 and HO-1 protein levels were assessed following a 24-h treatment period.

2.3. 8-Isoprostane, sFlt1, sEng, activin A and endothelin-1 measurement

8-isoprostane in placental explant and HUVEC culture supernatants was determined by enzyme immunoassay (Caymen Chemicals, Ann Arbor, MI). Levels of sFlt, sEng and activin A in placental explant culture supernatants and levels of endothelin-1 in HUVEC culture supernatants were all determined via enzyme-linked immunosorbent assay (ELISA; R&D systems). All assays were performed according to manufacturer's instructions.

2.4. Markers of endothelial cell activation

The markers of endothelial cell activation were determined in the HUVECs via flow cytometry (FACS). Briefly, HUVECs were mechanically scraped from their flasks prior to immunostaining with the following antibodies (antibody synonyms, antibody dilutions and catalogue numbers shown in parentheses): CD54-Pacific Blue (ICAM1, 1:100, 353109), CD106-PE (VCAM1, 1:50, 305805) CD62E-APC (E-selectin, 1:100, 336011). Immunostaining was compared with appropriate isotype controls. Antibodies and isotype controls were obtained from Biolegend (San Diego, CA). HUVECs were incubated on ice with either antibodies or isotype controls for 20 min prior to washing and fixing in 1% paraformaldehyde in FACS buffer. The markers of endothelial cell activation were then analyzed on a BD FACS Canto II (BD Biosciences, San Jose, CA). Analyses were performed using FlowJo cytometric analysis software (Oakland, OR).