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Sulforaphane improves endothelial function and reduces placental oxidative stress *in vitro*

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ABSTRACT

Introduction: The maternal endothelial dysfunction characteristic of preeclampsia arises, in part, from excessive placental production of anti-angiogenic factors, including soluble Flt-1, soluble endoglin and activin A, inducing oxidative stress. We assessed whether the antioxidant and NRF2-activator sulforaphane could mitigate endothelial and trophoblast dysfunction *in vitro*.

Methods: We induced dysfunction in human umbilical vein endothelial cells (HUVECs) with TNF-α, assessing endothelial activation and dysfunction (endothelin-1, vascular cell adhesion molecule; VCAM1, intracellular adhesion molecule; ICAM1, e-selectin and endothelial permeability) in the presence or absence of sulforaphane. We also assessed the effects of sulforaphane in mitigating hypoxic and hyperoxic injury in term placental explants by measuring secretion of anti-angiogenic factors. To assess the role of NRF2 we silenced NRF2 in HUVECs and primary trophoblast cells.

Results: Sulforaphane reduced TNF-α mediated HUVEC secretion of endothelin-1, VCAM1, ICAM1 and E-selectin, and prevented increased endothelial permeability. In placental explants, sulforaphane reduced the secretion of soluble Flt-1, soluble endoglin and activin A. Sulforaphane induced activation and nuclear translocation of NRF2 in HUVECs, inducing heme oxygenase 1. NRF2 silencing blocked some but not all of sulforaphane's effects in HUVECs. NRF2 silencing did not prevent sulforaphane's inhibition of trophobast secretion of soluble Flt-1 or activin A.

Conclusion: In reducing placental and endothelial oxidative stress, sulforaphane may offer a new adjuvant therapeutic approach for the treatment of preeclampsia.

1. Introduction

Preeclampsia remains a significant cause of maternal and perinatal morbidity and mortality, and is a leading cause of iatrogenic preterm birth [1]. The mainstay of care of women with preeclampsia is controlling maternal blood pressure to safely allow prolongation of pregnancy to improve fetal maturation [1,2]. This focus of management on the hypertension has not substantially changed in over 50 years [3]. However, over the last decade, insights into the mechanisms underlying the hypertension and maternal organ dysfunction have identified promising new avenues for other approaches to management [3].

In particular, inadequate placentation from the beginning of pregnancy is thought to result in sustained and progressive ischemic-reperfusion injury to the placenta across pregnancy [4,5]. This injury in turn underlies the excessive placental release of anti-angiogenic vasoactive substances, such as soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng) and activin A [6,7], that, via oxidative stress, induce systemic inflammation and endothelial damage, vasoconstriction, hypertension and other end-organ damage [7–11]. Recognition of inflammation and endothelial damage as key contributors to the development of preeclampsia has better enabled more precise pharmacological targeting as potential adjunctive therapeutic approaches [3].

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By protecting the endothelium, reducing systemic inflammatory stress and minimizing placental production of vasoactive compounds, halting disease progression is, perhaps, a realistic prospect.

Nuclear factor erythroid 2–related factor 2 (NRF2) is an endogenous inducer of cellular antioxidants [12]. Under physiological conditions bioavailable levels of NRF2 are regulated through cytosolic binding to Kelch-like-ECH-associated protein-1 (KEAP-1) by preventing its proteasomal degradation [12,13]. Exposure to oxidative stress induces modifications in KEAP-1, allowing NRF2 to translocate to the cell nucleus. There, NRF2 stimulates the antioxidant response element (ARE) leading to the transcription of a cassette of cellular antioxidants such as heme oxygenase-1, (HO-1), NAD(P)H dehydrogenase (NQO1) and glutathione S-transferase (GST) [12,14]. Exogenous stimulation of NRF2 has been shown to restore endothelial health and attenuate the damaging effects of vasoactive compounds in preeclampsia [12]. This suggests that the administration of a NRF2 activator may be an effective adjunctive therapy for preeclampsia [12,14].

Sulforaphane, a naturally occurring organosulfur present in cruciferous vegetables, is a NRF2 inducer that exerts both antioxidant and anti-inflammatory actions via the Nrf2/ARE pathway [15,16]. By inducing cysteine modifications to KEAP-1, sulforaphane preserves NRF2 stability, promotes cytosolic transcription, and facilitates nuclear translocation of NRF2 [13,17]. In various small animal models, sulforaphane has been shown to protect endothelial function and improve retinal, renal and liver function following ischaemic-reperfusion injuries [18–21]. It is also a promising therapy in cardiovascular disease such as chronic hypertension and atherosclerosis [22,23].

We hypothesize that sulforaphane may have utility as an adjunctive therapy for preeclampsia. Here we used *in vitro* models of preeclampsia to assess the endothelial and placental protective capacity of sulforaphane.

2. Materials and methods

2.1. Ethics

The Monash Health Human Research Ethics Committee approved the collection of placentae, umbilical cords and serum and informed written consent was obtained from each patient prior to collection. Healthy singleton term human placentae and cords for human umbilical vein endothelial cell (HUVEC), and trophoblastexplant and villous cytotrophoblast culture were collected at the time of caesarean section. Exclusion criteria were: smoking, alcohol and drug abuse, multiple pregnancies, < 38 weeks, BMI > 35, pre-existing hypertension, renal disease, diabetes, thyroid dysfunction, metabolic syndrome or preeclampsia in a previous pregnancy. Exclusions were made for the following medications; antihypertensive, aspirin, non-steroidal anti-inflammatory drugs or thyroid medications. Serum was obtained from women diagnosed with preeclampsia according to SOMANZ guidelines, [2] immediately prior to delivery.

2.2. Human umbilical vein endothelial cell culture

HUVECs were isolated from umbilical cords collected from healthy term pregnancies (n = 5–7) at the time of caesarean section, as previously described [24]. Briefly, cells were cultured in M199 medium (Gibco-BRL), 10% fetal calf serum (FCS), epidermal and fibroblast growth factors (10 ng/ml), heparin (90ug/ml), 1% streptomycin sulphate (100 μg/ml) and amphotericin B (0.25 μg/ml; Invitrogen). Flasks were coated with 0.2% gelatin before seeding with isolated HUVECs. Cells were incubated and expanded in 5% CO_2, 70% N_2, and 25% O_2 at 37 °C with cultures for experimentation taken from second and third passages.

2.3. Markers of endothelial cell activation

The markers of endothelial cell activation were quantified in HUVECs treated with sulforaphane (Sigma-Aldrich, St. Louis, MO, S6317-5MG; 5 μM, 10 μM, 20 μM) and/or TNFα (1 ng/ml, Thermo Fisher Scientific PHC3015) for 24 h, via flow cytometry, expressed as mean flow index (MFI). Briefly, HUVECs were mechanically scraped from their flasks prior to staining with one of the following antibodies, with synonyms, antibody dilutions and catalogue numbers shown in parentheses: CD54-Pacific Blue (intracellular adhesion molecule; ICAM1, 1:100, 353109), CD106-PE (vascular cell adhesion molecule; VCAM1, 1:50, 305805) CD62E-APC (E-selectin, 1:100, 336011) and staining was compared against their 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). Analysis was performed using FlowJo cytometric analysis software (Tree Star, Oakland, OR).

2.4. Endothelin-1 and 8-isoprostane production by HUVECs

Endothelin-1 (ET-1) levels were determined in the supernatants of HUVECs treated with sulforaphane (Sigma-Aldrich, St. Louis, MO, S6317-5MG; 5 μM, 10 μM, 20 μM) and/or TNFα (1 ng/ml) for 24 h via ELISA (DET100, R&D systems, Minneapolis, MN). Level of 8-isoprostane, a marker of oxidative stress, was measured in the same culture supernatants by enzyme immunoassay (Caymen Chemicals, Ann Arbor, MI). All assays were performed according to manufacturer's instructions

2.5. Endothelial cell permeability

Integrity of the endothelial cell monolayer following TNFα treatment was assessed via a FITC-dextran based permeability assay. HUVECs were plated in gelatinized polycarbonate transwell inserts (Corning, Oneonta, NY; 0.4 μM, 6.5 mm, 24 well plate) at a density of 50,000 cell insert, and incubated at 37 °C for 3 days to form a monolayer. Cells were then treated with recombinant TNFα at an optimized dose (100 ng/ml) in the presence or absence of sulforaphane (20 μM) for 24 h. At the end of the treatment period, culture media was removed from both the upper and lower chambers, and 1 mg/ml FITC-dextran (Sigma-Aldrich; MW 40,000) in culture media was added to the upper chamber, whilst fresh culture media was added to the lower chamber. Cells were then incubated for 1 h at 37 °C. Media was removed from the lower chamber and diluted 1:20 with PBS in a black-walled 96-well plate before fluorescence readings were obtained at 485 nm excitation and 535 nm emission.

Endothelial cell permeability following treatment with serum from women with preeclampsia (5% serum in M199 Medium supplemented with 1% antibiotics and 1% L-Glutamine) was measured using the 24-well *in vitro* vascular permeability assay kit (Merck Millipore, Billerica, MA). The assay was performed according to manufacturer's instructions. Endothelial cells treated with serum from gestation matched healthy pregnant women were used as controls.

2.6. Placental explant culture

Immediately after collection, several cotelydons were removed from the placenta and rinsed in chilled 1x Hank's Balanced Salt Solution (HBSS) to remove blood. Samples were submerged in 1x HBSS in a petri dish while villous tissue was gently dissected from major connective tissue and vasculature. Placental villous explants (50 mg) were then cultured on a 6 well plate with Medium 199 (Life Technologies, Carlsbad, CA) supplemented with 1% Antibiotics (Life Technologies)

and 1% L-Glutamine (Life Technologies).

One group of placental explants (n = 6) was treated with xanthine/xanthine oxidase (X/XO) (Sigma-Aldrich; 2.3 mM X and 15 mU/ml XO), to induce oxidative stress, in the presence or absence of sulforaphane (5 μM, 10 μM and 20 μM). Placental explants treated with media only were used as negative controls. These explants were cultured in 5% O_2 at 37 °C for 24 h. Another group of placental explants (n = 6) were exposed to 1% O_2 in the presence or absence of sulforaphane (5 μM, 10 μM and 20 μM) at 37 °C for 24 h. Placental explants exposed to 5% O_2 were used as negative controls. After 24 h of incubation, culture media was removed and stored at −80 °C with 0.005% butylated hydroxytoluene (BHT, Sigma-aldrich, St. Louis, MO; W218405) to prevent radical formation and degradation of 8-isoprostane.

2.7. Placental villous cytotrophoblast culture

Cotelydons were randomly excised from placentae (n = 6), the basal plate removed and samples washed in ice-cold 1x HBBS to remove residual blood and encourage villous segmentation. Samples were placed in a petri dish and submerged in 1x HBSS. Villi (20–25 g) were separated from vessels and exposed to three digestions in low glucose DMEM (Life technologies; 316000083) with 7.5 ml of 2.5% trypsin (Life Technologies; 15090046), 0.19 g of Dispase Grade II (Life Technologies; 17105–041) and 150uL of bovine DNase I Grade II (Sigma-Aldrich; 10104159001) for 15 min in a 37 °C water bath. Between each digestion supernatant was collected, filtered through a cell strainer and held at room temperature until all incubations were completed. Samples were centrifuged at 350 g, 4 °C for 5 min, the supernatant discarded and the pellet resuspended in complete trophoblast media (DMEM/F12 Life Technologies; 11330-057, 1% antimycotic-antibiotic Life Technologies 15; 140-122 and Life Technologies; 15240-062). Percoll gradient separation (5–70% GE Healthcare; 17-0891-01) was used with centrifugation at 1200 g, 4 °C for 20 min to isolate a layer of villous cytotrophoblasts. These were diluted in complete trophoblast media and plated at 0.7 million cells per well on a 24 well plate. All samples were incubated at 37 °C in 8% oxygen for 24 h to encourage adhesion and proliferation.

Samples were randomly divided into two treatment groups. The first X/XO (2.3 mM X and 15 mU/m XO) in the presence or absence of sulforapahane (20 μM) in 8% oxygen. The second group was placed in 1% oxygen in the presence or absence of sulforaphane (20 μM). Negative controls either received sulforaphane or not and did not undergo treatment. Samples were incubated at 37 °C in their respective oxygen incubators for a further 24 h.

2.8. Small interfering RNA transfection

HUVECs and placental explants were reverse transfected with single stranded small interfering RNA (siRNA) directed towards NRF2, scrambled sequence (negative control) or GAPDH (positive control) with the use of Lipofectamine RNAimax Reagent (all from Life Technologies, Carlsbad, CA), according to manufacturer's instructions. All experiments were completed within 4 days of transfection as described below. Samples were returned to 8% oxygen, 37 °C and incubated for a further 24 h.

2.9. Assays

Commercial ELISAs were used in accordance with manufacturer guidelines. Levels of sFlt-1 (R&D Systems, Minneapolis, Human VEGF R1/Flt1 DVR100B dilution: 1:25) and sEng (R&D Systems, Minneapolis Human Endoglin/CD105 DNDG00 dilution: neat) were tested in the supernatant of hypoxic samples while concentrations of 8-isoprostane (Cayman, Ann Arbour, 8-isoprostane 516,351 dilution 1:50) and activin A (R&D Systems, Minneapolis DAC00B dilution: 1:2) were evaluated in the supernatant of X/XO treated samples.

2.10. Protein extraction

HUVEC and placental supernatant was collected and stored at −80 °C with 0.005% BHT (Sigma-Aldrich) to prevent radical formation and degradation of 8-isoprostane. Protein was extracted using physical cell scraping in the presence of lysis buffer (made in house) and protease inhibitor (Sigma-Aldrich; 11697498001). Centrifugation was used to separate cellular debris and the resulting supernatant was stored at −80 °C prior to analysis with western blot.

2.11. Western blot

HUVEC cultures were assessed for NRF2 activation and antioxidant response by determining nuclear NRF2 translocation and HO-1 protein levels. Confirmation of siRNA knockdown was also performed using western blot for HO-1. Nuclear and cytoplasmic fractions were isolated using NE-PER reagents (Thermofisher) Protein levels were quantified using BCA. Western blots were performed as previously described [8]. Membranes were blocked with 5% skim milk in 1 x HBBS with 0.1% Tween-20 for 1 h prior to probing with antibodies. The primary antibody HO-1 (ab52947, 1:2000, Abcam) was diluted in blocking buffer and incubated at 4 °C overnight. Membranes were incubated in secondary anti-rabbit antibody (sc-2004, 1:10000, Abcam) with blocking buffer for one hour at room temperature, prior to imaging (biorad ChemiDoc, Western ECL substrate, 170–5060). Membranes were then washed and incubated in primary beta actin antibody for cytosoloic fractions (sc-47778, 1:5000, Abcam) or HDAC1 for nuclear fractions (ab7028, 1:2000, Abcam) and secondary anti-mouse (sc-2005, 1:20000, Abcam) each for one hour at room temperature.

2.12. Statistical analyses

Statistical analyses were performed using one-way ANOVA followed by multiple comparisons with a Tukey post-hoc test. Groups were considered to be significantly different if $P < 0.05$. All data were analysed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Sulforaphane increased NRF2 protein levels, NRF2 nuclear translocation and HO-1 protein levels in HUVECs

Treatment of HUVECs with TNF-α did not induce NRF2 nuclear translocation (Fig. 1A) or total NRF2 (Fig. 1B). Treatment of HUVECs with sulforaphane increased nuclear translocation of NRF2 3-fold with a corresponding 4-fold increase in total NRF2, compared with untreated and TNF-α alone treated HUVECs (Fig. 1A & C; $p < 0.01$, Fig. 1B & C; $p < 0.0001$). This was associated with an almost 4-fold increase in HO-1 protein levels compared to TNF-α treatment alone (Fig. 1D $p = 0.04$), confirmed by western blot (Fig. 1E).

3.2. Sulforaphane ameliorated endothelial cell dysfunction in vitro

Treatment of HUVECs with TNFα significantly increased the expression of ICAM1, VCAM1 and E-selectin ($P < 0.001$; Fig. 2A–C). These effects were mitigated by sulforaphane in a dose-dependent manner (Fig. 2A–C; $p < 0.01$). TNF-α treatment of HUVECs also increased expression of ET-1 ($p = 0.004$). Only the highest dose of sulforaphane (20 μM) significantly mitigated this effect (Fig. 2D, $p = 0.04$). At 20 μM sulforaphane also attenuated both TNF-α and preeclamptic serum stimulated increases in endothelial cell monolayer permeability (Fig. 2E; $p = 0.001$ & Fig. 2F; $p = 0.04$, respectively, expressed as fold change from control).

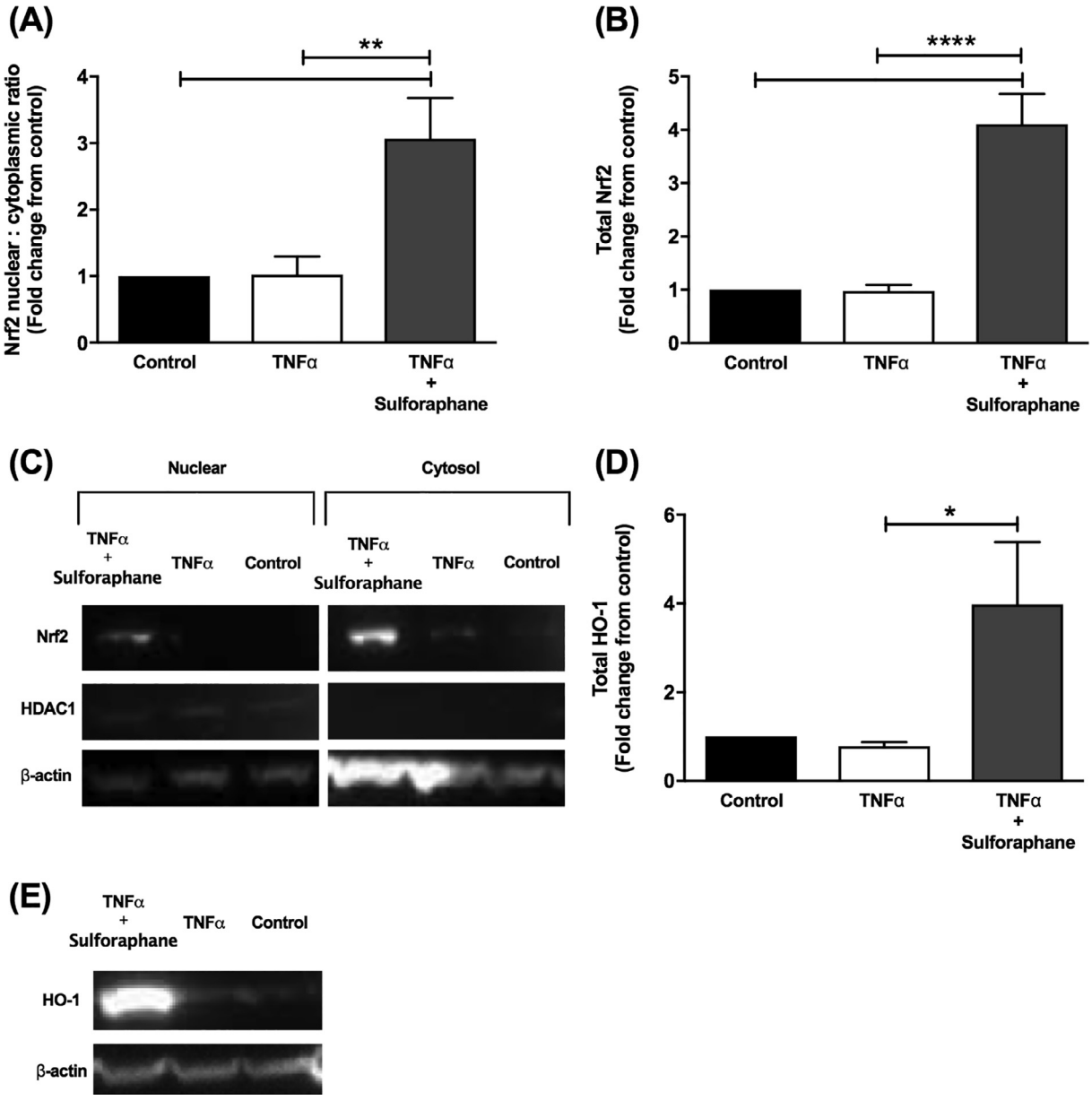

Fig. 1. Nrf2 and HO-1 protein expression in HUVECs treated with sulforaphane. (A) Nrf2 nuclear translocation and (B) total Nrf2 protein expression were measured 6 h after HUVECs were treated with media only, TNF-α (100 ng/ml) or TNF-α with 20 μM sulforaphane. (C) Representative western blot image for Nrf2 are shown with white space indicating noncontiguous lanes from the same blot. (D) Total HO-1 expression measured 24-hours after treatments and a (E) representative western blot image. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. n = 6 cell lines from independent placental donors.

3.3. Nrf2 knockdown blocked some of the endothelial cell effects of sulforaphane

To determine the involvement of NRF2 as a mediator of sulforaphane on *in vitro* endothelial function, we silenced NRF2 with siRNA. In the presence of NRF2 silencing sulforaphane neither increased HO-1 in HUVECs (Fig. 3A & B) nor prevented TNF-α induced increased endothelial monolayer permeability (Fig. 3C, expressed as fold change from control). NRF2 silencing prevented the TNF-α-induced increase in ICAM-1 (Fig. 3D) but it did not block sulforaphane's mitigation of TNF-α induction of VCAM-1 and E-selectin (Fig. 3E & 3F respectively; all $p < 0.05$). Silencing NRF2 blocked the TNF-α-induced increases in ET-1 and the oxidative stress marker 8-isoprostane, preventing any injury and therefore any assessment of sulforaphane (Fig. 3G & H respectively).

3.4. Sulforaphane altered placental explant and isolated trophoblast secretion of vasoactive compounds

We then sought to evaluate the effects of sulforaphane on the placental secretion of the vasoactive compounds sFlt-1 and soluble sEng following hypoxic injury (1% O_2). Both compounds are elevated in the serum of preeclamptic women and have been implicated in the endothelial dysfunction that underlies disease progression. Placental explant culture in 1% O_2 significantly increased sFlt-1 ($p = 0.04$) and sEng, ($p = 0.005$) secretion (Fig. 4A and 4B), an effect prevented by sulforaphane at 20 μM, but not at lower concentrations (sFlt-1: Fig. 4A, $p = 0.047$) and (sEng: Fig. 4B, $p = 0.02$).

Mimicking oxidative injury, exposure of placental explants to X/XO significantly increased activin A secretion ($p = 0.004$), an effect mitigated by sulforaphane treatment at 10 μM and 20 μM concentrations (Fig. 4C $p = 0.03$, $p = 0.047$).

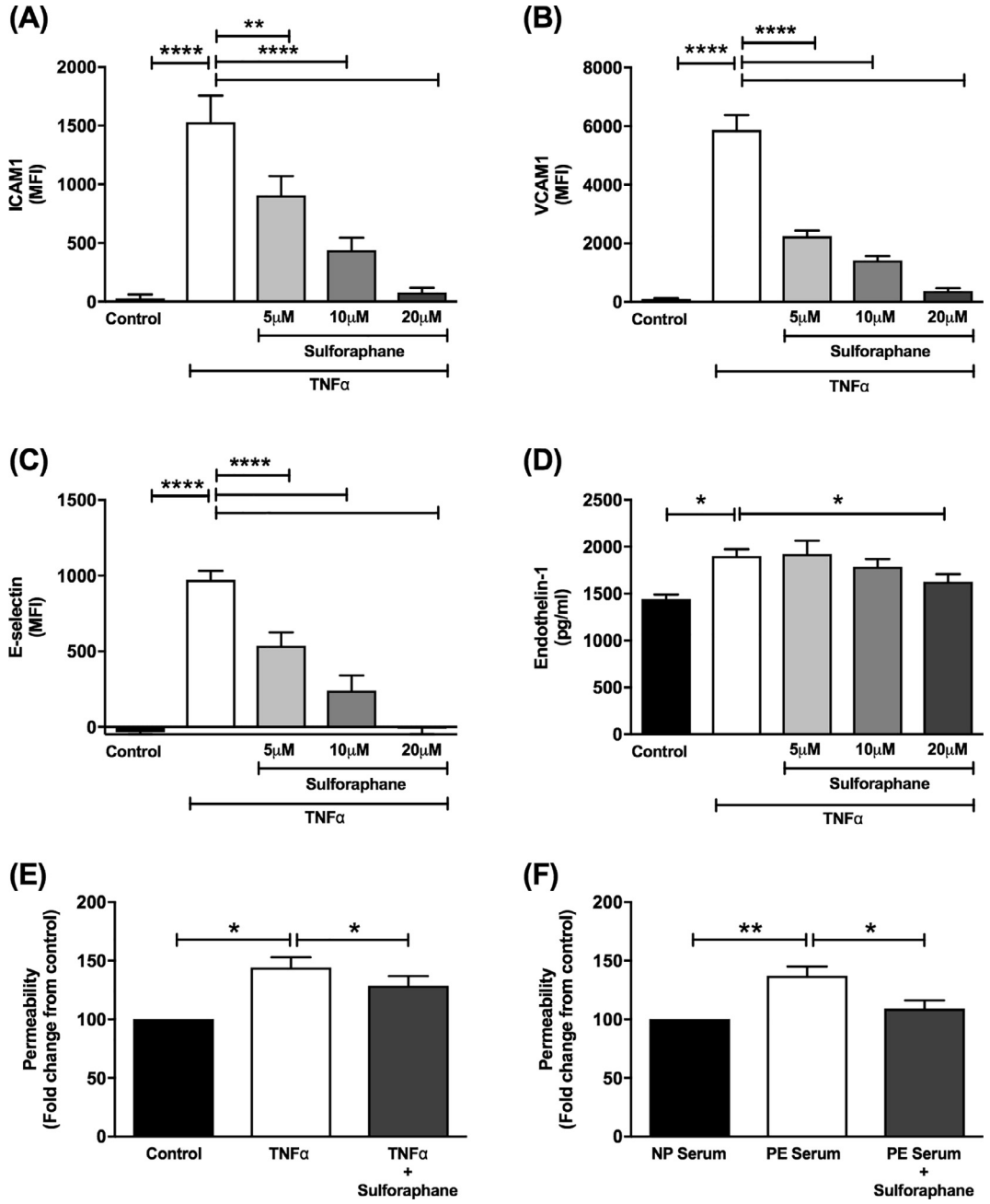

Fig. 2. Effect of sulforaphane treatment on hallmarks of endothelial dysfunction *in vitro*. Expression of (A) ICAM1, (B) VCAM1 and (C) E-selectin measured 6 h after HUVECs were treated with either media only, TNF-α (1 ng/ml figures a-d or 100 ng/ml figures e-f) or TNF-α with 3 different doses of sulforaphane (5 μM, 10 μM, 20 μM) as measured using flow cytometry. Protein levels of (D) endothelin-1 in culture supernatants collected 24-hours after treatment was measured using ELISA. (E) FITC-dextran permeability through HUVEC monolayers treated with either media only, TNF-α (100 ng/ml) or TNF-α and 20 μM sulforaphane following 24 h of treatment, expressed as fold change from control. (F) FITC-dextran permeability in HUVEC monolayers 24 h after treatment with either serum from normal pregnant women (NP serum), with serum from preeclamptic women (PE serum) or with PE serum and 20 μM sulforaphane, expressed as fold change from control. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. n = 5–7 cell lines from independent placental donors.

We then used isolated villous cytotrophoblasts to explore the effect of sulforaphane in ameliorating production of sFlt-1, sEng, activin A and 8-isporostane. Incubation in 1% O_2 did not significantly increase trophoblast production of sFlt-1 (Fig. 5A) or sEng (Fig. 5B). However, administration of sulforaphane (20 μM) significantly reduced sFlt-1 secretion, compared to normoxic controls (p = 0.0006) and hypoxic culture (p = 0.0003). There were no effects of sulforaphane on sEng secretion (p = 1.0).

Treatment of isolated trophoblasts with X/XO significantly increased secretion of both activin A (p = 0.006) and 8-isoprostane (p = 0.009). Sulforaphane (20 μM) prevented the increase in activin A (p = 0.02) but not 8-isoprostane.

To explore whether the effects of sulforaphane in villous cytotrophoblasts were mediated via the NRF2 pathway we used single stranded silencing RNA directed at NRF2 to block this pathway. Despite NRF2 silencing, sulforaphane continued to decrease sFlt-1 secretion from trophoblasts compared to both normoxic (p = 0.04) and hypoxic (p = 0.001) cultures. Similarly, NRF2 silencing did not block sulforaphane mitigation of X/XO-induced activin A secretion (p = 0.01). Paradoxically, we observed a rise in soluble endoglin (p = 0.007) when sulforaphane was administered to NRF2 silenced cells under hypoxic conditions (Fig. 5).

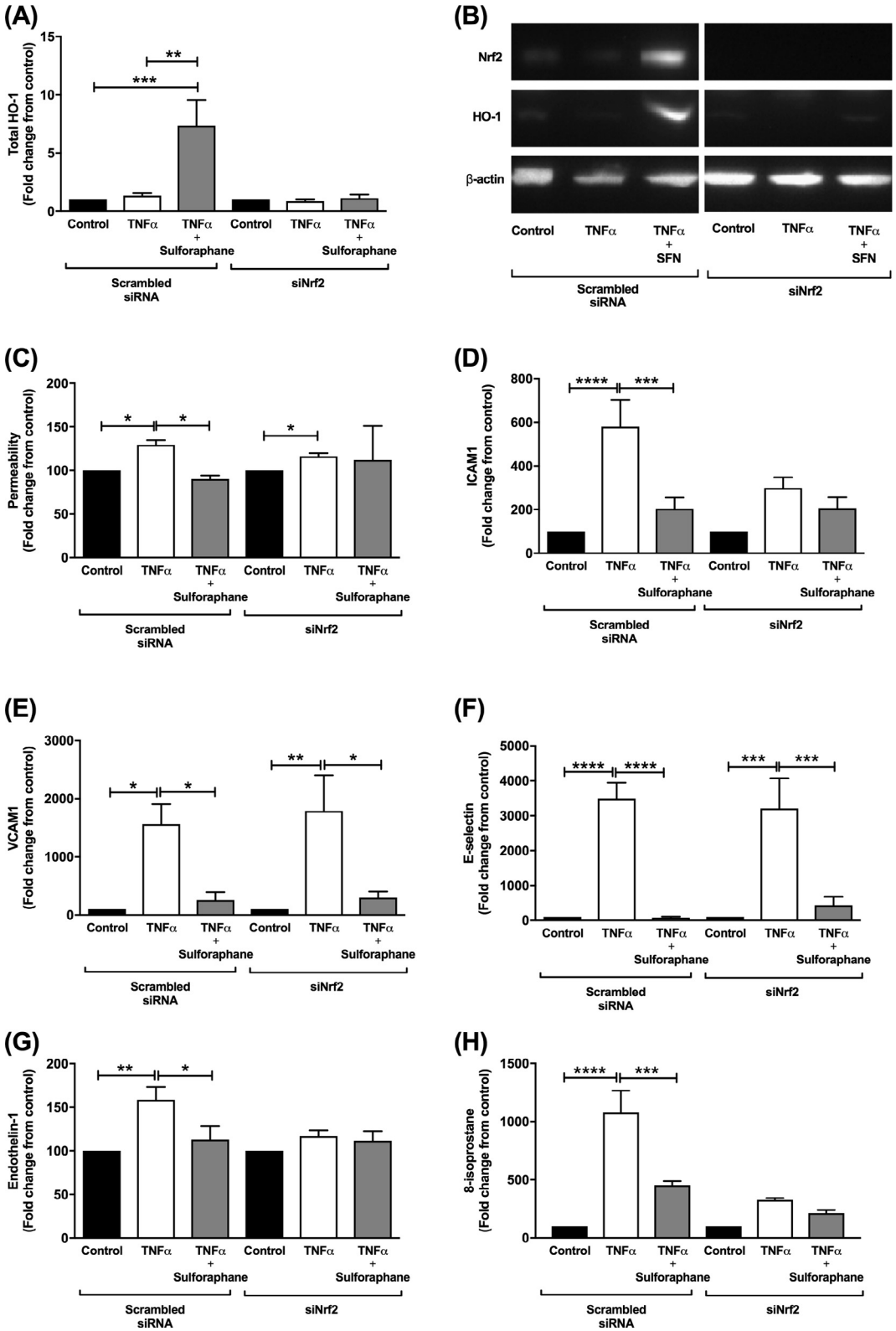

Fig. 3. Nrf2 knockdown. HUVECs were transfected with small interfering RNA directed towards Nrf2 (siNrf2) and treated with either media only, TNF-α only (1 ng/ml figures a,b, d-f and 100 ng/ml figure c) or TNF-α with 20 μM sulforaphane. HUVECs transfected with small interfering RNA containing a scrambled sequence (scrambled siRNA) and then exposed to the treatments were used as controls. (A) Total HO-1 protein expression in HUVECs 24-hours after treatment measured using western blot. (B) Representative images for HO-1 western blot. Additionally, after 24-hours FITC-dextran permeability though (C) HUVEC monolayers was measured, expressed as fold change from control. Expression of (D) ICAM1, (E) VCAM1 and (F) E-selectin was measured 6 h after treatment using flow cytometry. Levels of (G) endothelin-1 and (H) 8-isoprostane in HUVEC culture supernatants were measured 24 h after treatment using ELISA and EIA respectively. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. n = 5–6 cell lines from independent placental donors.

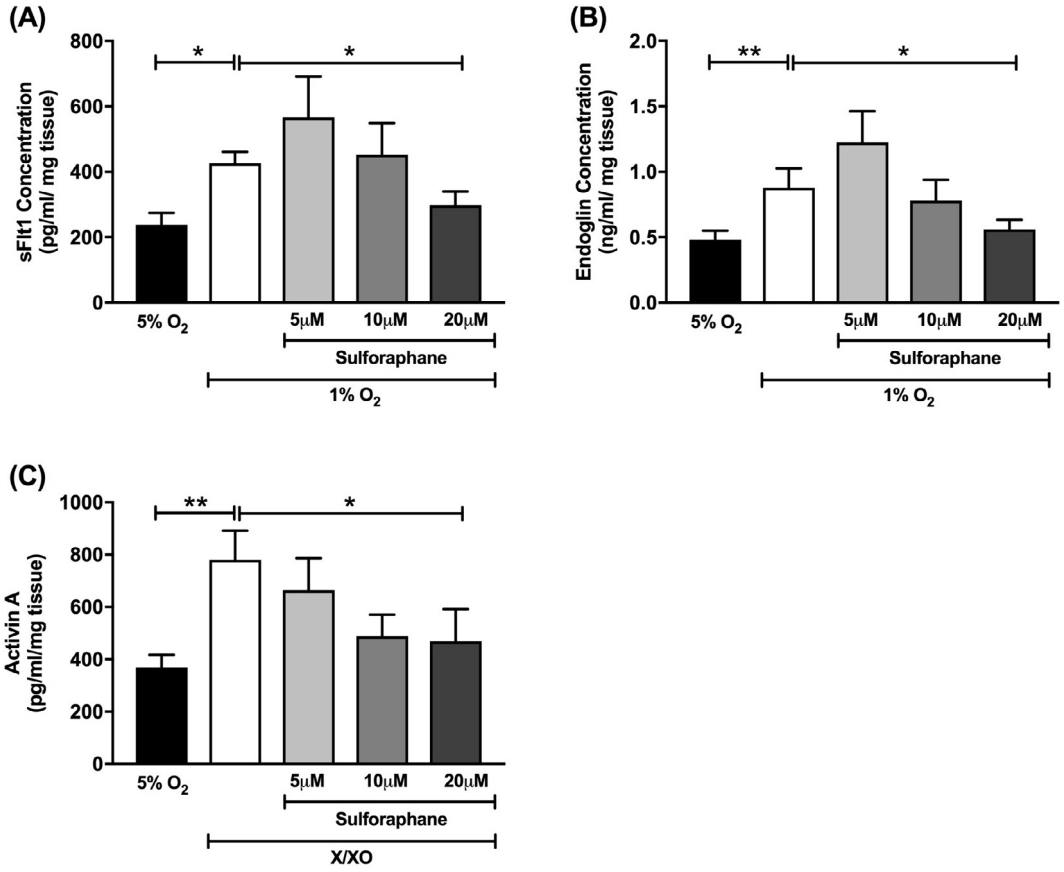

Fig. 4. Effect of sulforaphane treatment on cultured placental explants *in vitro*. Explants were incubated for 24 h in 5% oxygen 37˚c in either media only or media with X/XO solution, or in 1% oxygen 37˚c for 24 h, in the presence or absence of sulforaphane (5 μM, 10 μM and 20 μM). Levels of (A) sFlt-1 and (B) sEng were quantified using ELISA from culture media of hypoxic samples after 24 h of treatment. (C) The level of activin A quantified using ELISA from culture media of X/XO treated samples after 24 h of treatment. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. n = 6 explants from independent placental donors.

We saw no significant changes in HO-1 protein expression in any treatment group (data not shown).

4. Discussion

Here we have shown that sulforaphane is able to protect endothelial cells and reduce placental secretion of vasoactive compounds. Specifically, in endothelial cells sulforaphane reduced TNF-α induced endothelial monolayer permeability and the secretion of ET-1, ICAM, VCAM and E-selectin. In placental explants, sulforaphane reduced secretion of factors known to be upregulated by preeclampsia, including sFlt-1, sEng and activin A. While NRF2 silencing blocked the effects of sulforaphane in HUVECs, it did not prevent the sulforaphane-induced decrease in trophoblast secretion of sFlt-1 or activin A, suggesting that some of sulforaphane's actions operate through pathways other than NRF2.

Following the observation that oxidative stress was a likely common pathway causing the maternal endothelial dysfunction underlying preeclampsia, [25–27] there has been much interest in antioxidants as preventative therapies [28–31]. While the results from small clinical trials were promising [32], large randomized clinical trials of vitamin C and E failed to show benefit [33,34]. As vitamin C and E act in a downstream manner, reducing intracellular superoxide and inducing endothelial nitric oxide synthase (eNOS) activation, [35] it is possible that this post-transcriptional approach to antioxidant defense is too late in the disease cascade. Rather than an exogenous antioxidant approach, we suggest that promoting and harnessing endogenous defenses may be more effective.

This is not a new suggestion. Statins are known to induce the NRF2 pathway [36] and reduce markers of preeclampsia [37]. A pilot clinical trial suggested that pravastatin was safe in early pregnancy and may be an effective preventative. Results of larger trials are awaited. Resveratrol is another NRF2 activator that we, [38] and others, [39] have shown can reduce trophoblast secretion of anti-angiogenic factors sFlt-1, sEng and activin A, and can mitigate endothelial dysfunction *in vitro*. However, it was recently reported that resveratrol may adversely affect fetal pancreatic development [40]. Given these safety concerns we explored another NRF2 regulator, sulforaphane. Despite clinical evidence of the safety and efficacy of sulforaphane, this compound has yet to be evaluated in pregnant women or in models of preeclampsia [41–43]. Therefore, as a first step our intent was to evaluate sulforaphane *in vitro*, using concentrations of sulforaphane similar to therapeutic *in vitro* levels [41–43].

We confirmed that sulforaphane improves endothelial health in HUVECs treated with TNFα. Others have shown that sulforaphane reduced TNFα-induced expression of VCAM and ICAM in human endothelial cell types [44,45]. As with resveratrol, [38] sulforaphane mitigated the placental secretion of sFlt-1, sEng and activin A. This prompted us to undertake experiments with isolated villous cytotrophoblasts. However, simulating hypoxic-reperfusion placental injury by hypoxia (1% oxygen) did not increase sFlt-1 or sEng secretion from villous cytotrophoblasts as it did in placental explants [46,47]. This has been reported by others [48]. We believe that this is most likely due to abnormal syncytial formation under hypoxic conditions [49]. However, sulforaphane did significantly reduce the secretion of sFlt-1 and activin A. Taken together, our observations suggest that sulforaphane may

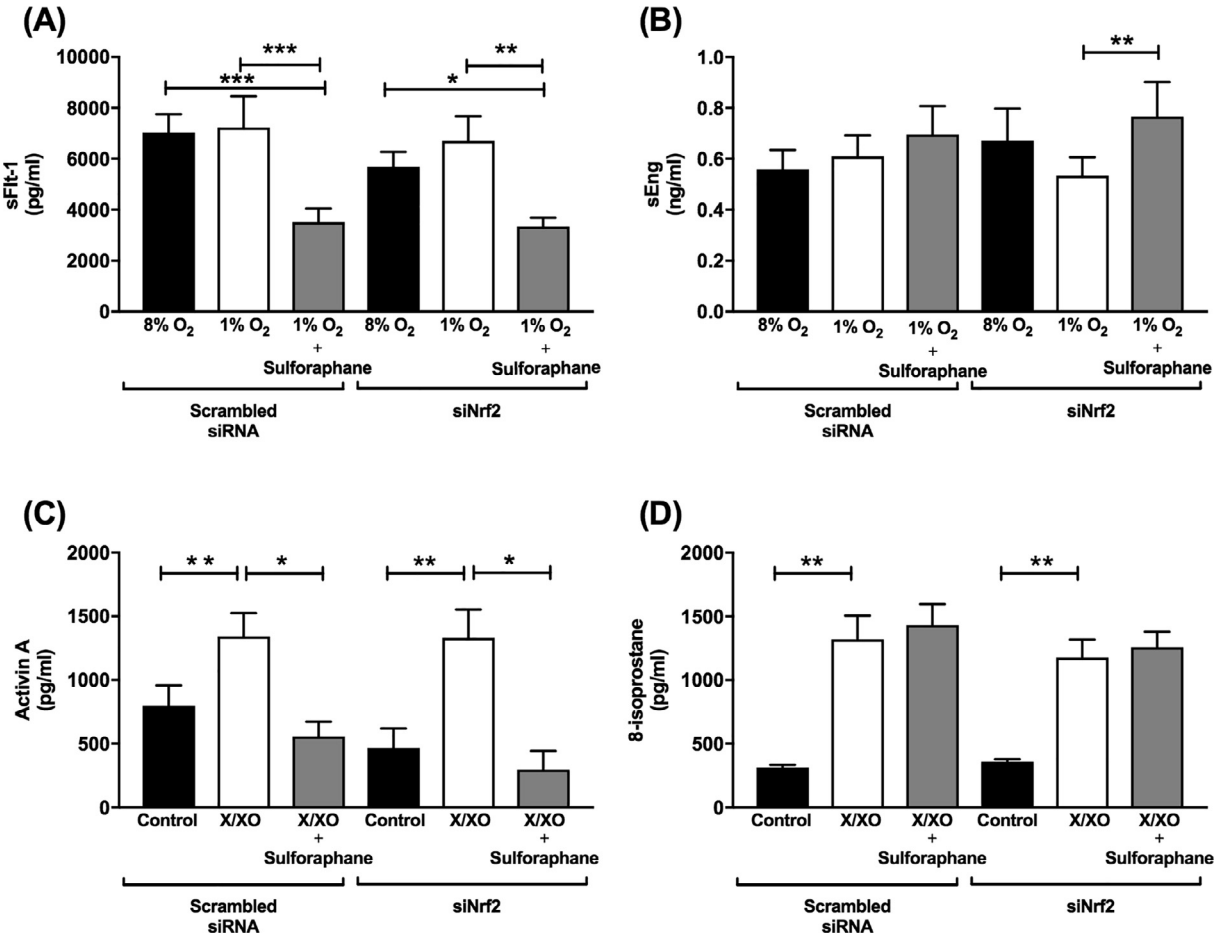

Fig. 5. Effect of sulforaphane on treated placental villous cytotrophoblasts. Trophoblasts were incubated for 24 h in 8% oxygen at 37˚c before silencing with single stranded mRNA silencing sequence directed at Nrf2 or scrambled negative sequence. After 48 h, samples were treated with either X/XO and returned to 8% oxygen at 37˚c, or moved to a hypoxic environment of 1% oxygen at 37˚c, in the presence of absence of sulforaphane (20 μM). At 72 h levels of (A) sFlt-1 and (B) sEng were quantified in hypoxic supernatant and (C) activin A and (D) 8-isoprostane measured in X/XO treated culture media. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. n = 6 placentae.

offer promise as a preventative and adjuvant treatment for pre-eclampsia and that clinical trials would be worthwhile.

A novel aspect to our study is that we silenced the NRF2 pathway to explore the involvement of NRF2 in sulforaphane's effects. As we did, others have shown that the sulforaphane-induced decrease in adhesion molecules expression was independent of NRF2 [44,45]. We also found that sulforaphane suppression of sFlt-1 and activin A secretion from villous cytotrophoblasts was maintained after NRF2 knockdown. This suggests that sulforaphane confers placental protection through mechanisms beyond the NRF2 pathway. Whether NRF2 silencing results in cell death has yet to be confirmed but we observed no difference in protein levels between treatment groups (data not shown) suggesting that this was not the case. The role of NRF2 signaling and HO-1 protein abundance in villous cytotrophoblasts remains unclear [39]. Studies advocating antioxidant-mediated cytoprotection via the NRF2/HO-1 pathway did not use villous cytotrophoblasts [50]. In fact, HO-1 activation in primary cytotrophoblasts did not confer cellular protection, nor did silencing HO-1 increase secretion of antiangiogenic compounds [51]. Therefore, in villous cytotrophoblasts, sulforaphane may act via alternate antioxidant pathways such as Rho A/ROCK and NF-κB signaling [45].

Sulforaphane influences TGF-β signaling, inhibiting the pro-inflammatory 2/3 pathway and stimulating Samd1/5/8 to reduce immune activation [52,53] and improve cellular function, independently of NRF2. That sEng secretion from cytotrophoblast cells increased under hypoxic conditions in the presence of sulforaphane following NRF2 knockdown further implicates TGF-β signaling. As a co-receptor for the TGF-β receptor, endoglin mediates a pro-angiogenic effect via the TGF-βRII/ALK1/Smad1/5/8 signaling cascade, a mechanism involved in vascular proliferation and migration [54,55]. This pathway is strongly linked to angiogenic maintenance through activation of eNOS and vessel dilation. Though the exact mechanisms remain unclear, sEng is believed to induce Smad 2/3 anti-angiogenic signaling, resulting in inflammation and endothelial dysfunction [55]. As such, by accentuating TGF-β 1/5/8 signaling, sulforaphane may induce a positive feedback mechanism to increase endoglin synthesis [56]. This mechanism is important given that TGF-β signaling has been implicated in structural abnormalities of preeclamptic placentae [57].

In summary, we have shown that sulforaphane is both endothelial protective, largely though not solely via NRF2 mediated antioxidant actions, and has protective effects in reducing the placental production of antiangiogenic compounds. However, the lack of ability of sulforaphane to mitigate all injury, in either endothelial cells or trophoblast, likely reflects the multiple pathways underlying such injuries, only some of which are targeted by sulforaphane. Similarly, that some of the actions of sulforaphane are not blocked by NRF2 silencing suggests other, as yet unknown, mechanisms of action. It is clear that the development of novel preventative and therapeutic agents in pre-eclampsia remains dependent on further elucidation of the fundamental pathogenesis of the disease. That said, we suggest that the data reported here support further study of sulforaphane as a potential adjuvant therapy for preeclampsia.

Conflict of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preghy.2019.02.002.

References

[1] J.P.S. A. Metin Gülmezoglu, Matthews Mathai et al, WHO recommendations for Prevention and treatment of pre-eclampsia and eclampsia, World Health Organisation Geneva, Switzerland, 2011.
[2] S.A. Lowe, L. Bowyer, K. Lust, L.P. McMahon, M.R. Morton, R.A. North, M.J. Paech, J.M. Said, Guideline for the management of hypertensive disorders of pregnancy, SOMANZ Guidelines, Society of Obstetric Medicine of Australia and New Zealand, 2017, pp. 5–7.
[3] C. Fenton, S.R. Hobson, E.M. Wallace, R. Lim, Future therapies for pre-eclampsia: beyond treading water, Aust New Zealand J Obstet Gynaecol 54 (1) (2014) 3–8.
[4] J.M. Roberts, C.A. Hubel, The two stage model of preeclampsia: variations on the theme, Placenta 30 (Suppl A) (2009) S32–S37.
[5] A.C. Palei, F.T. Spradley, J.P. Warrington, E.M. George, J.P. Granger, Pathophysiology of hypertension in preeclampsia: a lesson in integrative physiology, Acta Physiol. 208 (3) (2013) 224–233.
[6] S. Gurusinghe, E.M. Wallace, R. Lim, The relationship between Activin A and anti-angiogenic factors in the development of pre-eclampsia, Preg. Hypertens. 4 (1) (2014) 3–6.
[7] A. Wang, S. Rana, S.A. Karumanchi, Preeclampsia: the role of angiogenic factors in its pathogenesis, Physiology 24 (2009) 147–158.
[8] R. Lim, R. Acharya, P. Delpachitra, S. Hobson, C.G. Sobey, G.R. Drummond, E.M. Wallace, Activin and NADPH-oxidase in preeclampsia: insights from in vitro and murine studies, Am. J. Obstet. Gynecol. 212 (1) (2015) 86.e1-12.
[9] S. Mandang, U. Manuelpillai, E.M. Wallace, Oxidative stress increases placental and endothelial cell activin A secretion, J. Endocrinol. 192 (3) (2007) 485–493.
[10] S.R. Hobson, R. Acharya, R. Lim, S.T. Chan, J. Mockler, E.M. Wallace, Role of activin A in the pathogenesis of endothelial cell dysfunction in preeclampsia, Pregn. Hypertens. Int. J. Women's Cardiovasc. Health 6 (2) (2016) 130–133.
[11] S. Muttukrishna, J. Hyett, M. Paine, J. Moodley, N. Groome, C. Rodeck, Uterine vein and maternal urinary levels of activin A and inhibin A in pre-eclampsia patients, Clin. Endocrinol. 64 (4) (2006) 469–473.
[12] N. Kweider, C.J. Wruck, W. Rath, new insights into the pathogenesis of pre-eclampsia – the role of Nrf2 activators and their potential therapeutic impact, Geburtshilfe Frauenheilkd. 73 (12) (2013) 1236–1240.
[13] E. Kansanen, S.M. Kuosmanen, H. Leinonen, A.-L. Levonen, The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer, Redox Biol. 1 (1) (2013) 45–49.
[14] N. Kweider, B. Huppertz, M. Kadyrov, W. Rath, T. Pufe, C.J. Wruck, A possible protective role of Nrf2 in preeclampsia, Ann. Anat. = Anatomischer Anzeiger: official organ of the Anatomische Gesellschaft 196 (5) (2014) 268–277.
[15] Y. Zhang, P. Talalay, C.G. Cho, G.H. Posner, A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure, Proc. Natl. Acad. Sci. 89 (6) (1992) 2399–2403.
[16] M.A. Riedl, A. Saxon, D. Diaz-Sanchez, Oral sulforaphane increases Phase II antioxidant enzymes in the human upper airway, Clin. Immunol. (Orldo, Fla.) 130 (3) (2009) 244–251.
[17] D.D. Zhang, M. Hannink, Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress, Mol. Cell. Biol. 23 (22) (2003) 8137–8151.
[18] H. Pan, M. He, R. Liu, N.C. Brecha, A.C. Yu, M. Pu, Sulforaphane protects rodent retinas against ischemia-reperfusion injury through the activation of the Nrf2/HO-1 antioxidant pathway, PLoS One 9 (12) (2014) e114186.
[19] C.E. Guerrero-Beltrán, P. Mukhopadhyay, B. Horváth, M. Rajesh, E. Tapia, I. García-Torres, J. Pedraza-Chaverri, P. Pacher, Sulforaphane, a natural constituent of broccoli, prevents cell death and inflammation in nephropathy, J. Nutr. Biochem. 23 (5) (2012) 494–500.
[20] H.-Y. Yoon, N.-I. Kang, H.-K. Lee, K.Y. Jang, J.-W. Park, B.-H. Park, Sulforaphane protects kidneys against ischemia-reperfusion injury through induction of the Nrf2-dependent phase 2 enzyme, Biochem. Pharmacol. 75 (11) (2008) 2214–2223.
[21] H.-D. Zhao, F. Zhang, G. Shen, Y.-B. Li, Y.-H. Li, H.-R. Jing, L.-F. Ma, J.-H. Yao, X.-F. Tian, Sulforaphane protects liver injury induced by intestinal ischemia reperfusion through Nrf2-ARE pathway, World J. Gastroenterol. 16 (24) (2010) 3002–3010.
[22] L. Wu, M.H. Noyan Ashraf, M. Facci, R. Wang, P.G. Paterson, A. Ferrie, B.H. Juurlink, Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system, Proc. Natl. Acad. Sci. U.S.A. 101 (18) (2004) 7094–7099.
[23] G.S. Shehatou, G.M. Suddek, Sulforaphane attenuates the development of atherosclerosis and improves endothelial dysfunction in hypercholesterolemic rabbits, Exp. Biol. Med. 241 (4) (2016) 426–436.
[24] B. Baudin, A. Bruneel, N. Bosselut, M. Vaubourdolle, A protocol for isolation and culture of human umbilical vein endothelial cells, Nat. Protoc. 2 (3) (2007) 481–485.
[25] C.A. Hubel, Oxidative stress in the pathogenesis of preeclampsia, Proc. Soc. Exp. Biol. Med. 222 (3) (1999) 222–235.
[26] J.M. Roberts, C.A. Hubel, Is oxidative stress the link in the two-stage model of pre-eclampsia? Lancet 354 (9181) (1999) 788–789.
[27] J.C. Livingston, R. Chin, B. Haddad, E.T. McKinney, R. Ahokas, B.M. Sibai, Reductions of vascular endothelial growth factor and placental growth factor concentrations in severe preeclampsia, Am. J. Obstet. Gynecol. 183 (6) (2000) 1554–1557.
[28] L.C. Chappell, P.T. Seed, A.L. Briley, F.J. Kelly, R. Lee, B.J. Hunt, K. Parmar, S.J. Bewley, A.H. Shennan, P.J. Steer, L. Poston, Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomised trial, Lancet 354 (9181) (1999) 810–816.
[29] L.C. Chappell, P.T. Seed, F.J. Kelly, A. Briley, B.J. Hunt, D.S. Charnock-Jones, A. Mallet, L. Poston, Vitamin C and E supplementation in women at risk of pre-eclampsia is associated with changes in indices of oxidative stress and placental function, Am. J. Obstet. Gynecol. 187 (3) (2002) 777–784.
[30] D. Beazley, R. Ahokas, J. Livingston, M. Griggs, B.M. Sibai, Vitamin C and E supplementation in women at high risk for preeclampsia: a double-blind, placebo-controlled trial, Am. J. Obstet. Gynecol. 192 (2) (2005) 520–521.
[31] R. Rahman, P. Murthi, H. Singh, S. Gurusinghe, J.C. Mockler, R. Lim, E.M. Wallace, The effects of hydroxychloroquine on endothelial dysfunction, Pregn. Hypertens. 6 (4) (2016) 259–262.
[32] D.R. McCance, V.A. Holmes, M.J. Maresh, C.C. Patterson, J.D. Walker, D.W. Pearson, I.S. Young, Vitamins C and E for prevention of pre-eclampsia in women with type 1 diabetes (DAPIT): a randomised placebo-controlled trial, Lancet 376 (9737) (2010) 259–266.
[33] A. Rumbold, E. Ota, C. Nagata, S. Shahrook, C.A. Crowther, Vitamin C supplementation in pregnancy, Cochrane Database Syst. Rev. 9 (2015) Cd004072.
[34] A. Rumbold, E. Ota, H. Hori, C. Miyazaki, C.A. Crowther, Vitamin E supplementation in pregnancy, Cochrane Database Syst. Rev. 9 (2015) Cd004069.
[35] M.G. Traber, J.F. Stevens, Vitamins C and E: beneficial effects from a mechanistic perspective, Free Radical Biol. Med. 51 (5) (2011) 1000–1013.
[36] Y. Chigusa, K. Kawasaki, E. Kondoh, H. Mogami, M. Ujita, K. Fujita, K. Tatsumi, S. Takeda, I. Konishi, Simvastatin inhibits oxidative stress via the activation of nuclear factor erythroid 2-related factor 2 signaling in trophoblast cells, J. Obstet. Gynaecol. Res. 42 (1) (2016) 36–43.
[37] F.C. Brownfoot, S. Tong, N.J. Hannan, R. Hastie, P. Cannon, T.u.J. Kaitu'u-Lino, Effects of simvastatin, rosuvastatin and pravastatin on soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sENG) secretion from human umbilical vein endothelial cells, primary trophoblast cells and placenta, BMC Pregn. Childbirth 16 (2016) 117.
[38] S. Gurusinghe, A.G. Cox, R. Rahman, S.T. Chan, R. Muljadi, H. Singh, B. Leaw, J.C. Mockler, S.A. Marshall, P. Murthi, R. Lim, E.M. Wallace, Resveratrol mitigates trophoblast and endothelial dysfunction partly via activation of nuclear factor erythroid 2-related factor-2, Placenta 60 (2017) 74–85.
[39] N.J. Hannan, F.C. Brownfoot, P. Cannon, M. Deo, S. Beard, T.V. Nguyen, K.R. Palmer, S. Tong, T.u.J. Kaitu'u-Lino, Resveratrol inhibits release of soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin and improves vascular dysfunction – implications as a preeclampsia treatment, Sci. Rep. 7 (1) (2017) 1819.
[40] V.H. Roberts, L.D. Pound, S.R. Thorn, M.B. Gillingham, K.L. Thornburg, J.E. Friedman, A.E. Frias, K.L. Grove, Beneficial and cautionary outcomes of resveratrol supplementation in pregnant nonhuman primates, FASEB J. 28 (6) (2014) 2466–2477.
[41] J.F. Doss, J.C. Jonassaint, M.E. Garrett, A.E. Ashley-Koch, M.J. Telen, J.T. Chi, Phase 1 study of a sulforaphane-containing broccoli sprout homogenate for sickle cell disease, PLoS One 11 (4) (2016) e0152895.
[42] P.A. Egner, J.G. Chen, A.T. Zarth, D.K. Ng, J.B. Wang, K.H. Kensler, L.P. Jacobson, A. Munoz, J.L. Johnson, J.D. Groopman, J.W. Fahey, P. Talalay, J. Zhu, T.Y. Chen, G.S. Qian, S.G. Carmella, S.S. Hecht, T.W. Kensler, Rapid and sustainable detoxication of airborne pollutants by broccoli sprout beverage: results of a randomized clinical trial in China, Cancer Prevent. Res. 7 (8) (2014) 813–823.
[43] M. Kikuchi, Y. Ushida, H. Shiozawa, R. Umeda, K. Tsuruya, Y. Aoki, H. Suganuma, Y. Nishizaki, Sulforaphane-rich broccoli sprout extract improves hepatic abnormalities in male subjects, World J. Gastroenterol. 21 (43) (2015) 12457–12467.
[44] X.L. Chen, G. Dodd, C. Kunsch, Sulforaphane inhibits TNF-alpha-induced activation of p38 MAP kinase and VCAM-1 and MCP-1 expression in endothelial cells, Inflamm. Res. 58 (8) (2009) 513–521.

[45] C.-N. Hung, H.-P. Huang, C.-J. Wang, K.-L. Liu, C.-K. Lii, Sulforaphane inhibits TNF-α-induced adhesion molecule expression through the Rho A/ROCK/NF-κB signaling pathway, J. Med. Food 17 (10) (2014) 1095–1102.
[46] Y. Zhao, Y.F. Zheng, Q.Q. Luo, T. Yan, X.X. Liu, L. Han, L. Zou, Edaravone inhibits hypoxia-induced trophoblast-soluble Fms-like tyrosine kinase 1 expression: a possible therapeutic approach to preeclampsia, Placenta 35 (7) (2014) 476–482.
[47] O. Nevo, D.K. Lee, I. Caniggia, Attenuation of VEGFR-2 expression by sFlt-1 and low oxygen in human placenta, PLoS One 8 (11) (2013) e81176.
[48] Y. Gu, D.F. Lewis, Y. Wang, Placental productions and expressions of soluble endoglin, soluble fms-Like tyrosine kinase receptor-1, and placental growth factor in normal and preeclamptic pregnancies, J. Clin. Endocrinol. Metab. 93 (1) (2008) 260–266.
[49] B. Huppertz, J. Kingdom, I. Caniggia, G. Desoye, S. Black, H. Korr, P. Kaufmann, Hypoxia favours necrotic versus apoptotic shedding of placental syncytiotrophoblast into the maternal circulation, Placenta 24 (2–3) (2003) 181–190.
[50] N. Kweider, A. Fragoulis, C. Rosen, U. Pecks, W. Rath, T. Pufe, C.J. Wruck, Interplay between vascular endothelial growth factor (VEGF) and nuclear factor erythroid 2-related factor-2 (Nrf2): implications for preeclampsia, J. Biol. Chem. 286 (50) (2011) 42863–42872.
[51] S. Tong, T.J. Kaitu'u-Lino, K. Onda, S. Beard, R. Hastie, N.K. Binder, C. Cluver, L. Tuohey, C. Whitehead, F. Brownfoot, M. De Silva, N.J. Hannan, Heme oxygenase-1 is not decreased in preeclamptic placenta and does not negatively regulate placental soluble fms-like tyrosine kinase-1 or soluble endoglin secretion, Hypertension 66 (5) (2015) 1073–1081.
[52] C.J. Oh, J.Y. Kim, A.K. Min, K.G. Park, R.A. Harris, H.J. Kim, I.K. Lee, Sulforaphane attenuates hepatic fibrosis via NF-E2-related factor 2-mediated inhibition of transforming growth factor-beta/Smad signaling, Free Radical Biol. Med. 52 (3) (2012) 671–682.
[53] B.M. Kaminski, S.M. Loitsch, M.J. Ochs, K.C. Reuter, D. Steinhilber, J. Stein, S. Ulrich, Isothiocyanate sulforaphane inhibits protooncogenic ornithine decarboxylase activity in colorectal cancer cells via induction of the TGF-beta/Smad signaling pathway, Mol. Nutr. Food Res. 54 (10) (2010) 1486–1496.
[54] S. Venkatesha, M. Toporsian, C. Lam, J. Hanai, T. Mammoto, Y.M. Kim, Y. Bdolah, K.H. Lim, H.T. Yuan, T.A. Libermann, I.E. Stillman, D. Roberts, P.A. D'Amore, F.H. Epstein, F.W. Sellke, R. Romero, V.P. Sukhatme, M. Letarte, S.A. Karumanchi, Soluble endoglin contributes to the pathogenesis of preeclampsia, Nat. Med. 12 (6) (2006) 642–649.
[55] M. Varejckova, E. Gallardo-Vara, M. Vicen, B. Vitverova, P. Fikrova, E. Dolezelova, J. Rathouska, A. Prasnicka, K. Blazickova, S. Micuda, C. Bernabeu, I. Nemeckova, P. Nachtigal, Soluble endoglin modulates the pro-inflammatory mediators NF-kappaB and IL-6 in cultured human endothelial cells, Life Sci. 175 (2017) 52–60.
[56] T. Sanchez-Elsner, L.M. Botella, B. Velasco, C. Langa, C. Bernabeu, Endoglin expression is regulated by transcriptional cooperation between the hypoxia and transforming growth factor-beta pathways, J. Biol. Chem. 277 (46) (2002) 43799–43808.
[57] T. Ohmaru-Nakanishi, K. Asanoma, M. Fujikawa, Y. Fujita, H. Yagi, I. Onoyama, N. Hidaka, K. Sonoda, K. Kato, Fibrosis in preeclamptic placentas is associated with stromal fibroblasts activated by the transforming growth factor-β1 signaling pathway, Am. J. Pathol. 188 (3) (2018) 683–695.