Sofalcone Upregulates the Nuclear Factor (Erythroid-Derived 2)–Like 2/Heme Oxygenase-1 Pathway, Reduces Soluble fms–Like Tyrosine Kinase-1, and Quenches Endothelial Dysfunction
Potential Therapeutic for Preeclampsia

Kenji Onda, Stephen Tong, Anzu Nakahara, Mei Kondo, Hideaki Monchusho, Toshihiko Hirano, Tu’uhevaha Kaitu’u-Lino, Sally Beard, Natalie Binder, Laura Tuohely, Fiona Brownfoot, Natalie J. Hannan

Abstract—Preeclampsia is a severe complication of pregnancy, characterized by hypertension, oxidative stress, and severe endothelial dysfunction. Antiangiogenic factors, soluble fms–like tyrosine kinase-1 (sFlt-1) and soluble endoglin, play key pathophysiological roles in preeclampsia. Heme oxygenase-1 (HO-1) is a cytoprotective, antioxidant enzyme reported to be downregulated in preeclampsia. Studies propose that inducing HO-1 may also decrease sFlt-1 production. Sofalcone, a gastric antulcer agent in clinical use, is known to induce HO-1 in gastric epithelium. We aimed to investigate whether sofalcone induces HO-1 and reduces sFlt-1 release from primary human placental and endothelial cells and blocks endothelial dysfunction in vitro. We isolated human trophoblasts and endothelial cells (human umbilical vein endothelial cells) and also used uterine microvascular cells. We investigated the effects of sofalcone on (1) HO-1 production, (2) activation of the nuclear factor (erythroid-derived 2)–like 2 pathway, (3) sFlt-1 and soluble endoglin release, (4) tumor necrosis factor α–induced monocyte adhesion and vascular cell adhesion molecule upregulation, and (5) endothelial tubule formation. Sofalcone potently increased HO-1 mRNA and protein in both primary trophoblasts and human umbilical vein endothelial cells. Furthermore, sofalcone treatment caused nuclear translocation of nuclear factor (erythroid-derived 2)–like 2 and transactivation of other nuclear factor (erythroid-derived 2)–like 2 responsive genes (NQO1, TXN, and GCLC). Importantly, sofalcone significantly decreased the secretion of sFlt-1 from primary human trophoblasts. Sofalcone potently suppressed endothelial dysfunction in 2 in vitro models, blocking tumor necrosis factor α–induced monocyte adhesion and vascular cell adhesion molecule 1 expression in human umbilical vein endothelial cells. These results indicate that in primary human tissues, sofalcone can potently activate antioxidant nuclear factor (erythroid-derived 2)–like 2/HO-1 pathway, decrease sFlt-1 production, and ameliorate endothelial dysfunction. We propose that sofalcone is a novel therapeutic candidate for preeclampsia. (Hypertension. 2015;65:855-862. DOI: 10.1161/HYPERTENSIONAHA.114.04781.)

Key Words: heme oxygenase-1 ■ preeclampsia ■ sFLT-1 protein, human ■ sofalcone

Preeclampsia is a pregnancy specific condition affecting ≈5% to 8% of all pregnancy in women and a leading cause of maternal and perinatal morbidity and mortality. An imbalance between angiogenic and antiangiogenic factors leading to endothelial dysfunction is proposed to be a key mechanism of preeclampsia. Previous landmark studies identified that antiangiogenic factors, such as soluble fms–like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), play crucial roles in the development of preeclampsia. Administration of both sFlt-1 and sEng causes severe preeclamptic phenotype in rodents. Furthermore, sFlt-1 and sEng have also been shown to have predictive significance in clinic in women who go onto to develop preeclampsia. Heme oxygenase-1 (HO-1) is an inducible rate-limiting enzyme, which catalyzes free heme into carbon monoxide, free iron, and biliverdin. It is known that HO-1–derived carbon monoxide and biliverdin have strong anti-inflammatory and antioxidant properties. Transcription of HO-1 mRNA is

Received October 20, 2014; first decision November 8, 2014; revision accepted January 13, 2015.
The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.114.04781/-/DC1.
Correspondence to Natalie J. Hannan, Translational Obstetrics Group, Department of Obstetrics and Gynecology, Mercy Hospital for Women, University of Melbourne, Heidelberg, Victoria 3084, Australia. E-mail nhannan@unimelb.edu.au
© 2015 American Heart Association, Inc.

Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.114.04781
regulated by nuclear factor (erythroid-derived 2)-like 2 (Nrf2), an antioxidant transcription factor.6 Nrf2 plays a crucial role in induction of phase II antioxidant enzymes, including NAD(P)H quinone oxidoreductase 1 (NQO1), thioredoxin (TXN), glutamate-cysteine ligase catalytic subunit (GCLC), as well as HO-1, and therefore, it contributes to preventing cells and tissues from exposure to excessive oxidative stress. For decades, it has been postulated that oxidative stress is an important part of the pathophysiology of preeclampsia. Furthermore, there have been studies which propose that Nrf2 and HO-1 expression is reduced in preeclamptic placenta.7 In addition, it is believed that induction of HO-1 reduces sFlt-1 and sEng production. Thus, agents that upregulate HO-1 may be promising as a potential treatment for preeclampsia, given that they are likely to both increase antioxidant defenses and decrease sFlt-1 and sEng production.8 Sofalcone, 20-carboxymethoxy-4,4-bis(3-methyl-2-butenyloxy) chalcone, is structurally a chalcone derivative and is synthesized from sofadarine isolated from the root of the Chinese plant Sophora subprostrata, an antulcer agent that is classified as a gastric mucosa protector. Sofalcone exerts gastric protective action via pleiotropic mechanisms, including increasing gastric mucosal blood flow, gastric mucous glycoprotein, and endogenous prostaglandin in the stomach.9,10 Sofalcone is currently used for treatment of gastritis and gastric ulcer in clinic in Japan and South Korea. We have previously demonstrated that sofalcone is a potent inducer of HO-1 in gastric epithelium.11 Thus, we hypothesized that sofalcone can induce HO-1 (and other antioxidant genes) in placental tissues leading to a reduction of the antiangiogenic factors, such as sFlt-1 or sEng. We also investigated whether sofalcone ameliorates endothelial dysfunction induced by tumor necrosis factor α (TNF-α).

Materials and Methods

Reagents
Sofalcone (20-carboxymethoxy-4,4’-bis(3-methyl-2-butenyloxy) chalcone) was kindly provided by Taisho Toyama Pharma Co, Ltd (Tokyo, Japan). Cobalt protoporphyrin, a positive control for HO-1 induction, was obtained from Frontier Scientific (Logan, UT). Antibody for human HO-1 was purchased from Enzo Life Sciences (Tokyo, Japan). Nrf2 antibody (H-300) was from Santa Cruz Biotechnology (Dallas, TX). GAPDH antibody was obtained from Cell Signaling Technology (Danvers, MA). Histone H3 antibody was purchased from Millipore (Billerica, MA). Kits for enzyme-linked immunosorbent assay (ELISA) for the determination of sFlt-1 and sEng were purchased from R&D Systems (Minneapolis, MN). Sofalcone and cobalt protoporphyrin were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the medium was kept at 0.2% throughout experiments. Cell lines: THP-1 (human monocyte cell line), HUVEC (human umbilical vein endothelial cell line), and uterine microvascular (UtMV) cells were kindly gifted by Dr Jemma Evans.

Primary HUVEC Isolation
Umbilical cords were collected from normal term placentas, and the cord vein was cannulated and infused with PBS to wash away blood. Subsequently ≈10 mL (1 mg/mL) of collagenase (Worthington, Lakewood, NJ) was infused into the cord, and the cord was incubated at 37°C for 10 minutes. The disassociated cells were recovered. HUVECs were pelleted, resuspended, and cultured in M199 media (Life Technologies) containing 20% fetal calf serum, 1% antibiotic–antimycotic (100x; Life Technologies), 1% endothelial cell growth factor (Sigma), and 1% heparin.

Primary Trophoblast Isolation
Approximately 150 g of placental tissue was washed with sterile PBS, and maternal and fetal surfaces were removed. Placental cotyledons were scraped with a scalpel to dissociate placental villi from vessels. Placental tissue was washed with 0.9% NaCl to remove blood cells and then subjected to 3 20-minute digestion cycles with 0.25% trypsin and 0.2 mg/mL DNAse in enzyme digestion buffer containing 10x Hank buffered salt solution, sodium bicarbonate, HEPES, and deionized H.O. Cell suspensions were filtered and then separated by discontinuous Percoll gradient centrifugation. The layer containing trophoblasts was collected and then subjected to CD9 negative selection (to remove any contaminating cells).

Primary trophoblasts were cultured in DMEM high glutamax (Life Technologies) containing 10% fetal calf serum (Sigma, St Louis, MO) and 1% antiangiotic (Life Technologies) on fibronectin (10 μg/mL; BD Bioscience, Franklin Lakes, NJ) coated plates. Cells attached overnight and were then washed twice with sterile PBS.

Quantitative Reverse Transcription Polymerase Chain Reaction
Total RNA was extracted using RNeasy Total RNA extraction kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. cDNA conversion was performed with Superscript VILO cDNA synthesis kit (Life Technologies). Quantitative polymerase chain reaction (PCR) was run on CFX 384 (Bio-Rad, Hercules, CA). In brief, Taqman assays for HO-1 (HMOX1; Hs01110250_m1), NAD(P)H quinone oxidoreductase 1 (NQO1; Hs02512143_s1), thioredoxin (TXN; Hs01555214_g1), glutamate-cysteine ligase catalytic subunit (GCLC; Hs00155249_m1), vascular cell adhesion molecule (VCAM1; Hs01003372_m1), endothelin-1 (EDN-1; Hs00174961_m1), vascular endothelial growth factor A (VEGFA; Hs00900055_m1), and the housekeeping gene YWHAZ (Hs0344281_g1) were purchased from Life Technologies. PCR was performed under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute (40 cycles). Data were normalized against the housekeeping gene YWHAZ, as it has previously been shown to be one of the most stable house-keeping genes to study human placental gene expression.12,13 Importantly, we also observed that YWHAZ mRNA expression was stable across all our treatments, confirming the premise that it is an appropriate housekeeping gene. Relative gene expression was determined using ΔΔCt calculation method.

Western Blot Analysis
HUVEC protein lysates (20 μg) were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Blots were blocked with 5% skim milk followed by incubation with primary rabbit anti-HO-1 antibody (1:1000) overnight at 4°C, followed by incubation with secondary antirabbit-HRP antibody for 1 hour at room temperature. ECL prime reagent (GE healthcare Life Sciences, Pittsburgh, PA) was applied, and immunoreactive bands were visualized using a Chemidoc XRS (BioRad, Hercules, CA). Blots were stripped by Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA) and reprobed with GAPDH (1:5000) antibody, for protein loading control.
Extraction of Nuclear Fraction and Detection of Nrf2

Nuclear proteins were extracted from HUVECs using hypotonic buffer (10 mmol/L HEPES [pH 7.9], 1.5 mmol/L MgCl₂, 10 mmol/L KCl) and nuclear extraction buffer (20 mmol/L HEPES [pH 7.9], 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 420 mmol/L NaCl, and 25% wt/wt glycerol) by centrifugation. Protein concentration was quantified by a coomassie blue assay method (Thermo Fisher Scientific). Nuclear protein (20 μg) was separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Proteins were detected by immunoblotting with Nrf2 antibody (1:200) and H3 antibody (1:5000) as a loading control.

Enzyme Linked Immunosorbent Assay

Secretion of sFlt-1 and sEng in culture media was measured by ELISA kit (R&D Systems Minneapolis, MN) according to the manufacturer's information. Optical density was determined using a BioRad X-Mark microplate spectrophotometer (BioRad). Flt-1 and endoglin levels were determined using BioRad Microplate manager 6 software.

THP-1 Monocyte—HUVEC Adhesion Assay

THP-1 monocyte adhesion assay using endothelial cells was performed as follows. Primary HUVECs were seeded in 96-black wall plates with clear bottom to confluency. Cells were treated with TNF-α (10 ng/mL) for 2 hours followed by sofalcone treatment in the presence of TNF-α (10 ng/mL) for an additional 24 hours. HUVECs washed with PBS and THP-1 cells (1×10⁶ cells/mL) stained with calcein-AM were added and cocultured for 45 minutes. Unattached THP-1 cells were then washed out with PBS. The fluorescent intensity of calcein (incorporated in THP-1 cells) was measured using a FLUOstar OMEGA fluorescent plate reader (BMG labtech, Ortenberg, Germany) with 485/520 nm, and images were captured using an EVOS-FL microscope (Life Technologies).

Endothelial Tube–Forming Assay

Endothelial tube–forming assay was performed as follows. Flat bottomed 48-well tissue culture plates were coated with neat Matrigel (In Vitro Technologies, Noble Park, Victoria, Australia) and incubated at 37°C for 30 minutes to allow matrigel to polymerize. Isolated primary HUVECs were seeded at 60000 cells per well and incubated at 37°C for 14 to 16 hours to allow tube formation. Cells were then cultured in the presence of either TNF-α (10 ng/mL) alone, with both TNF-α (10 ng/mL) and sofalcone 50 μM, or control media for 8 to 12 hours. Tube formation was assessed, and images were captured using an EVOS-FL microscope (Life Technologies) at x4 magnification.

Cell Viability Assay (MTS)

Cell viability assay was performed using CellTiter 96-Aqueous One solution or CellTiter Blue Cell viability assay kit (Promega, Madison, WI) according to the manufacturer’s instructions.

Statistical Analysis

Statistical analysis was performed using 1-way ANOVA followed by multiple comparison with Dunnet or Bonferroni ad-hoc test where appropriate. Data were regarded statistically significant if the P values were <0.05. All data were analyzed using GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA).

Results

Sofalcone Increased HO-1 mRNA and Protein in Primary Trophoblasts and Endothelial Cells

First, we investigated whether sofalcone could induce HO-1 in primary trophoblasts, primary HUVECs, and a UtMV cell line. Primary trophoblasts and HUVECs were isolated and seeded for culture. Trophoblasts, HUVECs, and UtMV cells were treated with sofalcone (10, 20, and 50 μmol/L) for 24 hours, and mRNA

Figure 1. Sofalcone (SOFA) increases heme oxygenase-1 (HO-1) mRNA and protein in primary trophoblasts, primary human umbilical vein endothelial cells (HUVECs), and uterine microvascular (UtMV) cells. Cells were treated with sofalcone (10, 20, and 50 μmol/L) for 24 hours. Sofalcone significantly increased HO-1 mRNA in (A) primary trophoblasts (B) primary HUVECs, and (C) UtMV cells in a dose-dependent manner (error bars, mean±SEM [n=3, P<0.05]). D, Western blot analysis that showed sofalcone (10, 20, and 50 μmol/L) increased HO-1 protein (32 kDa) expression in primary HUVECs. Control includes 0.2% DMSO as a vehicle. Cobalt protoporphyrin (CoPP; 10 μmol/L) was included as a positive control. E, Fluorescence immunocytochemistry showed that sofalcone (50 μmol/L) increased HO-1 staining (green) in primary trophoblasts treated with sofalcone; nuclear counterstain DAPI (blue).
was collected and assessed by quantitative PCR. We observed that sofalcone dose dependently increased HO-1 mRNA expression in trophoblasts, HUVECs, and UtMV cells (Figure 1A–1C). Sofalcone (50 μmol/L) significantly increased HO-1 mRNA expression compared with the control group in both trophoblasts and HUVECs (P<0.05 for both). Western blot analysis demonstrated that sofalcone potently increased HO-1 protein expression in primary HUVECs treated for 24 hours. The effect on HO-1 protein induction by 20 μmol/L of sofalcone was comparable with that of 10 μmol/L of cobalt protoporphyrin (Figure 1D, cobalt protoporphyrin is a positive control, known to upregulate HO-1). Sofalcone also increased HO-1 protein in primary trophoblasts, which was confirmed by fluorescent immunocytochemical analysis (Figure 1E). Cell viability (MTS) confirmed that there was no difference with cell viability with treatment for trophoblasts and HUVECs; however, 50 μmol/L of sofalcone caused cell death in the UtMV cells, which was the reason for a top dose of 20 μmol/L to treat the UtMV cells (data not shown).

Sofalcone Facilitates Nrf2 Nuclear Accumulation and Increased Nrf2 Responsive Gene Expression in Primary Trophoblasts and Primary HUVECs

Next, we examined whether sofalcone could activate nuclear translocation of Nrf2, a transcription factor in trophoblasts and HUVECs (Figure 2). Nrf2 upregulates genes involved in antioxidant defenses, including HO-1. Western blot analysis revealed a marked increase in the amount of Nrf2 in the nuclear fraction of HUVECs with sofalcone treatment (50 μmol/L) for 6 hours (Figure 2A). Immunocytochemical analysis also demonstrated that Nrf2 increases in primary trophoblast nuclei with sofalcone (50 μmol/L) treatment for 6 hours (Figure 2B).

We next investigated whether sofalcone could increase specific genes known to be regulated by Nrf2 activation (NQO1, TXN, and GCLC). We observed significant dose-dependent increase in the expression of NQO1, TXN, and GCLC in primary trophoblasts treated with sofalcone for 24 hours (Figure 2C–2E; n=3; P<0.01 and P<0.05). Similarly, in primary HUVECs, expression of NQO1, TXN, and GCLC dose dependently increased with sofalcone treatment for 24 hours (Figure 2F–2H; n=3; P<0.05 and P<0.01). NQO1 and TXN mRNA was significantly enhanced with sofalcone treatment (Figure 2F and 2G). However, GCLC mRNA expression showed a dose dependent increase with sofalcone treatment, but it was not statistically significant (Figure 2H). We therefore conclude that sofalcone treatment results in the nuclear translocation of Nrf2 and upregulates transcription of antioxidant genes that are direct targets of Nrf2.
Sofalcone Upr egulates HO-1 and Decreases sFlt1

Sofalcone Reduced sFlt-1 Production in Primary Trophoblasts and UtMV Cells but Not Primary HUVECs

Next, we investigated whether sofalcone could reduce production of the antiangiogenic factor sFlt-1 (Figure 3). We first examined primary trophoblasts where cells were cultured for 24 hours and then treated with sofalcone (10, 20, and 50 μmol/L) for an additional 24 hours. We observed that sofalcone significantly decreased the sFlt-1 concentrations in the culture media (Figure 3A; n=3; P<0.05 and P<0.01). In primary trophoblasts, the levels of sEng production were below detection and therefore could not be assessed (data not shown). To induce higher levels of sEng, we added forskolin (20 μmol/L) for 48 hours, which did produce measurable levels of sEng. After forskolin treatment, primary trophoblasts were then treated with sofalcone (10, 20, and 50 μmol/L) for an additional 24 hours. However, sofalcone had no effect on sEng production (data not shown).

We next investigated the effect of sofalcone on sFlt-1 production by primary HUVECs (Figure 3B) and UtMV cells (Figure 3C). Primary HUVECs treated with sofalcone (10, 20, and 50 μmol/L) for 24 hours showed no significant reduction in the amount of sFlt-1 in the culture media. (Figure 3B; n=4). Sofalcone had no effect on sENG production by HUVECs (data not shown). UtMV cells treated with sofalcone (10 and 20 μmol/L) for 24 hours showed a significant reduction in sFlt-1 levels (Figure 3C; n=3; P<0.05). We also tested VEGF expression in both primary trophoblasts and HUVECs treated with sofalcone (10, 20, and 50 μmol/L). There was no significant difference in VEGF mRNA levels with sofalcone treatment (Figure S1A and S1B in the online-only Data Supplement; n=3).

Sofalcone Ameliorates TNF-α–Induced Endothelial Dysfunction

TNF-α is known to cause endothelial dysfunction in vitro.14–16 We investigated whether sofalcone can reduce TNF-α–induced endothelial dysfunction by examining the degree of monocyte adhesion to endothelial cells stimulated with TNF-α (Figure 4). TNF-α–treated HUVECs significantly increased THP-1 monocyte adhesion to HUVECs compared with the control (n=3; P<0.01). Sofalcone (10, 20, and 50 μmol/L) dose dependently decreased the amount of THP-1 monocyte adherence to HUVECs, a finding that was significant at 50 μmol/L (Figure 4A; n=3). Thus, sofalcone was able to decrease monocyte adhesion to HUVECs induced by TNF-α.

We investigated whether sofalcone could quench TNF-α–induced upregulation of VCAM1 in HUVECs (Figure 4B). Indeed, we found that sofalcone potently decreased VCAM1 expression at all doses (10–50 μmol/L), and at the top dose, VCAM1 mRNA levels were comparable with control expression (Figure 4B; n=3; P<0.01). We also examined whether sofalcone could decrease HUVEC expression of the vasoconstrictive molecule, endothelin-1. Sofalcone significantly reduced endothelin-1 expression at the top dose of 50 μmol/L in TNF-α–treated HUVECs.

Sofalcone Rescued TNF-α–Impaired Angiogenesis

We next examined whether sofalcone could rescue TNF-α–induced impairment of HUVEC tube formation. Tube formation assays are an established in vitro model of angiogenesis. Cells treated with TNF-α showed reduced numbers of tubes compared with the control (Figure 4D). However, sofalcone treatment was able to counteract the decrease in tube formation caused by TNF-α (Figure 4D).

Discussion

Preeclampsia is thought to result from shallow trophoblast invasion, which leads to excessive oxidative stress in the placenta, which subsequently causes increased release of antiangiogenic factors that cause maternal endothelial dysfunction.17 HO and its by products, carbon monoxide, and biliverdin/ bilirubin act efficiently against these disease conditions, and therefore, they are postulated to be therapeutic targets in preeclampsia.18,19

We have previously reported that sofalcone, which is currently used to treat gastric ulcers or gastritis in Japan and South Korea, can potently induce HO-1 expression in gastric epithelial cells and adipocyte cells.11,20 Given that HO-1 is a promising therapeutic target for preeclampsia,17,18,21–23 we set out to investigate the effects of this clinical drug in several pathophysiological models of preeclampsia in vitro.

First, we investigated whether sofalcone could induce HO-1 in primary trophoblasts, primary HUVECs, and a human UtMV cell line. Our quantitative PCR experiments showed that treatment of cells with sofalcone for 24 hours significantly increased HO-1 mRNA expression in primary trophoblasts, HUVECs, as well as the human UtMV cells. The
induction of mRNA correlated well with protein production, clearly demonstrating that sofalcone potently induced HO-1 in these human cells.

Nrf2 is a transcription factor known to be a key regulator of antioxidative genes, including HO-1, NQO1, TXN, and GCLC.24–28 In a nonstimulated state, Nrf2 protein is sequestered in the cytoplasm through binding to Keap1 protein.25 Once the Nrf2 pathway is activated, Nrf2 disassociates from Keap1 and moves into the nucleus where it can regulate gene transcription. In this study, we have clearly demonstrated, using both western blot analysis of nuclear protein extracts and immunocytochemical fluorescent localization, that sofalcone markedly increased the amount of Nrf2 nuclear translocation.

Next, we further investigated this effect by determining whether the increase in nuclear Nrf2 by sofalcone treatment enhanced Nrf2 responsive gene expression. Using quantitative PCR, we demonstrated that NQO1, TXN, and GCLC are increased in primary human trophoblasts and HUVECs. NQO1 is used as a key gene to determine Nrf2 function;27 furthermore, NQO1 has key antioxidant and cytoprotection properties, and its transcription is highly dependent on Nrf2 pathway. TXN also known as an important antioxidant gene regulated by Nrf2.28 Previously, it has been reported to be decreased in placenta in patients with preeclampsia.29,30 GCLC is a rate-limiting enzyme for glutathione synthesis.24 Previous studies have shown that Nrf2 activity is decreased in preeclamptic placenta and its activation might be useful to reduce oxidative stress in tissues.7,31

Both sFlt-1 and sEng are antiangiogenic factors that have been shown to cause endothelial dysfunction in preeclampsia. Cudmore et al.8 demonstrated that induction of HO-1 by adenovirus transfection or treatment with statins could reduce sFlt-1 and sEng production. Thus, next, we investigated whether treatment of primary human trophoblasts or HUVECs or UtMV cells with sofalcone could reduce sFlt-1 or sEng production. Our results clearly demonstrate that sFlt-1 secretion by primary trophoblasts or UtMV cells was significantly decreased with sofalcone treatment. However, there was no significant difference in sFlt-1 production in primary HUVECs or sEng production in both primary cells.

Taken together, these data suggest that potent HO-1 induction does not necessarily lead to a reduction in both sFlt-1 and sEng production. Furthermore, we tested VEGF expression with increasing doses of sofalcone treatment in both primary
trophoblasts and HUVECs, revealing that there was no change in VEGF mRNA levels.

Endothelial dysfunction is a key characteristic of preeclampsia and can be caused by a variety of factors, including reactive oxygen species, hypoxia, or cytokines, such as TNF-α or angiostatin II. Given that TNF-α has been shown to induce endothelial dysfunction in models of pregnancy hypertension and preeclampsia, we investigated whether sofalcone could reduce markers of endothelial dysfunction induced by TNF-α. We modeled endothelial dysfunction in vitro by treating HUVECs with TNF-α and showed that sofalcone treatment significantly quenched leukocyte adhesion to HUVECs stimulated with TNF-α. We demonstrated that this was likely through a significant reduction of VCAM-1 expression. We observed a significant decrease in the expression of the potent vasoconstrictor, endothelin-1, with the top dose of sofalcone in TNF-α–stimulated HUVECs. Furthermore, we also demonstrate that TNF-α–impaired angiogenesis was quenched with sofalcone. These results suggest that sofalcone potently ameliorates TNF-α–induced endothelial dysfunction.

A strength of this work is that we performed the majority of our functional experiments using primary human cells, instead of cell lines. Experimental results arising from primary human cells freshly isolated may be a better reflection of human biology than immortalized cell lines that have likely undergone multiple passages. Furthermore, primary cells provide better models for preeclampsia in vitro. However, a potential limitation of our study is that we isolated primary trophoblast cells from term placentas. We cannot exclude the possibility that the effects seen with sofalcone may not occur in placenta at more preterm gestations, although we consider this unlikely. Further studies would be required to determine whether there is in fact a differential gestational effect.

It is important to note that preclinical studies have shown that oral sofalcone administration into rats (1000 mg/kg/day) across several stages of pregnancy (before conception/early stage, organogenesis stage, and perinatal/lactational stage) or rabbits (400 mg/kg/day) in the organogenesis stage has no adverse effects on reproductive ability, embryo/fetal lethality, or malformation. Based on these results, sofalcone may be safe to administer to pregnant women after 24 weeks of pregnancy when organogenesis is complete. Importantly, preeclampsia is rarely diagnosed before 24 weeks of gestation, and treatment would not commence well after the majority of organogenesis is complete which is usually ≈13 weeks of gestation. Therefore, there is clearly scope for sofalcone to be used clinically as a treatment of severe preeclampsia.

In conclusion, we have shown that a gastric protective drug sofalcone activates Nrf2 and potently induces HO-1 expression in primary trophoblasts and endothelial cells. We further show that sofalcone reduced sFlt-1 production in primary human trophoblasts and ameliorated markers of endothelial dysfunction in vitro. As such, we have generated a body of preclinical data suggesting that sofalcone may be a promising therapeutic for preeclampsia.

**References**


**Sources of Funding**

The National Health and Medical Research Council of Australia provided salary support; ST no. 1050765, TKL no. 1062418, and NJH no. 628927.

**Disclosures**

None.

**Perspectives**

The current data show novel actions of the gastric protective drug sofalcone, including activation of Nrf2 and potent induction of HO-1 expression in human primary trophoblasts and endothelial cells. We further show that sofalcone reduced sFlt-1 production in primary human trophoblasts and ameliorated markers of endothelial dysfunction in vitro. As such, we have generated a body of preclinical data suggesting that sofalcone may be a promising therapeutic for preeclampsia.
Preeclampsia is a severe pregnancy complication characterized by serious hypertension.

What Is Relevant?

- Preeclampsia is a severe pregnancy complication characterized by serious hypertension.

What Is New?

- Novel actions of a gastric ulcer medication, sofalcone.
- Sofalcone quenches physiological markers of the severe pregnancy complication preeclampsia.

Summary

We propose that sofalcone should be considered as a novel therapeutic for preeclampsia.
Sofalcone Upregulates the Nuclear Factor (Erythroid-Derived 2)–Like 2/Heme Oxygenase-1 Pathway, Reduces Soluble fms–Like Tyrosine Kinase-1, and Quenches Endothelial Dysfunction: Potential Therapeutic for Preeclampsia
Kenji Onda, Stephen Tong, Anzu Nakahara, Mei Kondo, Hideaki Monchusho, Toshihiko Hirano, Tu'uhevaha Kaitu'u-Lino, Sally Beard, Natalie Binder, Laura Tuohy, Fiona Brownfoot and Natalie J. Hannan

Hypertension. 2015;65:855-862; originally published online February 9, 2015; doi: 10.1161/HYPERTENSIONAHA.114.04781

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/65/4/855

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2015/02/09/HYPERTENSIONAHA.114.04781.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Sofalcone up-regulates the nuclear factor (erythroid-derived 2)-like 2 /heme-oxygenase-1 pathway, reduces sFlt-1 and quenches endothelial dysfunction: a potential therapeutic for preeclampsia

Kenji Onda¹,², Stephen Tong¹, Anzu Nakahara², Mei Kondo², Hideaki Monchusho², Toshihiro Hirano², Tu’uhevaha Kait'u-Lino¹, Sally Beard¹, Natalie Binder¹, Laura Tuohey¹, Fiona Brownfoot¹ and Natalie J Hannan¹.

¹ Translational Obstetrics Group, Department of Obstetrics and Gynaecology, Mercy Hospital for Women, University of Melbourne, Heidelberg, Victoria, Australia, 3084; ² Department of Clinical Pharmacology, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan, 192-0392.

Running title; Sofalcone up-regulates HO-1 and decreases sFlt-1

Corresponding authors; Dr. Natalie J. Hannan, Translational Obstetrics Group, Department of Obstetrics and Gynaecology, University of Melbourne; Mercy Hospital for Women, Heidelberg, Victoria 3084 Australia. PH: +61-3-8458 4371 Fax: +31-3-8458 4380. Email: nhannan@unimelb.edu.au
Supplemental figure S1. Sofalcone (SOFA) does not alter VEGF expression in either primary trophoblast or primary HUVECs. SOFA (10, 20, and 50 mmol/L) did not alter VEGF mRNA levels in primary trophoblast (A) or primary HUVECs (B) (n=3, NS).