Protein Pump Inhibitors Decrease Soluble fms-Like Tyrosine Kinase-1 and Soluble Endoglin Secretion, Decrease Hypertension, and Rescue Endothelial Dysfunction

Kenji Onda,* Stephen Tong,* Sally Beard, Natalie Binder, Masanaga Muto, Sevvandi N. Senadheera, Laura Parry, Mark Dilworth, Lewis Renshall, Fiona Brownfoot, Roxanne Hastie, Laura Tuohy, Kirsten Palmer, Toshihiko Hirano, Masahito Ikawa, Tu‘uhevaha Kaitu‘u-Lino, Natalie J. Hannan

Abstract—Preeclampsia is a severe complication of pregnancy. Antiangiogenic factors soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin are secreted in excess from the placenta, causing hypertension, endothelial dysfunction, and multiorgan injury. Oxidative stress and vascular inflammation exacerbate the endothelial injury. A drug that can block these pathophysiological steps would be an attractive treatment option. Protein pump inhibitors (PPIs) are safe in pregnancy where they are prescribed for gastric reflux. We performed functional studies on primary human tissues and animal models to examine the effects of PPIs on sFlt-1 and soluble endoglin secretion, vessel dilatation, blood pressure, and endothelial dysfunction. PPIs decreased sFlt-1 and soluble endoglin secretion from trophoblast, placental explants from preeclamptic pregnancies, and endothelial cells. They also mitigated tumor necrosis factor-α–induced endothelial dysfunction: PPIs blocked endothelial vascular cell adhesion molecule-1 expression, leukocyte adherence to endothelium, and disruption of endothelial tube formation. PPIs decreased endothelin-1 secretion and enhanced endothelial cell migration. Interestingly, the PPI esomeprazole vasodilated maternal blood vessels from normal pregnancies and cases of preterm preeclampsia, but its vasodilatory effects were lost when the vessels were denuded of their endothelium. Esomeprazole decreased blood pressure in a transgenic mouse model where human sFlt-1 was overexpressed in placenta. PPIs upregulated endogenous antioxidant defenses and decreased cytokine secretion from placental tissue and endothelial cells. We have found that PPIs decrease sFlt-1 and soluble endoglin secretion and endothelial dysfunction, dilate blood vessels, decrease blood pressure, and have antioxidant and anti-inflammatory properties. They have therapeutic potential for preeclampsia and other diseases where endothelial dysfunction is involved. (Hypertension. 2017;69:457-468. DOI: 10.1161/HYPERTENSIONAHA.116.08408.)

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Preeclampsia affects 3% to 8% of pregnancies and is a leading cause of mortality during pregnancy. It is globally responsible for 60 000 deaths annually and far greater rates of fetal losses.1 Placental factors are released in excess into the maternal circulation, causing hypertension and endothelial dysfunction. This leads to multisystem organ injury to the maternal kidneys, liver, hematological system, and the brain (causing eclamptic seizures).1

The only treatment for preeclampsia is delivery of the placenta as this removes the source of placental-derived factors responsible for the maternal organ injury.1,2 In preterm preeclampsia, clinicians are often forced to deliver the fetus preterm to save the mother. However, this inflicts iatrogenic prematurity, which can lead to cerebral palsy, chronic disability, or death. A treatment that quenches the severity of the maternal disease could allow these pregnancies to safely advance to a gestation where neonatal outcomes are significantly improved. Severe preeclampsia at any gestation can progress rapidly over hours and become life-threatening. Therefore, a treatment that reduces the...
severity of preeclampsia at any gestation could improve clinical outcomes.

Factors released in excess from the placenta in preeclampsia thought to play an important role in causing endothelial dysfunction (and subsequent maternal organ injury) are the antiangiogenic factors soluble fms-like tyrosine kinase-1 (sFlt-1)\textsuperscript{3–6} and soluble endoglin (sENG).\textsuperscript{3–6} sFlt-1 is a splice variant of vascular endothelial growth factor receptor factor 1 and comprises only the extracellular domain.\textsuperscript{3,4} sFlt-1 binds to and sequesters circulating vascular endothelial growth factor, decreasing vascular endothelial growth factor signaling and its ability to promote vascular homeostasis.\textsuperscript{5} Furthermore, recent evidence suggests that sFlt-1 may induce hypertension by impairing endothelial nitric oxide synthase (eNOS) phosphorylation and increasing angiotensin II sensitivity.\textsuperscript{7} sENG shares sequence homology with the extracellular domain of full-length membrane-bound endoglin, a coreceptor that facilitates transforming growth factor receptor-\(\beta\)-1 and transforming growth factor receptor-\(\beta\)-3 signaling in endothelial cells. Like sFlt-1, sENG also antagonizes endothelial receptor signaling by competing with full-length endoglin, decreasing its ability to bind with its coreceptors to maintain vessel homeostasis.

The evidence linking sFlt-1 and sENG with the pathophysiology of preeclampsia is strong.\textsuperscript{3–5} Circulating levels are significantly elevated in women with preeclampsia and levels correlate with disease severity.\textsuperscript{1} Administering sFlt-1 to rats causes hypertension and proteinuria, hallmarks of preeclampsia.\textsuperscript{3} Adenoviral coadministration of both sFlt-1 and sENG to rats recapitulate the full spectrum of multigain injury seen in preeclampsia, including severe proteinuria, thrombocytopenia, elevated liver enzymes, and fetal growth restriction.\textsuperscript{9} Preeclampsia is also associated with placental and systemic oxidative stress\textsuperscript{5,10} and intravascular inflammation,\textsuperscript{2} which are thought to exacerbate the endothelial injury.

Therefore, an attractive candidate therapeutic for preeclampsia may be a drug that is considered safe in pregnancy and can do the following: (1) decrease sFlt-1 and sENG secretion, (2) decrease blood pressure, (3) decrease endothelial dysfunction, (4) upregulate endogenous antioxidant defenses, and (5) decrease secretion of inflammatory cytokines. As far as we are aware, no such drug has been reported.

Proton pump inhibitors (PPIs) are widely prescribed during pregnancy for symptomatic gastric reflux. Large epidemiological studies have shown that they are safe in pregnancy, even when administered during the first trimester.\textsuperscript{11,12}

It was reported that heme-oxygenase-1 (HO-1) may negatively regulate sFlt-1 secretion.\textsuperscript{13} Noting that it has also been reported that the PPI lansoprazole upregulates HO-1 in gastric mucosa,\textsuperscript{14} we hypothesized that PPIs might upregulate HO-1, which should then decrease sFlt-1 and sENG secretion. In this study, we report functional experiments on primary human tissues and mouse models that show PPIs have diverse actions that may mitigate the pathophysiology underlying preeclampsia. Our data identify PPIs as candidate therapeutics for preeclampsia.

Methods

Detailed Methods are available in Methods in the online-only Data Supplement.

Methods Overview

We performed functional experiments where we administered proton pump inhibitors to primary human tissues or cells and assessed their effects on sFlt-1 and sENG secretion, endothelial dysfunction (using in vitro assays), whole vessel dilation, and secretion of cytokines. To perform these experiments, we examined primary tissues from placenta (isolated cytotrophoblast cells or placental explant tissue from women diagnosed with preeclampsia), endothelial cells (human umbilical vein endothelial cells [HUVECs] and uterine microvascular cells), and whole vessels isolated from the omentum (maternal fatty apron tissue). All experiments were run with technical triplicates, and each experiment was repeated a minimum of 3× (using samples from different patients). We also performed in vivo experiments where we administered esomeprazole to a mouse model of preeclampsia (where sFlt-1 is overexpressed specifically in the placenta) and pregnant eNOS\textsuperscript{−/−} mice.

We obtained ethical approval to perform these studies. All women provided written informed consent.

Statistical Analysis

All in vitro experiments were performed with technical triplicates, and all experiments were repeated a minimum of 3×. Data were tested for normal distribution and statistically tested as appropriate. When 2 groups were compared a 1-way ANOVA (for parametric data) or Kruskal–Wallis test (for nonparametric data) followed by multiple comparison with Dunnet or Bonferroni ad hoc test where appropriate or post hoc analysis using either the Tukey (parametric) or Dunn test (nonparametric). When 2 groups were analyzed, either an unpaired \(t\) test (parametric) or a Mann–Whitney \(U\) test (nonparametric) was used. Data are expressed as mean fold change from control ± SEM.

Results

Proton Pump Inhibitors Decrease sFlt-1 Expression and Secretion From Placenta and Endothelium

In preeclampsia, excess secretion of sFlt-1 is thought to cause hypertension, endothelial dysfunction, and subsequent maternal organ injury. Administering PPIs (lansoprazole, rabeprazole, and esomeprazole) to primary trophoblast cells dose dependently reduced sFlt-1 secretion (Figure 1A, this ELISA detects all sFlt-1 variants) and reduced mRNA expression of the 2 major sFlt-1 variants present in placenta: sFlt-1 e15a and sFlt-1 i13 (Figure S1A and S1B in the online-only Data Supplement). They also reduced sFlt-1 secretion from placental explants obtained from normal (Figure S1C) and preeclamptic pregnancies (Figure 1B; see Table S1 for clinical information). PPIs also decreased sFlt-1 e15a protein secretion from preeclamptic placental explants (Figure 1C). sFlt-1 e15a (or sFlt-1 v14) is the main sFlt-1 variant in placenta\textsuperscript{13} that has only been described recently.\textsuperscript{15,16}

Endothelium is another major tissue source of sFlt-1. When administered to HUVECs, PPIs dose dependently reduced sFlt-1 secretion (Figure 1D) and mRNA expression (Figure S1D and S1E) of the 2 sFlt-1 variants, sFlt-1 e15a and sFlt-1 i13. When we compared 5 PPIs, we found esomeprazole and rabeprazole were the most potent in reducing sFlt-1 secretion from HUVECs (Figure 1E). PPIs also decreased sFlt-1 secretion from primary uterine microvascular cells that,
unlike HUVECs, are a mix of venous and arterial endothelium (Figure S1F). Placental hypoxia increases sFlt-1 secretion, and there is evidence that its release may be regulated by hypoxia inducible factor-1α (HIF-1α). PPIs markedly decreased hypoxia inducible factor-1α protein expression (Figure 1F), raising the possibility that their ability to decrease sFlt-1 secretion may be mediated through hypoxia inducible factor-1α.

Figure 1. Proton pump inhibitors (PPIs) decrease soluble fms-like tyrosine kinase-1 (sFlt-1) expression and secretion in placental and endothelial tissues, and hypoxia inducible factor-1α (HIF-1α) expression. Relative sFlt-1 levels in the media after 24-h treatment with PPIs administered to (A) primary trophoblast and (B) preeclamptic explants. C, Relative levels of sFlt-1 e15a (placental specific sFlt-1 variant) in the media from preeclamptic explants after PPI treatment. D, Relative sFlt-1 levels in human umbilical vein endothelial cells (HUVECs) after PPI treatment. E, IC₅₀ analysis (concentration of drug required to inhibit sFlt-1 secretion from baseline by 50%) for 5 PPIs administered to HUVECs, which demonstrates rabeprazole (Rab) and esomeprazole (Eso) are the most potent at reducing sFlt-1 secretion. F, Western blot and densitometric analysis of HIF-1α expression in HUVECs treated with PPIs at 100 μmol/L for 24 h. PPIs were administered at 5 to 100 μmol/L concentrations or as indicated. Lans indicates lansoprazole; Ome, omeprazole; and Pant, pantoprazole. Data are mean fold change from controls±SEM (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; n≥3).
Proton Pump Inhibitors Decrease sENG Expression and Secretion From Placenta and Endothelium

sENG is the second major antiangiogenic factor released in excess from the placenta in preeclampsia and may play a role in severe disease.\textsuperscript{5,8,9} PPIs reduced sENG secretion from primary trophoblast (Figure 2A), HUVECs (Figure 2B), uterine microvascular cells (Figure S2A), and placental explants from cases of preterm preeclampsia (Figure 2C). When we compared the ability of 5 PPIs to reduce sENG secretion from HUVECs, we identified rabeprazole and esomeprazole were the most potent (Figure 2D).

Interestingly, PPIs also induced proangiogenic \textit{vegfa} mRNA expression in primary trophoblast (Figure 2E) and HUVECs (Figure S2B), but did not upregulate its family member, placental growth factor (not shown).

At the same doses used in our PPI experiments (up to 100 μmol/L), pravastatin (proposed as a treatment for preeclampsia\textsuperscript{19,20}) did not significantly affect sFlt-1 or sENG secretion.

Figure 2. Proton pump inhibitors (PPIs) decrease soluble endoglin (sENG) secretion from placental and endothelial tissues and increase vascular endothelial growth factor (VEGF) expression. Relative sENG levels in the media after PPI treatment for 24 h in (A) primary trophoblasts, (B) human umbilical vein endothelial cells, and (C) preeclamptic explants. D, IC\textsubscript{50} (concentration of drug required to decrease sENG secretion from baseline by 50%) were calculated for 5 PPIs, which demonstrates esomeprazole (Eso) and rabeprazole (Rab) are the most potent at inhibiting sENG secretion. E, VEGFA mRNA expression in primary trophoblast treated with PPIs for 24 h. sENG and VEGFA mRNA expression were measured in the same samples as the experiments shown in Figure 1. Lans indicates lansoprazole; Ome, omeprazole; and Pant, pantoprazole.
from HUVECs (Figure S2C and S2D). We conclude that PPIs reduce secretion of sFlt-1 and sENG from primary placental and endothelial cells and increase both placental and endothelial vegfa mRNA expression.

Decrease in sFlt-1 and sENG Secretion Observed With Proton Pump Inhibitors Is Not Explained by Inhibition of V-ATPase

We next set out to examine possible molecular pathways to explain how PPIs may be inhibiting sFlt-1 and sENG secretion. As a medication for gastric reflux, PPIs were specifically designed to inhibit H+/K+ ATPase V-ATPase (a proton pump in the gastric epithelium) to decrease gastric acidity. Of note, the V-ATPase enzyme is also present in placenta.21-23 To examine whether the reduction in sFlt-1 and sENG secretion observed with PPI treatment is mediated through V-ATPase inhibition, we examined the effects of blocking V-ATPase by administering bafilomycin, a potent V-ATPase inhibitor.24 Indeed, bafilomycin dose dependently decreased sFlt-1 secretion by human trophoblast (Figure S3A). However, bafilomycin did not decrease intracellular levels of sFlt-1 protein or the mRNA expression of the sFlt-1 variants (sFlt-1 i13 and sFlt-1 e15a; Figure S3B through S3D). In contrast, we confirmed esomeprazole (added as a positive control in the same experiment) significantly decreased sFlt-1 and mRNA expression of the 2 sFlt-1 variants. Furthermore, bafilomycin did not decrease sFlt-1 secretion from HUVECs, and in fact significantly increased sENG secretion from HUVECs (Figure S3E and S3F). Given blocking V-ATPase with bafilomycin did not decrease intracellular sFlt-1 protein or mRNA expression or consistently reduce sFlt-1 and sEng secretion, it is unlikely that PPIs decrease the production of these antiangiogenic factors through V-ATPase.

Reduced sFlt-1 and sENG Secretion With Proton Pump Inhibitors Is Not Explained by Altered Expression of Cargo Export Proteins

ARF1 (ADP-ribosylation factor 1) and RAB11 (Ras-related Protein 11) are proteins involved in the secretion and transport of proteins from cells. Given Jung et al25 previously concluded that these proteins facilitate sFlt-1 secretion from cells, we examined whether PPIs may decrease sFlt-1 secretion by inhibiting the expression of these proteins. Treating trophoblast with esomeprazole in fact increased ARF1 expression (Figure S4A and S4B) and did not affect RAB11 expression (Figure S4C and S4D). Thus, it seems unlikely that these molecules are involved in the decrease in sFlt-1 secretion induced by PPIs.

Proton Pump Inhibitors Vasodilate Whole Human Vessels Ex Vivo and Decrease Blood Pressure in an Animal Model of Preeclampsia

Vasoconstriction and hypertension are hallmarks of preeclampsia. We next examined whether PPIs have vasoactive properties. We isolated maternal omental arteries obtained at caesarean section from normal and preeclamptic women and performed pressure myography ex vivo. We first induced vasoconstriction by incubating arteries in U46619 (thromboxane agonist [thromboxane is elevated in preeclampsia]) and then added cumulative doses of esomeprazole. Esomeprazole caused potent dilation of arteries from both preeclamptic and normal pregnancies (Figure 3A and 3B; see Table S2 for clinical characteristics of the preeclampsia cohort). The vasodilatory effects of esomeprazole seemed to be mediated through the endothelium because vessels denuded of the endothelium did not dilate in response to esomeprazole (Figure 3C). To obtain in vivo evidence that PPIs are vasoactive, we added esomeprazole to a transgenic preeclampsia mouse model where human sFlt-1 is overexpressed specifically in the placenta.27 As expected, a significant increase in blood pressure (both systolic and diastolic) was observed in untreated mice from E16.5.27 However, daily administration of 150 μg of esomeprazole (which approximately equates to a 30-mg dose in humans) from E8.5 completely abrogated this increase in blood pressure (Figure 3D). There was a nonsignificant trend toward decreased maternal proteinuria in mice treated with esomeprazole compared with controls, and there were no differences in measurements of various fetal or placental parameters (Figure 3A through 3E). There was also a nonsignificant trend toward a decrease in the blood pressure of nonpregnant female mice administered esomeprazole when compared with mice receiving vehicle (Figure 3F). We confirmed the presence of human sFlt-1 in the mouse serum obtained at the time of sacrifice (Figure 3G). The fact that there was no difference in sFlt-1 levels in this animal model given sFlt-1 is expected given it is autonomously expressed by the lentiviral plasmid.

Proton Pump Inhibitors May Be Promoting Vasodilation by Modulating eNOS and Endothelin-1 Expression

eNOS is an enzyme in the endothelium that produces NO, a potent vasodilator that signals to the underlying vascular smooth muscle. Furthermore, it has recently been shown that sFlt-1 may be directly inducing hypertension in preeclampsia by impairing eNOS phosphorylation.7 We examined whether PPIs may be inducing vasorelaxation via the eNOS pathway. When we treated HUVECs with esomeprazole (Figure 4A), there was a significant increase in phosphorylated eNOS (p-eNOS, the active form). Cotreatment of tumor necrosis factor-α (TNF-α; a proinflammatory cytokine that circulates in excess in preeclampsia28 and likely contributor to endothelial dysfunction29) with esomeprazole and rabeprazole at different doses all induced a nonsignificant increase in p-eNOS protein (Figure S6A and S6B). These data suggest the PPIs upregulate p-eNOS expression. To obtain further evidence for this, we next administered esomeprazole to pregnant mice lacking eNOS (eNOS−/−). These mice are chronically hypertensive, which persists while they are pregnant.30 In contrast to the striking decrease in blood pressure seen in wild-type mice where sFlt-1 was overexpressed in placenta (Figure 3D), there was only a modest and nonsignificant decrease in blood pressure with esomeprazole treatment when the eNOS gene is absent (Figure 4B). Our data suggest that esomeprazole has vasodilatory properties and can decrease blood pressure in vivo (animal model) and ex vivo (whole maternal vessels from women with preeclampsia). Furthermore, this may be mediated through the endothelium, where p-eNOS may play a role.
Endothelin-1 is potent vasoconstrictor released from the endothelium into the circulation and is increased in preeclampsia. Administering PPIs to HUVECs reduced endothelin-1 mRNA expression (Figure 4C) and protein secretion (Figure 4D). PPIs also decreased endothelin-1 mRNA expression in uterine microvascular endothelial cells (Figure S6C).
Proton Pump Inhibitors Rescue Endothelial Dysfunction In Vitro

Given the importance of endothelial dysfunction in preeclampsia,2,5 another therapeutic strategy may be to identify drugs that act at the level of the endothelium to reduce dysfunction. PPIs potently blocked TNF-α–induced upregulation of vascular cell adhesion molecule-1 in HUVECs (Figure 5A; Figure S7A) and primary uterine microvascular cells (Figure S7B). PPIs also decreased vascular cell adhesion molecule-1 expression in HUVECs when preeclamptic serum was used instead of TNF-α to induce endothelial dysfunction (Figure S7C).

Given vascular cell adhesion molecule-1 promotes leukocyte adhesion to the endothelium, we performed a monocyte adhesion assay. PPIs dose dependently decreased TNF-α–induced leukocyte adhesion to HUVECs (Figure 5B). We next performed endothelial tube-forming experiments and found that esomeprazole rescued TNF-α–induced tube disruption (Figure 5C). In addition, we examined endothelial cell migration using the xCELLigence assay, which allows cell migration to be continuously monitored in real time. Treating HUVECs with sFlt-1 reduced cell migration, but this was rescued with the addition of either esomeprazole or lansoprazole (Figure 5D). Therefore, we found that PPIs rescued endothelial dysfunction in multiple in vitro assays.

Proton Pump Inhibitors Upregulate Expression of Endogenous Antioxidant Proteins

Excessive oxidative stress and inflammation is likely to exacerbate the placental and endothelial dysfunction that
occurs in preeclampsia. Therefore, reducing oxidative stress and inflammation may help decrease disease severity. Administering PPIs to primary trophoblast cells induced nuclear translocation of the transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), to the nucleus (Figure 6A). One of the targets of Nrf2 is HO-1, which has antioxidant and cytoprotective actions. PPIs potently induced HO-1 expression in primary trophoblast (Figure 6B; Figure S8A), placental explants from women with preterm preeclampsia (Figure 6C), HUVECs (Figure 6D; Figure S8B), and uterine microvascular cells (Figure S8C). They also upregulate NAD(P)H (nicotinamide adenine dinucleotide phosphate-oxidase) dehydrogenase.

Figure 5. Proton pump inhibitors (PPIs) decrease endothelial dysfunction in in vitro assays. A, Vascular cell adhesion molecule-1 (VCAM) expression in human umbilical vein endothelial cells (HUVECs) with tumor necrosis factor (TNF)-α treatment and increasing doses of rabeprazole (Rab) and esomeprazole (Eso). B, Monocyte adhesion to HUVECs after TNF-α treatment and increasing doses of lansoprazole (Lans), rabeprazole (Rab), and esomeprazole (Eso). C, Endothelial tube formation assay. TNF-α disrupts HUVECs tube formation, and this was rescued with eso (100 μmol/L). D, HUVEC migration (xCELLigence) assay: soluble fms-like tyrosine kinase-1 (sFlt-1; 125 ng/mL) reduced migration, but this was rescued by administering either Eso or Lans. PPIs were administered at 5 to 100 μmol/L concentrations, or as indicated. Data are mean±SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (n≥3).
quinone 1 and thioredoxin in HUVECs and trophoblasts, both antioxidant molecules that are Nrf2 targets (Figure S8D and S8E). These data demonstrate that PPIs upregulate endogenous antioxidant molecules in placenta and endothelium.

Proton Pump Inhibitors Decrease Secretion of Cytokines From Placenta and Endothelium
Adding PPIs to placental explants from preterm preeclamptic pregnancies reduced secretion of cytokines, including interleukin-1β, interleukin-6, interleukin-10, and C-C motif chemokine ligand (CCL), CCL2 and CCL7 (Figure S9A). Furthermore, PPIs reduced secretion of cytokines from endothelial cells treated with either TNF-α or preeclamptic serum to induce endothelial dysfunction (Figure S9B and S9C). These data suggest that PPIs can decrease cytokine secretion from placenta and endothelium.

Discussion
In this study, we have identified PPIs as candidate treatments for preeclampsia. They appear to have diverse biological actions that could be useful to block important pathophysiological processes thought to occur in preeclampsia.\textsuperscript{2,5} PPIs decrease secretion of sFlt-1 and sENG from primary trophoblast, placental explants from normal and preeclamptic pregnancies, and from 2 types of primary endothelial cells. The PPI esomeprazole vasodilates human vessels from normal and preeclamptic pregnancies and reduces blood pressure in a transgenic mouse model where human sFlt-1 is overexpressed in placenta.

The ability of esomeprazole to vasodilate vessels ex vivo and reduce blood pressure in vivo may be mediated through the endothelium, perhaps by increasing p-eNOS expression. Indeed, for our preeclampsia animal model, we note that sFlt-1 is autonomously expressed in the placenta meaning it is unlikely PPIs can directly decrease placental sFlt-1 secretion in that experiment. Furthermore, PPIs seem to block endothelial dysfunction in many in vitro assays. Finally, PPIs upregulate endogenous antioxidant molecules and decrease secretion of cytokines from placental tissues and endothelial cells.

We believe that our study has several strengths. We performed a significant body of functional experiments on 7 types of primary human tissues where we administered many PPIs at various doses. Importantly, we included vessels and placenta from cases of preterm preeclampsia and biological replicates were performed on samples from different patients. Hence we believe that the functional evidence we have generated is strong.

Although we have generated evidence suggesting that PPIs may alter molecular signaling involved in vessel dilation, we have not uncovered a unifying molecular mechanism to explain how PPIs have such diverse biological effects (ranging from vessel dilatation, decreasing sFlt-1 and sENG secretion...
to decreasing endothelial dysfunction). It is unclear whether PPIs are exerting these effects by acting on one, or multiple molecular targets.

We have explored relevant pathways to determine the molecular mechanism by which PPIs decreased sFlt-1 and sENG. Initially, we embarked on these studies with the hypothesis that PPIs may upregulate HO-1 and may reduce sFlt-1 and sENG secretion given it had previously been proposed that HO-1 was central to preeclampsia and that HO-1 regulates sFlt-1 and sENG production. However, we have since published data demonstrating that HO-1 is not decreased in preeclampsia and does not regulate placental sFlt-1 or sENG secretion. As such, we do not believe that the effects of sFlt-1 and sENG are mediated by the ability of PPIs to upregulate HO-1. Instead, we examined V-ATPase enzyme (given PPIs were designed to inhibit this proton pump) and the ARF1 and RAB11 cargo proteins (given a previous report suggesting they shuttle sFlt-1 out of cells). We were unable to show that they mediate the decrease in sFlt-1 and sENG secretion induced by PPIs. We have ongoing studies to try to determine the molecular mechanisms behind the actions of PPIs, specifically the decrease in antiangiogenic factors, given such a discovery might yield new insights into new drug targets.

Ghebremariam et al reported that the PPI omeprazole reduced eNOS and p-eNOS (active eNOS) expression and reduced nitric oxide release from isolated veins. They presented a qualitative blot (without densitometric analysis) to suggest that omeprazole decreased eNOS protein expression, and experiments demonstrating omeprazole impaired vascular reactivity. Furthermore, they showed administrating lansoprazole to nonpregnant mice increased circulating asymmetrical dimethylarginine (antagonist of eNOS) although blood pressure was not reported. In contrast, we generated a body of data to show the opposite that PPIs upregulate p-eNOS and induce vasodilation both ex vivo and in vivo. We administered different doses of esomeprazole or rabeprazole with or without TNF-α stimulation, and in all cases, there was a significant increase or a trend toward an increase in p-eNOS protein (quantitative densitometric analysis). Importantly, we also found that esomeprazole potently vasodilated whole maternal blood vessels and reduced blood pressure in an animal model of preeclampsia. Finally, we observed a possible trend toward a decrease in blood pressure when we administered esomeprazole to nonpregnant mice, but there was certainly no evidence that the blood pressure increased. It is difficult to reconcile our findings with Ghebremariam et al. Despite these conflicting findings, we suggest that clinical studies to explore the potential of PPIs to treat preeclampsia are well justified, given our preclinical data suggest that PPIs seem to have other biological effects (besides vasorelaxation) that may also be beneficial in treating preeclampsia and there is strong epidemiological evidence to suggest PPIs are safe when administered during pregnancy (as discussed below).

We note that it is difficult to know how much drug is seen locally at target tissues. However, we would make some observations. First, the surface area of the target tissues (vascular endothelium and placenta) is vast and easily accessible to circulating drugs. Second, we would anticipate that some decrease in sFlt-1 and sENG may be sufficient to affect the disease progression given these factors are present in normal pregnancies. It may not be necessary to block their secretion entirely. Third, we treated our mouse model (where sFlt-1 was overexpressed in the placenta) with esomeprazole at a dose that equates to ~30 mg daily dosing in humans (using a formula published by Reagan-Shaw et al), and this completely abrogated an increase in blood pressure. Finally, we performed multiple dose–response studies using multiple PPIs to show that the various biological responses are present at a range of doses. Importantly, the doses we used are in the micromolar range; these doses were chosen because the PPIs are found in the circulation at these concentrations, thus we anticipate that this will be the range that is clinically effective. It may be worthwhile undertaking further in vitro studies using drug doses within the nanomolar range as these might shed further clues as to the mechanisms by which PPIs are exerting the diverse biological effects we have observed.

Recent data have emerged combining microarray or ultrasound findings with histopathology defining further subtypes of preeclampsia, highlighting the heterogeneity of the disease. Leavey et al found specific correlations with microarray data (from large cohorts of preeclamptic placentas) with clinical findings and propose subclasses of preeclampsia distinct to the canonical disease (which is driven by antiangiogenic factors). They identified a cluster of patients they coined immunologic preeclampsia where the main drivers of the disease may be immunologic. It is therefore possible that PPIs might be less effective for subtypes of preeclampsia where antiangiogenic factors play less of a role. As the field of preeclampsia subtyping matures, it may be feasible to stratify response to PPIs in future clinical trials with preeclampsia subtyping.

There have been few orally available treatment options identified for preeclampsia that have reached clinical trials. In regards to treating preeclampsia, the field has perhaps been most focused on the potential use of pravastatin, a drug designed to inhibit cholesterol synthesis. There is preclinical evidence that pravastatin may have been useful to treat preeclampsia. A recent clinical trial of pravastatin to treat preterm preeclampsia has ceased recruitment and the results have yet to be published (unique identifier: ISRCTN23410175). A pharmacokinetic study of 20 participants has just been reported (unique identifier: NCT01717586). However, a potential drawback in using pravastatin is that it has been assigned category X classification by the Food and Drug Administration. Although we agree that there are valid arguments to support the use of statins to treat pregnancies complicated by preterm preeclampsia, a drug that is thought to be safe in pregnancy will be more clinically acceptable.

In contrast to pravastatin, large epidemiological studies have demonstrated that PPIs are safe in pregnancy, where they are widely prescribed to relieve symptomatic gastric reflux in pregnancy. In 2010, Pasternak and Hviid examined the first trimester exposure, a time in pregnancy where there is significant organogenesis and the fetus is most vulnerable to teratogens. Examining 5082 pregnancies exposed to PPIs and 840968 that were not exposed, they concluded that the first trimester exposure to PPIs was not associated with an increased risk of major birth defects. A systematic review published in 2009 (1530 pregnancies exposed to PPIs and
133,410 nonexposed controls) did not identify an increase in congenital abnormalities. A subsequent large study examining 112,022 pregnancies (of which 11,86 were exposed to PPIs) confirmed no increase in congenital anomalies, fetal growth restriction, or adverse neonatal outcomes (including preterm birth or low Apgar scores). Thus, large cohort studies indicate that PPIs are safe during pregnancy.

Given our preclinical data, and the reassuring epidemiological data suggesting that PPIs are safe, it would seem justifiable to proceed to clinical trials to examine whether PPIs could be used to either prevent preeclampsia or treat women diagnosed with preeclampsia. As such, we are currently recruiting women with preterm preeclampsia to a phase II randomized clinical trial, The PIE trial (Preeclampsia Intervention With Esomeprazole). We will examine whether esomeprazole can safely prolong gestation in women diagnosed with preterm preeclampsia and improve fetal, maternal, and neonatal outcomes.

Perspectives

We have shown in preclinical studies that PPIs potently decrease placental release of sFlt-1 and sEng, decrease endothelial dysfunction, are vasodilatory and have antioxidant and anti-inflammatory properties. They may represent a potential therapeutic option for preeclampsia and perhaps other diseases where there is significant endothelial dysfunction. If PPIs are found to be useful to treat or prevent preeclampsia, they may significantly reduce maternal and perinatal mortality globally.

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Disclosures

None.

References


Proton pump inhibitors rescue endothelial dysfunction, upregulate anti-inflammatory genes, and decreases cytokine secretion from endothelial cells.

What Is Relevant?

- Preeclampsia is a serious complication of pregnancy characterized by hypertension, endothelial dysfunction, and multiorgan injury.

Novelty and Significance

- Inflammation and oxidative stress exacerbate the endothelial injury.
- A drug that is safe in pregnancy that can block these processes could be used to treat or prevent preeclampsia.

Summary

We propose proton pump inhibitors are candidate drugs to prevent or treat preeclampsia, but clinical trials are needed.
Proton Pump Inhibitors Decrease Soluble fms-Like Tyrosine Kinase-1 and Soluble Endoglin Secretion, Decrease Hypertension, and Rescue Endothelial Dysfunction
Kenji Onda, Stephen Tong, Sally Beard, Natalie Binder, Masanaga Muto, Sevvandi N. Senadheera, Laura Parry, Mark Dilworth, Lewis Renshall, Fiona Brownfoot, Roxanne Hastie, Laura Tuohey, Kirsten Palmer, Toshihiko Hirano, Masahito Ikawa, Tu'uhevaha Kaitu'u-Lino and Natalie J. Hannan

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PROTON PUMP INHIBITORS DECREASE SFLT-1 AND SOLUBLE ENDOGLIN SECRETION, DECREASE HYPERTENSION AND RESCUE ENDOTHELIAL DYSFUNCTION

Authors
Kenji Onda¹,² PhD, Stephen Tong¹ PhD, Sally Beard¹ BSc, Natalie Binder¹ PhD, Masanaga Muto³, Sevvandi N Senadheera⁴ PhD, Laura Parry⁴ PhD, Mark Dilworth⁵,⁶ PhD, Lewis Renshall⁵,⁶ BSc, Fiona Brownfoot¹ MBBS, Roxanne Hastie¹ BSc, Laura Tuohey¹ BSc, Kirsten Palmer¹ PhD, Toshihiko Hirano² Masahito Ikawa³ PhD, Tu'uhevaha Kaitu'u-Lino¹ PhD, and Natalie J Hannan¹ PhD.

¹Translational Obstetrics Group, Department of Obstetrics and Gynaecology, Mercy Hospital for Women, University of Melbourne, Heidelberg, Victoria, Australia, 3084; ²Department of Clinical Pharmacology, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan; ³Research Institute for Microbial Diseases, Osaka University, Japan; ⁴School of Biosciences, University of Melbourne, Parkville, Victoria, Australia. ⁵Maternal and Fetal Health Research Centre, Institute of Human Development, University of Manchester, ⁶St Mary's Hospital, Central Manchester University Hospitals NHS Trust, Manchester Academic Health Science Centre, Manchester, United Kingdom.

* These authors contributed equally to this work.

Short title: Proton pump inhibitors to treat preeclampsia.

Keywords: Preeclampsia, treatment, proton pump inhibitors, endothelial dysfunction, hypertension, pregnancy

Corresponding author: Professor Stephen Tong, Translational Obstetrics Group, Department of Obstetrics and Gynaecology, University of Melbourne; Mercy Hospital for Women, Heidelberg, 3084 Victoria, Australia. Ph: +61-3-8458 4377 Fax: +31-3-8458 4380. Email: stong@unimelb.edu.au
Supplementary Methods

Tissue collection. Ethical approval was obtained for this study from the Mercy Health Human Research Ethics Committee. Women presenting to the Mercy Hospital for Women, Melbourne gave informed written consent for tissue or blood collection. Placentas, umbilical cords and omentum were obtained from normal term pregnancies (>38 weeks gestation) at elective cesarean section for functional studies. Placentas, omentum and serum were obtained from pregnancies complicated by early-onset, preterm preeclampsia (requiring delivery <34 weeks gestation). Severe preeclampsia was diagnosed in accordance with American College of Obstetricians and Gynecologists guidelines published in 2013 (http://www.acog.org/Resources-And-Publications/Task-Force-and-Work-Group-Reports/Hypertension-in-Pregnancy).

Preparation of tissue for functional studies. Placental tissue, omental fat and umbilical cords were collected within 30 min of delivery and washed in sterile phosphate buffered saline (PBS). Samples for mRNA and protein extraction were collected, snap frozen and stored at -80°C. Samples for ex vivo studies were prepared as detailed below.

Primary cytotrophoblast isolation. As described previously \(^1, 2\) 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 then subjected to three 20 minute digestion cycles with 0.25% trypsin and 0.2 mg/ml DNAse in Enzyme Digestion Buffer containing 10x Hanks Buffered Salt Solution, sodium bicarbonate, HEPES and deionised H\(_2\)O. Cell suspensions were filtered and then separated using a discontinuous Percoll gradient centrifugation. The layer containing cytotrophoblasts are then collected and subjected to a CD9 negative selection step, to remove contaminating non-trophoblast cells. Primary cytotrophoblasts were cultured in DMEM high Glutamax (Life Technologies) containing 10% Fetal Calf Serum (Sigma, St Louis, United States) and 1% anti-anti (Life Technologies) on fibronectin (10 µg/mL; BD Bioscience, USA) coated plates. Viable cells attached overnight and were then washed twice with sterile PBS to remove non-viable cells and cell debris.

Isolation and culture of placental explants. Small pieces of villous tissue were cut from the mid-section of the placenta to avoid the maternal and fetal surfaces. These were thoroughly washed with PBS and allowed to equilibrate at 37°C for 1 hour in DMEM containing 1% anti-anti and 10% fetal calf serum. Tissue explants were then dissected into small fragments of 1-2 mm size and three pieces put into each well of a 24 well plate.

Primary human endothelial cell isolation. The cord vein of umbilical cords of normal term placentas was cannulated and infused with PBS to wash out fetal blood. Next, approximately 10ml (1mg/ml) of collagenase (Worthington, Lakewood, New Jersey) was infused into the cord followed by incubation at 37°C for 10 minutes. The dissociated HUVEC cells were recovered by pelleting and re-suspension followed by culture in M199 media (Life Technologies) containing 20% fetal calf serum, 1% anti-anti and 1% endothelial cell growth factor (Sigma) and 1% heparin. Primary human uterine microvascular cells (UtMVECs) were obtained from Lonza (Clonetics®Myometrial Uterine Microvascular Cell Systems; Lonza Walkersville, Inc. USA).

Human whole vessel artery isolation. Omental biopsies from resected tissue were washed several times overnight in ice-cold Krebs' physiological solution (PSS) containing 112 mM NaCl, 25 mM NaHCO\(_3\), 4.7 mM KCl, 1.2 mM MgSO\(_4\), 7H\(_2\)O, 0.7 mM KH\(_2\)PO\(_4\), 10 mM HEPES, 11.6 mM D-glucose and 2.5 mM CaCl\(_2\).2H\(_2\)O (pH 7.4) to remove anesthetic. Whole omental arteries were carefully cleaned of connective and adipose tissue. The average inner lumen diameter at 60 mmHg in zero calcium was 275 ± 15 µm (mean ± SEM) in vessels of normal pregnant (n = 16) and 248 ± 30 µm in preeclamptic pregnant (n = 6) patients. Experiments were performed <20 hours post-surgery.
Proton pump inhibitors (PPI) in vitro experiments. Isolated primary human cytotrophoblast, preeclamptic explants (<34 weeks gestation), human umbilical vein endothelial cells (HUVECs) and uterine microvascular endothelial cells were treated with lansoprazole (Lans), rabeprazole (Rab), esomeprazole (Eso) (5-100 µM) for 24 h. Baflomycin was used to block the proton pump (via V-ATPase). Isolated primary human cytotrophoblast and HUVECs were treated with baflomycin (baf) (1-100 nM) for 24 h. In order to detect soluble endoglin a separate isolation of primary placental cytotrophoblast cells were stimulated by forskolin (20 mM) treatment. As a positive control to for HO-1 induction\textsuperscript{2,3} cells/tissue was treated with cobalt protoporphyrin (CoPP (10 µM)) as specified in the figures (Frontier Scientific (Logan, UT)).

Explants were initially cultured for 24 h without treatment. The explants were then treated with PPIs (5-100 µM) and cultured for further 24 h. Media was then collected and analysed by ELISA. Concentration of sFlt-1 and sENG after treatment was normalized to tissue weight.

Pravastatin in vitro experiments. Isolated primary HUVECs were treated with pravastatin (prav; 5, 50 and 100 µM) for 24 h, and were run concurrently with the PPIs experiments, where the same tissues were treated (5-100 µM).

Endothelial dysfunction rescue studies. Endothelial cells (HUVECs or UtMVECs) were treated with either TNFα (10 ng/mL) or preeclamptic patient serum (5% of total volume of media) to induce dysfunction. Conditioned media, total RNA, whole cell protein lysate or nuclear protein lysate (in the case of HIF-1α western blot) was collected (and stored at -80 ºC) for subsequent analysis.

Monocyte to HUVEC adhesion assay. Primary HUVECs were seeded in 96-well black wall plates with clear bottom to confluency. Cells were treated with TNFα (10 ng/ml) for 2 h followed by PPI treatment in the presence of TNFα (10 ng/ml) for a further 24 h. HUVECs were washed with PBS and THP-1 cells (1 x 10\textsuperscript{5} cells/ml) stained with calcein-AM were added and co-cultured for 45 minutes. Unattached THP-1 cells were then washed out with PBS. 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 formation assay. Flat bottomed 48-well tissue culture plates were coated with neat Matrigel (In Vitro Technologies, Vic, Aus) and incubated at 37°C for 30 minutes to allow matrigel to polymerize. Isolated primary HUVECs were seeded and incubated at 37°C for 14-16 h to allow tube formation. Cells were then cultured in the presence of either TNFα (10 ng/ml) alone; with both TNFα (10 ng/ml) and PPIs 5-100 µM; or control media for 8-12 h. Tube formation was assessed and images were captured using an EVOS-FL microscope at x4 magnification.

Whole vessel pressure myography. Isolated omental arteries were cannulated on glass micropipettes and mounted in a pressure myograph chamber (Living Systems Instrumentation, Burlington, VT, USA) with continuous superfusion of PSS (37°C) at a rate of 4 ml/min. In the absence of intraluminal flow, arteries were pressurized (60 mmHg) and diameter measured through video microscopy (Diamtrak software, Adelaide, SA, Australia). Prior to starting each experiment, the viability of the smooth muscle was tested with 100 mM potassium physiological saline solution (KPSS). Endothelial function was confirmed with 10\textsuperscript{-5} µM bradykinin (BK) applied for 2 min in arteries pre-constricted with the thromboxane agonist 9,11-dideoxy-9a,11α-methanoepoxy prostaglandin F\textsubscript{2α} (U46619; Cayman Chemical, Ann Arbor, MI, USA). Arteries were then submaximally preconstricted (~70% of maximal response to KPSS) with U46619\\textsuperscript{5} and vascular reactivity to increasing concentrations of esomeprazole (Eso) or vehicle (ethanol), 1 nM-300 µM was measured. The influence of Eso on endothelial vascular reactivity was evaluated in endothelium-denuded arteries by passing a 1 ml air bolus through the lumen while mounted in the arterial chamber as previously described\textsuperscript{5}. The endothelium was considered to be denuded if the response to BK (10 µM) was diminished by >95%. Smooth muscle integrity was tested in response to the endothelium-independent, nitric oxide donor, SNP (10 µM). In functional studies, diameter
measurements were expressed as a percentage of maximal diameter determined in Ca²⁺-free Krebs.

**ELISAs.** Conditioned media from primary cytotrophoblast, placental explants and endothelial cells (HUVECs and UtMVECs) was assessed using ELISA for the presence of the following soluble factors: soluble Flt-1 (sFLT); DuoSet VEGF R1/Flt-1 kit (R&D systems by Bioscience, Waterloo, Australia), soluble endoglin (sENG); DuoSet Human Endoglin CD/105 (R&D systems); Endothelin-1 (ET-1); (Quantikine endothelin-1; (R&D systems), and sFLT-e15a variant; using our own in house assay.

Mouse urine was assessed for creatinine to albumin ratio using the mouse creatinine and mouse albumin assays (Crystal Chem, IL 60515 US).

Optical density for ELISAs was determined using a BioRad X-Mark microplate spectrophotometer (BioRad), Protein levels determined using BioRad Microplate manager 6 software.

**Cytokine assays.** The cytokines, IL-1β, IL-6 and IL-10, and the chemokines, CXCL8, CCL2, CCL5, CCL7 and CX3CL1 were measured using quantitative Milliplex Luminex (MilliPlex MAP Human Cytokine Panel 8-plex, Millipore, Melbourne, Victoria, Australia) assays according to the manufacturer’s instructions. As described previously, 96-well Milliplex plates were pre-wet with 200 µl assay buffer (provided by the manufacturer) for 10 minutes and then aspirated using a vacuum manifold. Standards and samples (25 µl) were added to appropriate wells, followed by the addition of assay beads. Plates were incubated overnight for 16-18 h with mild agitation at 4°C; the fluid was then removed by vacuum and the wells were washed twice with wash buffer. Detection antibodies were added to each well, and incubated for 1 h at room temperature (RT), the fluorescent conjugate Streptavidin-Phycoerythrin was added to each well and plates incubated for 30 min at RT. Fluid was then removed by vacuum and wells washed twice. Analysis of each sample was performed in duplicate. Identical positive and negative quality controls are included on each assay in duplicate.

**Immunocytochemistry.** HO-1 and Nrf2 were localized by immunocytochemistry in primary cytotrophoblasts collected from control pregnancies. In brief, cells were treated with the PPIs (Lans, Rab and Eso); 6 hours for Nrf2 and 24 hours for HO-1. Media was removed, cells were washed with phosphate-buffered saline pH 7.6 (PBS). Fixed with ice cold 70% ethanol (in PBS) and air dried until staining. Cells were rehydrated for 10 min in PBS. Cells were incubated overnight at 4°C with either anti-HO-1 antibody (ADI-SPA-896; ENZO) or anti-Nrf2 antibody (H-300; Santa Cruz (Dallas, US) at 10 µg/ml in 1%BSA/PBS. For isotype controls, primary antibody was substituted with rabbit IgG. Staining was visualized using a fluorescent secondary antibody Alexafluor488 (Invitrogen) and nuclear staining was visualized with DAPI. Sections were then wet mounted and images captured using EVOS-FL microscope.

**Quantitative RT-PCR.** Total RNA was extracted from isolated trophoblast, human umbilical vein endothelial cells (HUVEC) and uterine microvascular endothelial cells (UtMVECs) and placental tissue using the RNaseasy mini kit (Qiagen, Valencia, CA) and quantified using a Nanodrop ND 1000 spectrophotometer (NanoDrop technologies Inc, Wilmington, DE). 0.2 µg of RNA was converted to cDNA using Superscript VILO cDNA synthesis kit (Life technologies, Mulgrave, Australia) as per manufacturer guidelines.

Quantitative PCR was performed using Taqman gene expression assays for: Heme oxygenase-1 (HMOX1; HO-1) and nuclear factor (erythroid-derived 2)-like 2 (NFE2L2; Nrf2), VEGFA, TXN, NQO1, VCAM1, ET-1 (EDN1), HIF1α, ARF1 and RAB11 qPCR was performed on the CFX 384 (Biorad, Hercules, CA) using FAM-labeled Taqman universal PCR mastermix (Applied Biosystems) with the following run conditions: 50°C for 2 minutes; 95°C for 10 minutes, 95°C for for 15 seconds, 60°C for 1 minute (40 cycles). The sFlt-1 splice variants i13 and e15a were measured with SYBR Green Master mix (Applied Biosystems) using primers specific for each variant as previously published. All data were normalized to an appropriate house-keeping gene (isolated cells normalized to: GAPDH and YWHAZ; placental tissue to: TOP1 and CYC1) as an internal control and calibrated against
the average Ct of the control samples. The results were expressed as fold change relative to controls. All samples were run in triplicate.

**Extraction of Nuclear fraction to detect nuclear proteins.** Nuclear proteins were extracted from HUVECs using hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl) and nuclear extraction buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 420 mM NaCl, and 25% w/w glycerol) with centrifugation. Protein concentration was quantified by a coomassie blue assay method (Thermo Fisher Scientific). 20µg of nuclear protein was separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membrane. Proteins were detected by western immunoblotting as detailed below.

**Western Blot Analysis.** 20 µg of either whole cell or nuclear protein lysates were separated on 10% polyacrylamide gels with wet transfer to PVDF membranes (Millipore, Billerica, MA). Membranes were blocked prior to incubation overnight with the primary antibody (see Table S1 for specific protein details: HIF-1α, VCAM1, HO-1, eNOS, p-eNOS, HO-1, GAPDH). Bands were visualized using a chemiluminescence detection system (GE Healthcare Life Sciences) an ChemiDoc XRS (BioRad, Hercules, CA). Relative densitometry was determined using QuantityOne software (BioRad). Loading controls (see Table) were used for densitometric analysis.

Cell viability (MTS) assays. Cell viability was assessed at matching doses and culture duration by MTS assay (Promega), as per manufacturer’s instructions. Treatment of primary cytotrophoblasts, human umbilical cord vein endothelial cells (HUVECs) and uterine microvascular (UtMVs) with 0-100µM of any of the PPIs used in this study did not reduce cell viability.
Western blot details

<table>
<thead>
<tr>
<th>Protein</th>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF1α</td>
<td>Anti-HIF1α (NB-100-105)</td>
<td>Novus Biologicals Littleton, Colorado</td>
<td>1:500</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Anti-VCAM1 (SC-1504)</td>
<td>Santa Cruz Biotechnology, Dallas, Texas</td>
<td>1:200</td>
</tr>
<tr>
<td>eNOS</td>
<td>Anti-eNOS/NOS Type III (610298)</td>
<td>Becton Dickinson Biosciences, Franklin Lakes, New Jersey</td>
<td>1:500</td>
</tr>
<tr>
<td>p-eNOS</td>
<td>Anti-eNOS (pT495), Phospho-Specific (612706)</td>
<td>Becton Dickinson Biosciences</td>
<td>1:200</td>
</tr>
<tr>
<td>HO-1</td>
<td>Anti-HO-1 (ADI-SPA-896)</td>
<td>ENZO Life Sciences, Farmingdale, New York</td>
<td>1:500</td>
</tr>
<tr>
<td>ARF1</td>
<td>Anti-ARF1 (ab232524)</td>
<td>Abcam, Cambridge, UK</td>
<td>1:1000</td>
</tr>
<tr>
<td>RAB11</td>
<td>Anti-RAB11 (71-5300)</td>
<td>Life Technologies, CA, USA</td>
<td>1:250</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Anti-GAPDH 14C10 (2118)</td>
<td>Cell Signalling Technology, Danvers, MA</td>
<td>1:5000</td>
</tr>
<tr>
<td>Histone 3</td>
<td>Anti-Histone 3 (04-928)</td>
<td>Merck Millipore, Billerica, Massachusetts</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Endothelial migration assay (xCELLigence). To determine whether PPIs could rescue disrupted endothelial migration the xCELLigence system (Roche diagnostic, New South Wales, Australia) was used. Recombinant vascular endothelial growth factor (VEGF) (Biologic, scientific, NSW, Australia) 12.5 ng/ml was placed in the lower receiver plate as the chemoattractant. HUVECs were treated with ± 125 ng/ml of recombinant sFlt-1 and either 100 µM of lansoprazole or esomeprazole. 40,000 cells/ well were placed in the upper chamber of the insert device. HUVEC migration was assessed by measuring the electrical impedance every minute for 2 h, and then every hour for a total of 48 h.

Murine model of lentiviral transduction of human sFlt-1 in the placenta. We followed our protocol described previously\(^\text{10}\). Wild-type B6D2F1 females were superovulated by i.p. injection of pregnant mare’s serum gonadotropin (5 IU) followed by human chorionic gonadotropin (5 units) 48 h later and then mated with wild-type B6D2 F1 males. Two to four cell-stage embryos were collected from the females at 1.5 d after copulation and then incubated in KSOM medium for 2 d to obtain blastocysts. Zona’s were removed by Tyrode’s solution (Sigma), and blastocysts were incubated individually for 5 h in 4 µL drops of medium containing lentiviral vectors (1000 ng of p24/mL) to overexpress human sFlt-1 (LVhsFLT1)\(^\text{10}\). The transduced blastocysts were washed three times and then transferred into pseudopregnant ICR females (via delivery to the top of the uterine horn, while under Avertin (Tri bromoethanol anesthesia). A total of 10 blastocysts were transferred into each uterine horn. Animals were also administered the analgesic carprofen. All animal experiments were
approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University.

On E8.5, animals were randomly assigned into either control or treated groups. Those in the treated group were given 150 µg of esomeprazole sodium (Abcam, UK) by i.p. injection. The dose of 150 µg was chosen as this equates to a 30 mg dose in humans, which is clinically relevant (clinical dose for PPIs range between 20mg – 160mg). We calculated 150 ug of esomeprazole sodium equates to a human dose of 30 mg by using the body surface area (BSA) normalization method published by Reagan-Shaw et al.\(^\text{11}\) Esomeprazole was given daily to mice until E18.5. Control animals were given vehicle (PBS) by i.p. injection daily (on E8.5-18.5). All mice were humanely euthanised at E18.5.

Mice were killed on E18.5 and tissue/samples were harvested. Placentas and fetuses were collected and weighed and fetal crown to rump length was measured. Blood was collected by cardiac puncture and urine were collected on E18.5. Blood samples were allowed to clot and were centrifuged to prepare serum samples. Concentrations of total human sFlt-1 were measured in mouse serum by ELISA. Urine albumin and creatinine concentrations were measured by ELISA.

Blood pressure in the sFlt-1 placental overexpressed model was measured by the tail-cuff method with BP98A (Softron). The mice were acclimatized and gently restrained, blood pressure was measured after their behavior, heart rates, and blood pressure had stabilized. After stabilization, both systolic and diastolic blood pressure was recorded at least 5 times and up to 10 times, until the stabilization was broken. The mean of both the systolic and diastolic blood pressures measured as above was used for further statistical analysis.

eNOS\(^{-/-}\) mouse model. Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986 under Home Office licence PPL 40/3385. The Local Ethical Review Process of the University of Manchester approved all protocols. Endothelial nitric oxide synthase knockout mice (eNOS\(^{-/-}\)), stock number 002684, were purchased from Jackson Laboratories (Maine, USA).

eNOS\(^{+/+}\) female mice (10 to 16 weeks old) were mated with eNOS\(^{-/-}\) males (10 weeks to 6 months old). Embryonic day 0.5 of gestation (E0.5) was determined by the discovery of a copulation plug (term = E19.5). All animals were provided with nesting material and housed in cages maintained under a constant 12 h light/dark cycle at 21-23 °C with free access to food (Beekay Rat and Mouse Diet, Bantin & Kingman, Hull, UK) and water. On E7.5, animals were randomly assigned into either control or treated groups. Those in the treated group were given 0.4 mg/ml Esomeprazole sodium (Abcam, UK) via drinking water; control mice received drinking water and vehicle only. This dose was chosen based on our previous study where we administered a dose of another drug (sildenafil citrate) in the drinking water and observed a physiological effect.\(^\text{12}\) Mice were dosed until E18.5 with a fresh bottle made up daily. In total, 7 eNOS\(^{-/-}\) mice were placed on vehicle and 9 were placed on Esomeprazole treatment. All mice were humanely euthanised at E18.5.

Systolic blood pressure (sBP) was measured using the non-invasive blood pressure system (model LE5001, PanLab, Spain) at three time-points; approximately 7 days prior to mating (non-pregnant, NP) and at E10.5 and E17.5. All mice were trained prior to these time-points which involved exposure to the restraining tube and sham measurements following inflation of the tail cuff. On experimental days, mice were transferred to a room with a temperature between 22-24°C and left to acclimatise for 45 minutes before measurements were recorded. First, a small rodent restraining tube was placed on a Thermopad heated mat (Harvard Apparatus, Kent, U.K.). Mice were not forced to enter the rodent restrainer but when entered were secured with the tail exposed for measurements. The tail-cuff occlusion device and pulse transducer apparatus were placed on the mouse tail for at least 15 minutes prior to recording sBP measurements.\(^\text{13}\) Heart rates were monitored and no sBP measurements were taken when the heart rate exceeded 600 beats per minute. Twenty sBP measurements were taken in total in each conscious restrained mouse (for a maximum of 15 minutes). The
median sBP readings were then calculated. Mean sBP for each group was then calculated and used for statistical analyses.

**Statistical analysis.** All *in vitro* experiments were performed with technical triplicates and all experiments were repeated a minimum of three times. Data was tested for normal distribution and statistically tested as appropriate. When three or more groups were compared a One-way ANOVA (for parametric data) or Kruskal-Wallis test (for non-parametric data) followed by multiple comparison with Dunnet or Bonferroni ad-hoc test where appropriate or post-hoc analysis using either the Tukey (parametric) or Dunn’s test (non-parametric). When two groups were analysed, either an unpaired t-test (parametric) or a Mann-Whitney test (non-parametric) was used. Data is expressed as mean fold change from control ±SEM.

To analyze the whole vessel pressure myography experiments concentration response curves from omental arteries were fitted to a sigmoidal curve using nonlinear regression to calculate the sensitivity of each agonist (pEC$_{50}$) or maximum relaxation ($E_{\text{max}}$). Data were differentially analyzed as one-way ANOVA with post-hoc analysis using Dunnett’s test or Student’s independent t-test as appropriate.

Blood pressure measurements for the both mouse models were assessed by a 2-way repeated measures ANOVA, which assessed both gestational and treatment effects.
Supplementary References


Supplementary Tables

Supplementary Table S1. Clinical characteristics of participants who provided preeclamptic placental explant tissues.

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age - yrs</td>
<td>39.5 (30, 41.5)</td>
</tr>
<tr>
<td>Gestation at Delivery - weeks</td>
<td>30 (28, 31.8)</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>26 (23, 39)</td>
</tr>
<tr>
<td>Parity (%)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>25%</td>
</tr>
<tr>
<td>1</td>
<td>50%</td>
</tr>
<tr>
<td>2</td>
<td>25%</td>
</tr>
<tr>
<td>Highest SBP prior to delivery (mmHg)</td>
<td>177.7 (147.5, 196.3)</td>
</tr>
<tr>
<td>Highest DBP prior to delivery (mmHg)</td>
<td>107.5 (93.8, 110)</td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td>0</td>
</tr>
<tr>
<td>Caeserean</td>
<td>100%</td>
</tr>
<tr>
<td>Birth weight (grams)</td>
<td>1384 (816, 1752)</td>
</tr>
<tr>
<td>Birth weight centile (%)</td>
<td>20 (0.3, 65.5)</td>
</tr>
<tr>
<td>Placental pathology</td>
<td>100% infarction, 100% decidual vasculopathy.</td>
</tr>
</tbody>
</table>

n=4. Median and interquartile range shown, unless another parameter is specified.
Supplementary Table S2. Clinical characteristics of participants who provided omental arteries.

<table>
<thead>
<tr>
<th>Clinical Characteristic</th>
<th>Median and IQR</th>
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<tr>
<td>Maternal Age - Yrs</td>
<td>31 (29, 32)</td>
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<td>Gestation at Delivery - weeks</td>
<td>30 (26.5, 30.5)</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>30 (22, 39.5)</td>
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<tr>
<td>Parity no. (%)</td>
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<tr>
<td>0</td>
<td>80%</td>
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<tr>
<td>1</td>
<td>0%</td>
</tr>
<tr>
<td>≥2</td>
<td>20%</td>
</tr>
<tr>
<td>Highest SBP prior to delivery (mmHg)</td>
<td>175.0 (165.0, 180.0)</td>
</tr>
<tr>
<td>Highest DBP prior to delivery (mmHg)</td>
<td>110.0 (100.0, 115.0)</td>
</tr>
<tr>
<td>Mode of delivery</td>
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<tr>
<td>Caeserean section</td>
<td>100%</td>
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<tr>
<td>Birth weight (grams)</td>
<td>1201 (809, 1573)</td>
</tr>
<tr>
<td>Birth weight centile (%)</td>
<td>16.2 (0.7, 56.3)</td>
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</table>

n=5. Median and interquartile range shown, unless another parameter is specified.
Supplementary Figure S1. Proton Pump Inhibitors (PPIs) reduce sFlt-1. PPIs [lansoprazole (Lans), rabeprazole (Rab), esomeprazole (Eso)] administered to primary trophoblast cells for 24h reduced mRNA expression of (A) sFlt-1 e15a and (B) sFlt-1 i13, (C) decreased sFlt-1 secretion from placental explants. PPIs administered to primary HUVECs for 24 hours (D) decreased sFlt-1 e15a and (E) sFlt-1 i13 mRNA expression. PPIs reduced sFlt-1 secretion (F) from uterine microvascular endothelial cells. PPIs were administered at 5-100 μM, or otherwise indicated. Data are mean % change from control ± SEM (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). (n≥3)
Supplementary Figure S2. Proton Pump Inhibitors (PPIs) reduce sENG. (A) PPIs [lansoprazole (Lans), rabeprazole (Rab), esomeprazole (Eso)] reduced secretion of sENG from uterine microvascular endothelial cells treated for 24 h. (B) PPIs increased VEGF mRNA expression in HUVECs. Pravastatin (prav) administered to HUVECs at 5-100 μM concentrations did not significantly alter (C) sFlt-1 or (D) sENG secretion. PPIs were administered at 5-100 μM, or otherwise indicated. Data are expressed as the mean % change from control ± SEM (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). (n≥3)
Supplementary Figure S3. Proton Pump Inhibition in primary trophoblast and endothelial cells.

The V-ATPase inhibitor bafilomycin (Baf) and the PPI esomeprazole (Eso) were compared. Isolated trophoblast and HUVECs were cultured with Baf (1-100nM) or Eso (100μM). (A) Trophoblast secretion of sFlt-1 was significantly reduced with Baf and Eso. (B) Intracellular sFlt-1 protein production was reduced in trophoblast treated with Eso, but not Baf. Expression of the sFlt-1 isoforms (C) sFlt-1-i13 and (D) sFlt-1-e15a were significantly reduced in trophoblast treated with Eso, but not Baf. (E) HUVEC secretion of sFlt1 was significantly reduced with Eso treatment, but not Baf. Data are mean % change from control ± SEM (*p<0.05; ***p<0.001; ****p<0.0001).
Supplementary Figure S4. Proton Pump Inhibitors alter cargo protein expression. Esomeprazole (Eso) (100μM) significantly upregulated ARF1 (A) mRNA and (B) protein expression in isolated trophoblast cells. There was no difference in RAB11 mRNA (C) or protein expression (D). Data are mean % change from control ± SEM (*p<0.05).
Supplementary Figure S5. Various physiological measurements in the mother, placental and fetus taken from pregnant mice where sFlt-1 was overexpressed in the placenta. (A) Albumin:creatinine ratio measured from the mother’s urine (n=8 Ctrl; n=7 eso), (B) fetal weights (sFlt-1 n=50; sFlt-1+Eso n=66), (C) placental weights (sFlt-1 n=50; sFlt-1+Eso n=66), (D) fetal:placental ratio (sFlt-1 n=50; sFlt-1+Eso n=66) and (E) fetal crown-rump length (sFlt-1 n=50; sFlt-1+Eso n=66). (F) blood pressure; non-pregnant mice. (G) Maternal sFlt-1 levels measured in mouse plasma (n=8 Ctrl; n=9 eso. Ctrl – control, eso – esomeprazole treated animals.
Supplementary Figure S6. Effects of PPIs on phosphorylated eNOS and endothelin-1 expression. (A) Western blot densitometric analysis of phosphorylated eNOS:total eNOS (p-eNOS:eNOS) ratio in HUVECs pre-treated with TNF-α (10ng/ml) followed by co-treatment with esomeprazole (eso), or (B) rabeprazole (Rab). (C) Endothelin-1 (ET-1) mRNA expression in uterine microvascular endothelial cells with TNF-α (10ng/ml) treatment and PPIs (lansoprazole (Lans), Rab, esomeprazole (Eso), at 5-100 μM concentrations). Comparisons were made against TNF-α controls. Data are mean ± SEM (*p<0.05; ****p<0.0001). (n≥3)
**Supplementary Figure S7.** PPIs decrease VCAM-1 expression in endothelial cells. VCAM-1 mRNA expression in (A) HUVECs and (B) uterine microvascular endothelial cells treated with TNF-α (10 ng/ml) and PPIs [lansoprazole (Lans), rabeprazole (Rab), esomeprazole (Eso)]. (C) VCAM-1 mRNA expression in HUVECs treated with the serum from patients with preeclampsia and PPIs at 100 µM. PPIs were administered at 5-100 µM, or otherwise indicated. Data are expressed as the mean % change from control (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001) (n≥3).
Supplementary Figure S8. PPIs up-regulate anti-oxidant genes. HO-1 mRNA expression in: (A) primary trophoblast cells treated with PPIs [lansoprazole (Lans), rabeprazole (Rab), esomeprazole (Eso)] (B) HUVECs treated with Lans, Rab, Eso, omeprazole (ome) and pantoprazole (pant) (C) Uterine microvascular endothelial cells treated with Lans, Rab and Eso. (D) mRNA expression of NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1) in HUVECs treated with Lans and Eso. (E) mRNA expression of Thioredoxin (TXN) in primary trophoblast cells treated with Lans and Eso. PPIs were administered at 5-100 μM for 24 h, or otherwise indicated. Data is expressed as mean % change from control ± SEM (*p<0.05; **p<0.01; *** p<0.001; **** p<0.0001). (n=3)
**Supplementary Figure S9.** Proton pump inhibitors decrease cytokine secretion in placental and endothelial tissues. (A) Cytokine concentrations in the media of preeclamptic placental explants treated with esomeprazole (Eso). Cytokine concentrations in the media of HUVECs treated with either (B) preeclamptic patient serum (5% of media) or (C) TNF-α (10ng/ml) to induce endothelial dysfunction ±. Comparisons were made for each individual cytokine between the TNF-α alone, versus TNF-α and Eso treatment groups. Eso administered at 100 μM. Data is mean fold change from control ± SEM. IL-interleukin; CCL/CX3CL-chemokines. ** p<0.01; *** p<0.001; **** p<0.0001. (n ≥ 3)