RESEARCH ARTICLE

Bioflavonoid luteolin prevents sFlt-1 release via HIF-1α inhibition in cultured human placenta

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Abstract

Preeclampsia (PE) is a serious hypertensive complication of pregnancy and is a leading cause of maternal death and major contributor to maternal and perinatal morbidity, including establishment of long-term complications. The continued prevalence of PE stresses the need for identification of novel treatments which can target prohypertensive factors implicated in the disease pathophysiology, such as soluble fms-like tyrosine kinase 1 (sFlt-1). We set out to identify novel compounds to reduce placental sFlt-1 and determine whether this occurs via hypoxia-inducible factor (HIF)-1α inhibition. We utilized a commercially available library of natural compounds to assess their ability to reduce sFlt-1 release from primary human placental cytotrophoblast cells (CTBs). Human placental explants from normotensive (NT) and preeclamptic (PE) pregnancies were treated with varying concentrations of luteolin. Protein and mRNA expression of sFlt-1 and upstream mediators were evaluated using ELISA, western blot, and real-time PCR. Of the natural compounds examined, luteolin showed the most potent inhibition of sFlt-1 release, with >95% reduction compared to vehicle-treated. Luteolin significantly inhibited sFlt-1 in cultured placental explants compared to vehicle-treated in a dose- and time-dependent manner. Additionally, significant decreases in HIF-1α expression were observed in luteolin-treated explants, suggesting a mechanism for sFlt-1 downregulation. The ability of luteolin to inhibit HIF-1α may be mediated through the Akt pathway, as inhibitors to Akt and its upstream regulator phosphatidylinositol-3 kinase (PI3K) resulted in significant HIF-1α reduction. Luteolin reduces anti-angiogenic sFlt-1 through inhibition of HIF-1α, making it a novel candidate for the treatment of PE.

KEYWORDS
flavones, hypoxia, placenta, preeclampsia, sFlt-1

Abbreviations: ANOVA, analysis of variance; CTB, cytotrophoblast; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HIF-1α, hypoxia-inducible factor-1α; Hsp, heat shock protein; mTOR, mammalian target of rapamycin; NT, normotensive; p70S6K, p70S6 kinase; PE, preeclampsia; phospho, phosphorylated; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase 1; SEM, standard error of the mean; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

1 | INTRODUCTION

Preeclampsia (PE) is a common pregnancy disorder occurring after 20 weeks of gestation characterized by new-onset hypertension, proteinuria, and end-organ damage linked to endothelial and vascular dysfunction.[1] Worldwide, PE affects ~7 million women each year, resulting in over 500 000 fetal deaths and 70 000 maternal deaths, with a higher mortality rate in the United States compared to other developed countries.[2–6] Major risk factors associated with PE occurrence include prior history of PE, chronic hypertension, obesity, diabetes mellitus, multiple gestations, and antiphospholipid syndrome.[7,8] Moreover, PE is associated with substantial long-term risk for cardiovascular disease, cerebrovascular disease,[9,10] and renal dysfunction[11] in mothers and increased risk for cardiovascular disease and metabolic syndrome in their children.[12,13] Currently, there are no pharmacological treatments, and the only effective treatment strategy for PE requires delivery of the baby and removal of the placenta, which can often occurs prematurely.[1]

The current paucity of effective treatments for PE likely is due to the complex pathophysiology of the disease. Disease pathogenesis is believed to progress in two stages beginning with abnormal formation of the placental vasculature in the first trimester resulting in placental ischemia and hypoxia. Abnormal placental development drives the systemic vascular dysfunction through release of anti-angiogenic factors, such as soluble fms-like tyrosine kinase 1 (sFlt-1), ultimately leading to the clinical manifestations of PE during the second stage in the late second and third trimesters.[7,14,15]

sFlt-1 is the soluble receptor for vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), which are important for the maintenance of vascular health. Excess sFlt-1 exerts its pathological actions by quenching bioavailable levels of VEGF and PlGF, causing endothelial and vascular dysfunction culminating in systemic vasoconstriction and hypertension. sFlt-1 is produced and secreted by the placenta in normal pregnancy, with inappropriate upregulation in PE such that circulating levels are elevated leading up to PE onset.[16–19] Its direct ability to promote hypertension in pregnancy has been demonstrated by overexpression of sFlt-1 producing PE-like symptoms in animal models.[17,20–22] Similarly, hypoxia-inducible factor 1α (HIF-1α) is highly expressed in preeclamptic placentas,[23] and HIF-1α overexpression in pregnant mice is associated with hypertension and increased sFlt-1 expression.[24–26] Studies have shown that sFlt-1 antagonism or depletion in cell culture[27] as well in vivo animal models of PE led to improved clinical symptoms.[28–30] Removal of sFlt-1 using apheresis recently demonstrated promise in reducing maternal blood pressure as well as in prolonging pregnancies in women with preterm PE,[31,32] suggesting that sFlt-1 reduction may provide relief from PE symptoms.

Currently, no approved treatments can safely prolong pregnancies or reduce dysregulated sFlt-1 levels in women with PE, making the delivery the sole treatment option, contributing to adverse neonatal outcomes. Thus, identifying a safe therapeutic for PE that can target anti-angiogenic factors implicated in the pathogenesis of PE is a major unmet need for women worldwide. Bioflavonoids are present in many plants, including their fruits and vegetables, and have been well established for their antioxidant and anti-inflammatory effects.[33–35] Regular consumption of bioflavonoids or their sources is associated with a reduced risk of chronic cardiovascular and neurodegenerative diseases.[36] While flavonoids, such as quercetin and puerarin, have also been studied for their potential to reduce blood pressure in PE animal models, it is unclear whether any of these compounds are inhibitors of sFlt-1.[37–39] Therefore, the aim of this study was to identify if bioflavonoids are inhibitors of sFlt-1 and determine its mode of action, such as inhibition of the HIF-1α pathway.

2 | MATERIALS AND METHODS

2.1 | Protocol for obtaining human placentas

Placental tissue was collected from normotensive (NT) or preeclamptic patients delivered at the University of Chicago Medical Center. PE was diagnosed according to the American College of Obstetricians and Gynecologists (ACOG) guidelines.[1] Patients with a history of diabetes, chronic hypertension, renal disease, or multiple gestations were excluded from this study. The Institutional Review Board approved using all study-related materials at the University of Chicago (Institutional Review Board No. #14-1532).

2.2 | Primary cytotrophoblasts isolation and culture for initial screening of natural compounds to inhibit sFlt-1

Primary cytotrophoblast cells (CTBs) were isolated from placentas collected from NT and PE patients and cryopreserved for subsequent culture experiments as described.[40] CTBs were cultured in medium 199 (Corning; Cat# 10-060-CV; Manassas, VA) with 5% fetal bovine serum (FBS) (Corning; Cat# 35-015-CV; Manassas, VA) and 1% penicillin–streptomycin (Corning; Cat#

30-002-CI; Manassas, VA). For screening experiments, cells were thawed and plated in a 96-well flat-bottom plate (Microtest 96; Becton Dickinson; Franklin Lakes, NJ). Prior to screening experiments, the medium was removed and replaced with fresh medium with either a drug library (Enzo Life Sciences; Cat# BML-2865; Farmington, NY) compound or vehicle dimethyl sulfoxide (DMSO) (Sigma-Aldrich; Cat# D8418; St. Louis, MO) and incubated in standard culture conditions, with 5% CO_2–95% room air (21% O_2) at 37°C, for 72 h, as previously described.[41] The compounds of the library were provided at a stock concentration of 2 mg/mL, which was diluted 1:100 in culture medium for a concentration of 20 μg/mL. Additional information about the product library can be seen in Table S1. At the end of the experiment, cell culture supernatant was collected for analysis.

2.3 | Placental villous explant cultures

Placental villous explant tissues were cultured in a complete medium, followed by RNA and protein extraction as described previously.[41,42] Villous biopsies (2 cm³) were excised from the maternal surface, midway between the chorionic and basal plates, within 30 min of delivery, and decidual layers were carefully removed. Tissue was dissected into 0.5 cm³ explants and thoroughly rinsed with phosphate-buffered saline (PBS) to ensure removal of maternal blood and placed in a 24-well flat-bottom plate (Falcon multi-well tissue culture plate; Becton Dickinson) containing 1 mL of conditioned medium 199 for 72 h under standard tissue culture conditions (room air with 5% CO_2) or hypoxia (5% CO_2, 2% O_2, 93% N_2) in a humidified cell culture incubator with varying concentrations of luteolin (1, 5,10 μM) (Sigma-Aldrich; Cat# L9283; St. Louis, MO), Akt IV inhibitor (Santa Cruz Biotechnology; Cat# sc-203 809; Dallas, TX), LY294002 (Sigma-Aldrich; Cat# 440202; Temecula, CA), or DMSO control. Concentrations of luteolin for these experiments were based on published in vitro experiments.[43,44] After 72 h, the explants were removed, blotted with sterile cotton gauze, and flash-frozen along with corresponding conditioned media for storage at −80°C. Experiments were duplicated on explants from each NT and PE patient.

2.4 | Enzyme-linked immunosorbent assay (ELISA) for sFlt-1

Collected media were applied to a human VEGF receptor 1 (VEGFR1) Quantikine ELISA Kit (R&D Systems; DVR 100B; Minneapolis, MN, USA) to quantify sFlt-1 secreted by primary trophoblast cells in culture. The manufacturer's specifications approve of using culture media on this assay, and the instructions were followed. The sensitivity of this ELISA to detect sFlt-1 was reported to be 5 pg/mL, with an intra-assay coefficient of variation of 2.6%–3.8% and an inter-assay coefficient of variation of 7.0%–8.1%.

2.5 | Immunoblotting

Tissue from placental explants (described above) were homogenized, and total protein was collected.[45,46] Briefly, protein quantification was assessed using Pierce BCA Protein Assay Kit (ThermoFisher; Cat# 23225; Scientific, Waltham, MA), and equal quantities (50 μg total protein) were resolved on a 4%–20% SDS gel and transferred to a nitrocellulose membrane. Western blots of sFlt-1 in culture medium were performed using equal amounts of heparin agarose-enriched medium as previously described[41] and similarly transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk in tris-buffered saline-tween (TBS-T) (0.05% tween) for 1 h and incubated with the primary antibodies against phosphorylated (ser473) Akt (Cell Signaling; Cat# 4051S; Danvers, MA), total Akt (Cell Signaling; Cat# 4685; Danvers, MA), HIF-1α (BD Biosciences; Cat# 610958; Sparks, MD), VEGFR1 (sFlt-1) (Abcam; ab32152; Waltham, MA), and β-Actin (BD Biosciences; Cat# 612656; Sparks, MD) at 1:1000 in 1% milk in TBS-T overnight at 4°C. After washing the membrane with TBS-T, goat anti-mouse (ThermoFisher Scientific; Cat# 31430; Waltham, MA) or goat anti-rabbit (ThermoFisher Scientific; Cat# 31460; Waltham, MA) secondary antibodies (1:5000) in 1% milk in TBS-T were added for 1 h at room temperature. Proteins were detected using enhanced chemiluminescent reagents and quantified using ImageJ (NIH) to collect densitometry data.

2.6 | Quantitative real-time PCR

RNA was extracted from placental explants ($n = 5$) using TRIzol (Invitrogen, Carlsbad, CA) and transcribed into cDNA using a high-capacity cDNA kit (Applied Biosystems, Carlsbad, CA). Reverse transcription–PCR was performed as previously described[47] and using a custom primer (Applied Biosystems; Carlsbad, CA) for sFlt-1 (forward primer: TCAGAGGTGAGCACTGCAACA; reverse primer: CATTCCTTGTGCTTTTAAATTTGGA) and a commercially available primer for HIF-1α (Catalog# Hs00936371; Life Technologies, Carlsbad, CA). Relative mRNA gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method as described.[48]

2.7 | Statistical analysis

Data from control DMSO (NT or normoxic) samples were averaged and used for normalization. Data are presented as means ± standard error of the mean (SEM), and analyses were completed using Prism (GraphPad; San Diego, CA). Statistically significant differences between means were assessed by t-test and two-way analysis of variance (ANOVA). The Tukey post hoc test was applied for ANOVA models. Differences were considered statistically significant at $p < .05$.

3 | RESULTS

3.1 | Natural library screen for sFlt-1 inhibitors

A library of 502 natural compounds, including 11 bioflavonoids, was screened using primary placental CTB cells treated with the compounds (20 μg/mL) or vehicle (DMSO) for 72 h. Several compounds reduced sFlt-1 protein expression released into the medium (Figure 1). Of these, luteolin was the most effective at reducing sFlt-1 expression at 97%, followed by other flavonoids apigenin (89%), naringenin (82%), and hesperetine (79%), relative to the vehicle control. In previous studies, luteolin has been shown to provide protective effects using in vitro and in vivo models of coronary artery disease, atherosclerosis, and heart
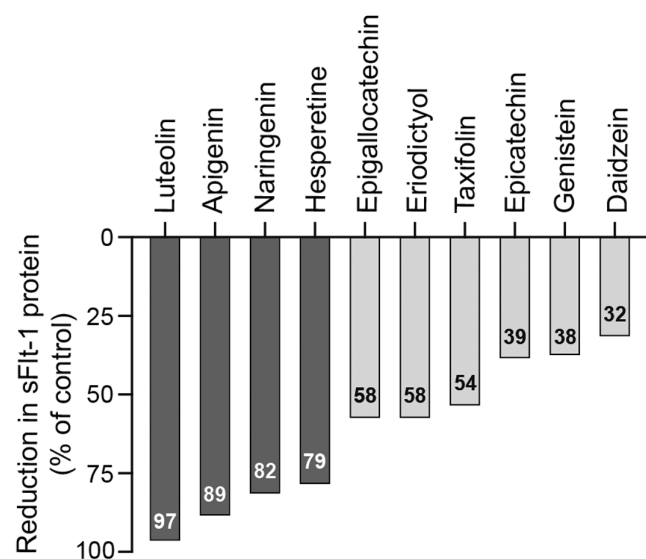
failure[49]; thus, we focused on luteolin to further investigate its potential in mediating the pathophysiology of PE.

3.2 | Dose- and time-dependent effects of luteolin on sFlt-1 expression in NT and PE placenta

Placental explants collected from NT and PE patients at delivery were treated with 0–10 μM luteolin for 72 h to determine the dose–response of luteolin in reducing sFlt-1. In both NT and PE samples, luteolin significantly decreased sFlt-1 protein expression at 5 and 10 μM by Western blot (Figure 2A,B). Similarly, a time-course study of placental explants treated with 5 μM luteolin for 0–72 h demonstrated that placental sFlt-1 increases over time in control samples, and this increase is diminished with luteolin treatment after 72 h ($p < .001$; Figure 2C). Furthermore, sFlt-1 secretion in conditioned medium from both NT and PE placental explants was significantly reduced ($p < .0001$ and $p < .01$, respectively) following luteolin treatment (5 μM, 72 h) compared with control explants as detected by western blot (Figure 2D,E).

3.3 | Effect of luteolin on sFlt-1 and HIF-1α protein and mRNA expression

Previous work has suggested that sFlt-1 expression is regulated by HIF-1α[23,50]; thus, we evaluated whether the reduction in sFlt-1 expression by luteolin was associated with a decrease in HIF-1α. Placental explants from NT and PE patients were treated with 5 μM luteolin for 72 h. sFlt-1 and HIF-1α protein expression were significantly higher in placental explants from PE patients as compared to tissue from NT patients ($p < .05$; Figure 3A), which is consistent with previous work.[17,51] PE placental tissue treated with luteolin had significantly decreased expression in HIF-1α and sFlt-1 ($p < .01$) relative to control. NT placental tissue treated with luteolin had significantly reduced HIF-1α expression ($p < .05$). Luteolin treatment significantly reduced sFlt-1 mRNA ($p < .05$); however, no significant differences were observed in HIF-1α mRNA ($p = .15$; Figure 3B). These results suggest that luteolin reduces HIF-1α protein expression but not transcription, thereby decreasing sFlt-1 transcription and protein expression.

FIGURE 1 Natural product library to reduce sFlt-1 release from primary placental CTBs. CTBs were isolated from normal placentas and plated in 96-well plates. CTBs were treated with each bioflavonoid at 20 μg/mL (~10–20 μM) or DMSO vehicle for 72 h in normoxia. sFlt-1 protein was quantified in the conditioned medium using ELISA. Data are the percentage of the sFlt-1 reduction compared to the control (vehicle-treated) conditioned medium.

3.4 | Examining the impact of luteolin on upstream mediators of HIF-1α expression

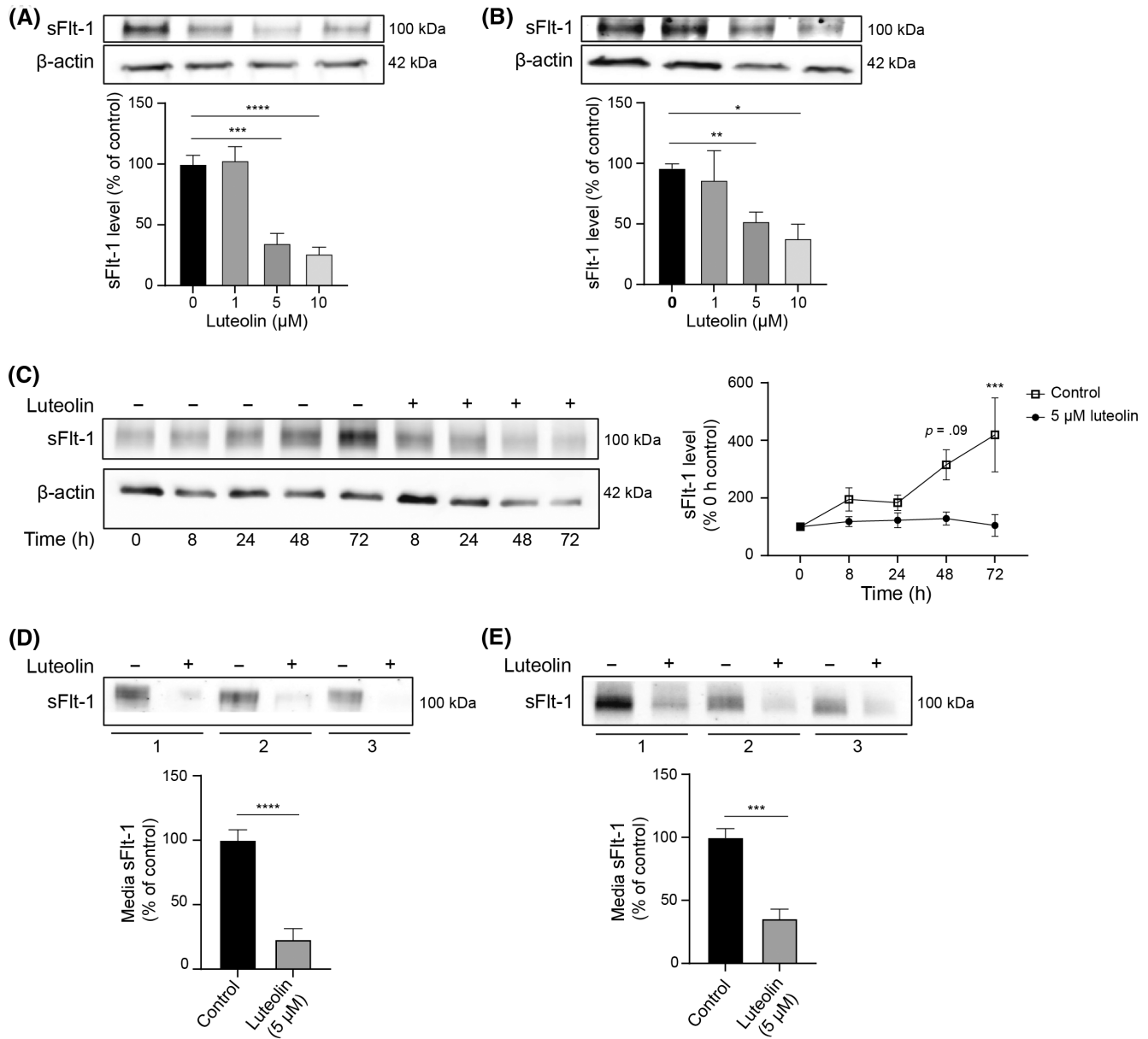sFlt-1 regulation may occur through upstream regulation by mTOR and downstream effector, p70S6K, which is

FIGURE 2 Luteolin inhibits sFlt-1 production in placental explants. Representative western blots of placental explants from normotensive (A) and preeclamptic (B) patients treated with luteolin for 72 h show a dose-dependent decrease in sFlt-1, demonstrating significance with 5-10 µM treatment compared to DMSO control. Over time, explants from normotensive patients have significantly increased sFlt-1 production when treated with DMSO control, but levels of sFlt-1 remain unchanged over time when treated with 5 µM luteolin (C). Similarly, sFlt-1 released into the media from normotensive (D) and preeclamptic (E) explants are significantly decreased with 5 µM luteolin treatment compared to DMSO control. $N = 6$; *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$.

mediated upstream by Akt signaling and epidermal growth factor receptor (EGFR).[52] Luteolin treatment (5 µM, 72 h) had a nonsignificant trend to decrease phospho-Akt (Ser473) expression in both NT and PE explants ($p = .07$ and $p = .053$, respectively; Figure 4). To further evaluate this pathway, we assessed HIF-1α expression after treatment with Akt inhibitors and luteolin. We utilized placental explants from NT patients in normoxia (21% O_2) and hypoxia (2% O_2) to mimic the conditions of PE, and increased HIF-1α expression in hypoxia was confirmed

in all experiments. Decreased HIF-1α protein expression was observed with treatment using a direct Akt inhibitor (5 µM) compared with control ($p < .05$; Figure 5A). The Akt inhibitor combined with luteolin demonstrated a reduction in HIF-1α similar to 10 µM luteolin, suggesting that luteolin is potentially capable of inhibiting this pathway through additional mechanisms. Similarly, treatment with an inhibitor of upstream phosphoinositide 3 kinase (PI3K) (LY294002, 10 and 50 µM) resulted in significantly decreased HIF-1α expression compared with control
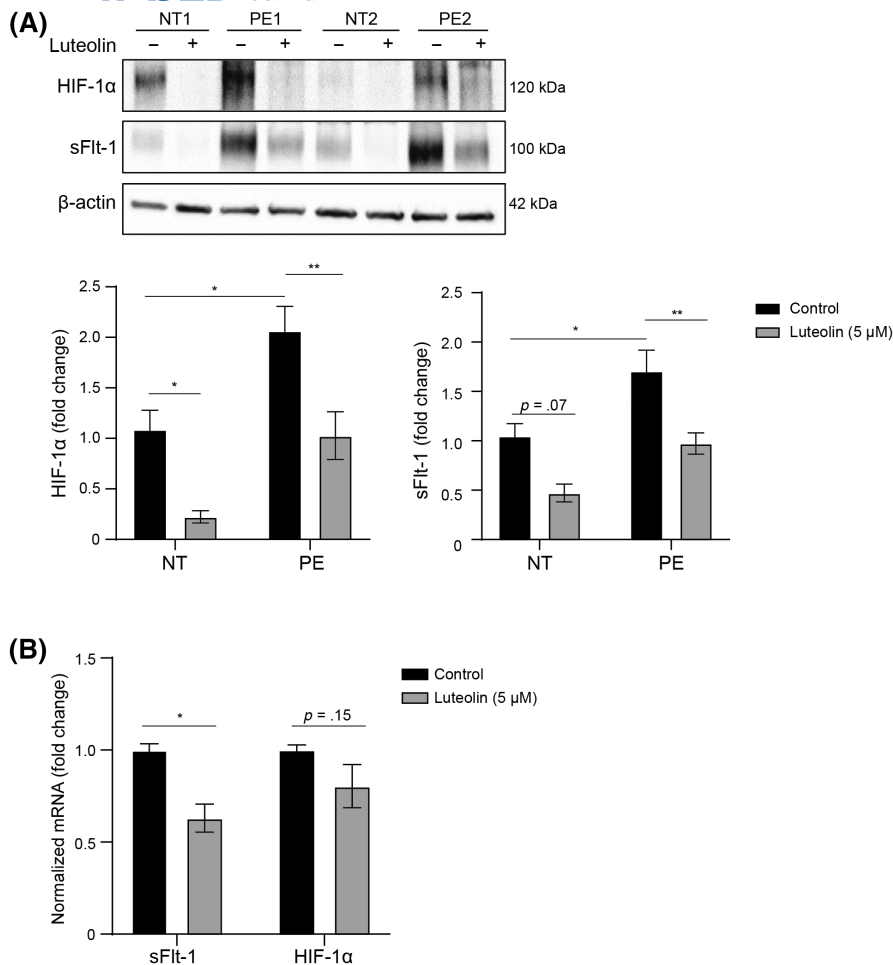
FIGURE 3   Luteolin decreases placental HIF-1α and sFlt-1 expression. Representative western blots of placental explants from normotensive (NT) or preeclamptic (PE) patients have significantly reduced levels of HIF-1α expression when treated with 5 µM luteolin compared to DMSO control. This decrease in HIF-1α is associated with a near-significant decrease in sFlt-1 in normotensive explants and a significant sFlt-1 decrease in preeclamptic explants (A). RT-PCR of mRNA from NT explants treated with luteolin demonstrates a significant reduction in sFlt-1 expression and a nonsignificant decrease in HIF-1α (B). $N = 5$; *$p < .05$, **$p < .01$.

under hypoxic conditions ($p < .01$ and $p < .001$, respectively; Figure 5B). No changes were observed in normoxic samples.

4 | DISCUSSION

In this study exploring novel compounds to target sFlt-1 production in PE, we report several key findings: (1) we identified that luteolin, a naturally occurring bioflavonoid, can reduce sFlt-1 protein levels, (2) luteolin also regulates HIF-1α expression, and (3) luteolin decreases HIF-1α through the Akt pathway. Given the evidence that sFlt-1 plays a key role in PE pathogenesis and that inhibition of sFlt-1 has been shown to improve PE symptoms and signs in human pregnancies, our data suggest that luteolin can potentially be used as a therapy for PE.

PE is a multisystem disease, and in this study, we aimed to see how luteolin would impact the factors leading to endothelial and vascular dysfunction in PE. Increased activation of HIF-1α leading to inappropriate upregulation of sFlt-1 is a common pathway observed in PE.[23,50] Increased sFlt-1 antagonizes VEGF and PlGF, creating angiogenic imbalance, causing maternal hypertension and end-organ damage.[17] Reduction of sFlt-1 from maternal circulation by plasmapheresis resulted in decreased blood pressure and proteinuria and extended human pregnancy before delivery.[31] These findings highlight the importance of targeting the sFlt-1 pathway. However, apheresis is an expensive and invasive procedure that requires extensive expertise and increases the risk for complications, such as uncontrolled bleeding and infection, thus limiting its utility in most clinical settings. Although other strategies, such as siRNA, are being developed as a therapy for PE,[53] natural compounds offer an advantage in their inexpensive and stable capsule formulations. Upon screening over 500 natural compounds to reduce sFlt-1 expression, we identified luteolin as having the most robust inhibition, prompting our study.

Luteolin is a natural bioflavonoid found in many plants, fruits, vegetables, teas, and herbs and is thus consumed regularly.[54] It has been shown to protect against reactive oxygen species, restoring normal nitric oxide production and mitochondrial function,[55] as well as nuclear factor-κB-mediated inflammation.[56] Several flavonoids have been studied in pathological settings, including cardiovascular disease and hypertension,[44,57,58] infections,[54,59] and mitigating the negative effects of chemotherapies.[60,61]
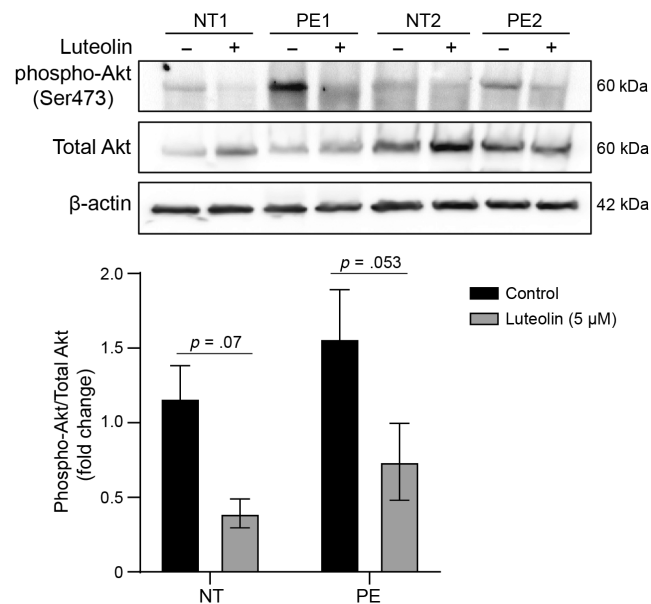
FIGURE 4 The effect of luteolin on phospho-Akt and total Akt expression. Representative western blots of placental explants from normotensive and preeclamptic patients treated with luteolin show a near-significant decrease in the ratio of Phospho-Akt to total Akt, suggesting that this pathway may be relevant in the inhibition of HIF-1α and sFlt-1. $N = 5$.

Given the broad spectrum of beneficial effects that flavonoids have been found to exert, they have also been recently studied for the potential treatment of PE.[37–39]

In placentas from both NT and PE pregnancies, we evaluated the ability of luteolin to prevent sFlt-1 expression under various conditions. Luteolin significantly attenuated sFlt-1 production at 5 μM and 10 μM, which is consistent with previously reported in vitro doses of luteolin.[43,44] Although the half-life of luteolin is approximately 5 h in vivo,[49] we observed that luteolin inhibited sFlt-1 production over time and significantly inhibited sFlt-1 after 72 h, suggesting that the mechanisms of luteolin degradation are not present in human placental tissue in vitro. Because sFlt-1 production has been linked to HIF-1α expression,[50] we also assessed whether this was a pathway through which luteolin acted. Placental tissue from NT and PE patients had significantly decreased HIF-1α expression with luteolin. Several pathways upregulate HIF-1α, including Akt,[62] and our study shows decreased phospho-Akt in placental samples treated with luteolin, suggesting that luteolin inhibits this pathway.

Utilizing NT placentas, we examined the effect of Akt pathway inhibitors compared to luteolin treatment in
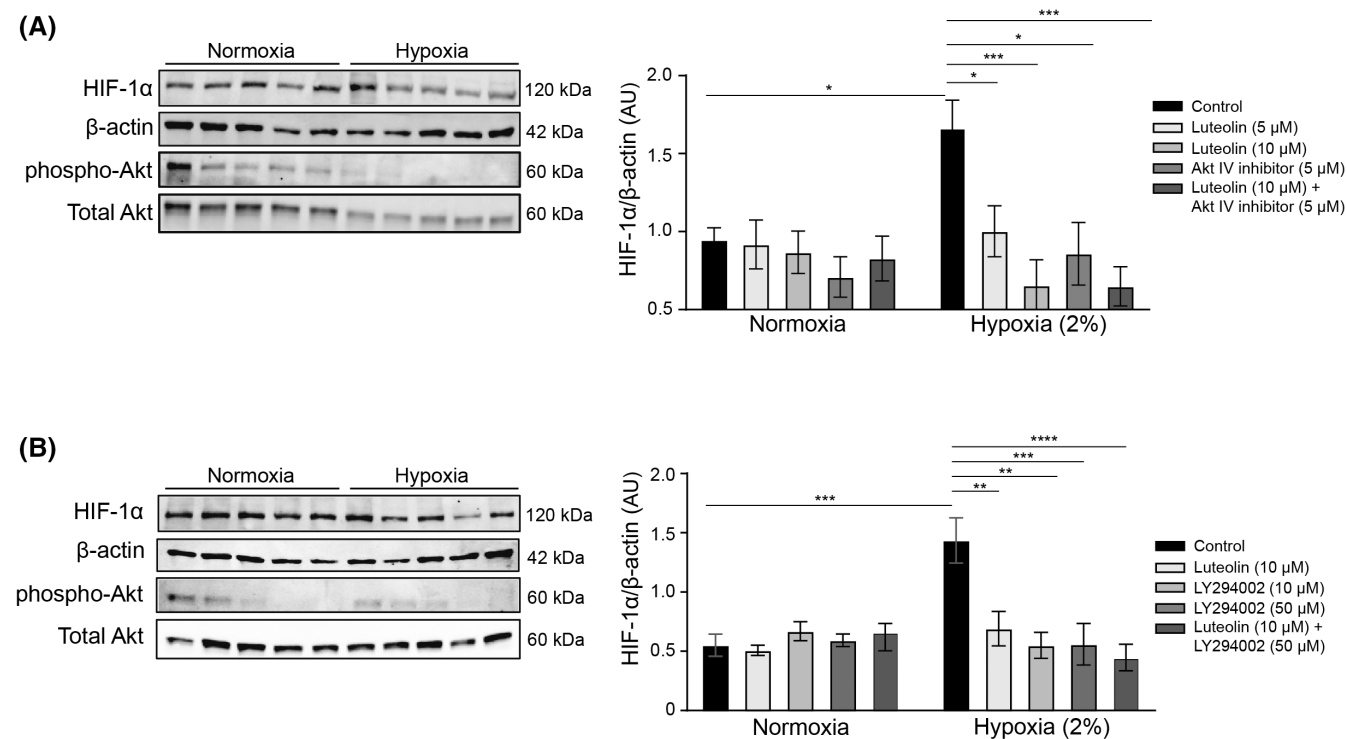

FIGURE 5 Luteolin decreases HIF-1α through Akt pathway. Representative western blots of explants from normotensive patients were treated in normoxic and hypoxic conditions with luteolin, Akt Inhibitor IV, or a combination of the two (A). We also compared luteolin treatment with PI3K inhibitor LY294002 or a combination of the two (B). We confirmed that phospho-Akt was decreased as a result of the treatment, and HIF-1α expression was measured and normalized to β-Actin (samples in blots are in the same order as listed in the graph). As expected, there was a significant increase in HIF-1α in hypoxia. However, treatment with luteolin, Akt inhibitor, and both concentrations of LY294002 resulted in a significant decrease of HIF-1α expression in hypoxia. Of note, samples in blots are in the same order as listed in the figures, and separate blots were used for the measurement of phospho-Akt and total Akt. $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$; $N = 5$.

normoxia and hypoxia, allowing us to examine samples from the same tissue under conditions mimicking NT and PE, respectively. Treatment with either luteolin or Akt IV inhibitor significantly decreased HIF-1α expression. Luteolin's ability to reduce HIF-1α expression more than the Akt IV inhibitor under hypoxia suggests that luteolin potentially inhibits HIF-1α through additional pathways. Similar reductions in HIF-1α were observed when treated with the upstream PI3K inhibitor, LY294002. In addition to the PI3K/Akt pathway, luteolin may inhibit HIF-1α expression through alternate mechanisms. Our group previously showed that ouabain inhibits HIF-1α expression by preventing its stabilization by heat shock proteins.[41] Along with pathways promoting the degradation of HIF-1α, these are potential mechanisms whereby luteolin might regulate the expression of HIF-1α, which will be explored further in future experiments.

Our current studies are limited to luteolin treatment in vitro to determine the effects on the anti-angiogenic protein sFlt-1 and its regulators. Luteolin has shown promise in reducing reactive oxygen species and inflammation. Still, its ability to mitigate these factors in PE has not been explored and will be a focus of future experiments. Although we have previously shown that luteolin causes vasodilation of uterine arteries in pregnant rats,[63] in vivo animal studies are ongoing to evaluate the effect of luteolin on sFlt-1 production, uteroplacental perfusion, blood pressure, and safety in pregnancy. In summary, these experiments provide compelling evidence that luteolin is a potent inhibitor of sFlt-1 production and secretion in the human placenta.

5 | CONCLUSION

Bioflavonoids, such as luteolin, are readily available and inexpensive compounds and represent an exciting potential therapeutic for PE. Here, we observed significant decreases in sFlt-1, HIF-1α, and phospho-Akt with luteolin treatment in human placental explants, suggesting promise for its use. However, future studies are necessary to determine the ability of luteolin to target these pathways and reduce PE symptoms in preclinical animal models.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.