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Aspirin inhibits expression of sFLT1 from human cytotrophoblasts induced by hypoxia, via cyclo-oxygenase 1

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Abstract

Introduction—Elevated circulating soluble FLT1 (sFLT1) levels in preeclampsia may play a role in its development. Aspirin is recommended for prevention of preeclampsia. We hypothesized that aspirin may inhibit the production of sFlt1.

Methods—Placentas from women with and without preeclampsia were collected. Primary cytotrophoblasts (CTBs) were cultured from normal placentas and treated with aspirin, sc-560, a COX1 inhibitor or celecoxib, a COX-2 inhibitor. The expression of *sFLT1*, *FLT1*, *COX1*, *COX2* was studied. The effect of aspirin on sFlt1 expression was also studied in HEK293 cells and in HTR-8/SVNeo cells.

Results—The expression of *sFLT1* was increased in preeclamptic placentas compared to control placentas and the expression and release of sFLT1 increased in CTBs exposed to 2% O₂ compared to controls. Aspirin at 3 and 12 mM concentration reduced the expression and release of sFLT1 in CTBs. Aspirin also inhibited sFlt1 expression from HTR-8/SVNeo and HEK293 cells. Sc-560, but not celecoxib, reduced sFLT1 expression and release from CTBs. Aspirin and sc-560 also reduced hypoxia-induced *FLT1* mRNA expression and inhibited *COX1* mRNA in CTBs.

Discussion—This study confirms that *sFLT1* expression is increased in preeclamptic placentas and in CTBs exposed to hypoxia. Aspirin inhibits the production *sFLT1* in CTBs and in HTR-8/SVNeo. Sc-560 recapitulated the effects of aspirin on sFLT1 expression and release in CTBs suggesting that the aspirin effect may be mediated via inhibition of COX1. The study increases

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our understanding of the mechanisms regulating sFlt1 expression and provides a plausible explanation for the effect of aspirin to prevent preeclampsia.

Keywords

gene expression; angiogenesis; placenta; preeclampsia; cytotoxicity

Introduction

Preeclampsia affects 2–8% of pregnancies with substantial maternal morbidity and mortality (1). Although dysfunction of the placenta is widely accepted as leading to disease, the pathogenesis of preeclampsia is poorly understood. The clinical manifestations of preeclampsia reflect widespread endothelial dysfunction, with vasoconstriction leading to hypertension and disruption of the glomerular capillary barrier leading to heavy proteinuria (2).

Circulating anti-angiogenic factors may play a key role in the pathogenesis of preeclampsia. The vascular endothelial growth factor (VEGF)¹ receptor fms-like tyrosine kinase 1 (FLT1) is a transmembrane protein that binds with high affinity to VEGF and placental growth factor (PlGF) and is required for normal angiogenesis. Several N-terminal variants arise from alternate transcripts of FLT1 that share a common VEGF binding site but lack a transmembrane domain and are hence secreted as soluble FLT1 (sFLT1) (3). These include the classic form of sFLT1, sFLT1-i13, as well as a recently evolved novel form of sFLT1, called sFlt1-e15a or sFlt-14 (4, 5). These forms of sFLT1 are naturally occurring VEGF/PlGF antagonists. We, and others, have recently identified a novel sFLT1 which is post-translationally cleaved from FLT1 (6, 7). This cleaved form of sFLT1 is also released into the extracellular milieu and can function as a VEGF antagonist.

sFLT1 is expressed by endothelial cells and trophoblasts and plays a major role in regulating placental angiogenesis (2, 8). sFLT1 levels increase during pregnancy but those who go on to develop preeclampsia begin this increase earlier in gestation and reach higher levels (9–11). Free PlGF and VEGF levels fall concurrently with the rise in sFLT1, which may be related to binding to sFLT1 (12–14). Administration of sFLT1 to pregnant rats leads to a preeclampsia-like phenotype with massive proteinuria and hypertension (9, 15). The proteinuria and hypertension induced by sFLT1 excess can be reversed or improved by infusion of VEGF or by inducing PlGF expression (16, 17). In a pilot study, reducing sFLT1 by extracorporeal adsorption appeared to transiently reduce hypertension and proteinuria in a cohort of preeclamptic women (18). Collectively, these data indicate that preeclampsia may be caused by excessive placental production of sFLT1 and reducing sFLT1 may help ameliorate preeclampsia.

In the developing preeclamptic placenta, the normal process of trophoblast invasion and remodeling of the uterine spiral arteries is impaired (19). This results in reduced placental

¹Vascular endothelial growth factor, VEGF; placental growth factor, PlGF; fms-like tyrosine kinase 1, Flt1; soluble fms-like tyrosine kinase 1, sFlt1; cytotrophoblast, CTB; cyclooxygenase, COX; poly ADP-ribose polymerase, PARP; Dulbecco's modified Eagle's medium, DMEM; fetal bovine serum, FBS; serum free media, SFM; polyvinylidene fluoride, PVDF;

perfusion, increased oxidative stress and inflammation. Apoptosis is an essential feature of normal placental development but is exaggerated with placental disease (2). Previous studies have demonstrated that hypoxia increases the apoptosis of human CTBs and induces an excess of sFLT1 (20, 21). Induction of uterine ischemia increases systemic sFLT1 release in rodents and non-human primates which in turn causes hypertension and proteinuria (22, 23).

A number of studies have been conducted to identify therapies for preeclampsia (24, 25). Aspirin has been the subject of many studies and meta-analyses have demonstrated a modest but significant reduction in the risk of pre-eclampsia with the use of low dose aspirin (26, 27). Aspirin inhibits cyclooxygenase (COX) activity in an irreversible manner which, in turn, inhibits prostaglandin and thromboxane synthesis (28). There are two isozymes of COX encoded by distinct gene products: a constitutive COX1 and an inducible COX2. COX2 is not usually expressed under normal conditions in most cells, but is induced during inflammation (29). Aspirin affects COX1 more than COX2 (28).

The effect of aspirin on the expression of sFLT1 and FLT1 in CTBs has not been previously examined. In this study, we examined the hypoxia-induced expression of sFLT1 in primary cultured human CTBs. We then tested the effect of aspirin and COX1 and COX2 inhibitors on the production of sFLT1 from CTBs.

Methods

Materials

Aspirin, celecoxib, trypsin, DNase and Percoll were purchased from Sigma-Aldrich (St. Louis, MO). Dispase was purchased from B&D Bioscience (San Jose, CA). 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (sc-560) was purchased from EMD Millipore (Billerica, MA). The ELISA Kit for human sVEGFR1 and anti-Flt1 antibody (AF321) were obtained from R&D Systems (Minneapolis, MN). Cleaved poly ADP-ribose polymerase (PARP) antibody, anti-caspase-3 and HRP-conjugated goat anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA). Anti-actin antibody (sc-1616) and HRP-conjugated donkey anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). The LDH cytotoxicity kit was purchased from Thermo-Fisher Scientific (Rockford, IL). Ham's F-10, Waymouth's medium, high Glucose Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Life Technologies (Grand Island, NY). C-terminal GFP-tagged open reading frame (ORF) clone of full length human VEGFR2 was purchased from OriGene (Rockville, MD). Recombinant adenovirus expressing FLT, sFLT1-i13, sFLT1-e15a and control virus were generated by the vector Core Facility at the University of Iowa and has been previously described (4, 6).

Placenta collection and tissue preparation

All clinical samples were collected at University of Iowa Hospitals under a study protocol approved by the IRB of University of Iowa (Project # 201007740 and 200910784). Placentas were collected from patients with preeclampsia at different gestational ages and control placentas were collected from patients with spontaneous preterm or term delivery

and these were used to prepare placental lysates to compare sFLT1 expression. Preterm delivery was defined as a vaginal or cesarean delivery that occurred prior to 37^{0/7} weeks gestation and term delivery as any delivery that occurred after 37^{0/7} weeks. The diagnostic criteria for preeclampsia were based on the ACOG practice bulletin regarding preeclampsia published in 2002 and includes the development of hypertension and proteinuria after the 20th week of pregnancy in a woman whose blood pressure has previously been normal together with the excretion of 0.3 g or more of protein in a 24-hour urine specimen or a protein creatinine ratio >0.3 (30). Exclusion criteria were the presence of an intrauterine infection, autoimmune disease, pregestational renal disease and multiple gestation. Normal placentas at 37 – 40 weeks were used for primary CTB culture.

For preparation of placental lysates, chorionic and decidual surfaces were removed from the placenta, and tissue was sampled from placental lobules. Each tissue sample was rinsed thoroughly with sterile PBS solution three times. Whole cell lysates of trophoblast layer from placental tissue were prepared with NP40 cell lysis buffer (1% NP-40, 150mM NaCl and 50mM Tris, pH 7.2) using a tissue homogenizer.

Cell culture

Primary CTBs were isolated by the trypsin-deoxyribonuclease-dispase/Percoll method as described previously (4, 31, 32). We have previously reported that over 95% of the resulting cell population stains positive with Cytokeratin 7, a marker for CTBs (4). The cells were plated at a density of 3×10^5 per cm² in Ham's F10/Waymouth (HW, 1:1 vol/vol) media containing 10% FBS. After allowing cells to attach for 24 hr, the media was changed to serum-free HW media. For hypoxia experiments, the cells were transferred into hypoxia chambers (Billups-Rothenberg, Del Mar, CA), filled with 5% CO₂/8% O₂/87% N₂ or 5% CO₂/2% O₂/93% N₂ for a total of 48 hr. The day after transfer to hypoxia chambers, the culture medium was switched to serum free media (SFM) containing treatment reagents. 24 hr later, conditioned media was processed for ELISA and cells were used for total RNA extraction for qPCR or used to prepare protein lysates for western blotting.

COS-7 cells were maintained in DMEM containing 10% FBS and 1% penicillin-streptomycin. COS-7 cells were transduced with recombinant adenovirus expressing FLT1, sFLT1-i13, sFLT1-e15a or control virus (30×10^7 plaque forming units/100mm dish) in SFM. Further processing of conditioned media and preparation of whole cell lysates from monolayers for western blotting were performed as previously described.(6)

The HTR-8/SVNeo cell line was a gift from Charles Graham and was grown in DMEM/F12 (1:1) with 10% serum and then exposed to aspirin or vehicle for 24 hr and cell monolayer used for RNA extraction. HEK293 cells were transiently transfected with VEGFR2 or an empty plasmid, pCDNA3 using Lipofectamine 3000 from Life Technologies (Grand Island, NY) according to the manufacturer's protocol. The day after transfection, cell cultures were switched to serum and antibiotic-free media containing aspirin or vehicle for 24 hr and then conditioned media collected and used in an sFlt1 ELISA.

Western blotting and ELISA

Cultured cells were washed with cold PBS and lysed in 2× Laemmli buffer (3% sodium dodecyl sulfate, 12% glycerol, 50 mM Tris, pH 6.8 and 80 mM dithiothreitol) containing protease inhibitor cocktail from Roche Applied Science (Indianapolis, IN). Whole cell lysates of trophoblast layer from placental tissue were prepared with NP40 cell lysis buffer (1% NP-40, 150mM NaCl and 50mM Tris, PH 7.2) using a tissue homogenizer. In some cases conditioned media from cultured cells were concentrated using Amicon Ultra centrifugal filters from EMD Millipore (Billerica, MA). Equal amounts of cell lysate and concentrated media were subjected to SDS-PAGE and resolved proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (EMD Millipore). PVDF membranes were incubated sequentially with primary antibodies at 1:1000 overnight and HRP-conjugated secondary antibodies for an hour. Signals were detected with SuperSignal West Pico/Femto Chemiluminescent Substrate from Thermo Fisher Scientific (Rockford, IL), and the image was captured using VisionWorks™LS image acquisition and analysis software and the EC³ imaging system from UVP LLC (Upland, CA). Stripping of membranes for repeated blotting was done using 0.2 M NaOH. Quantitation of sFLT1 from conditioned media was performed by an sFLT1 ELISA kit (Quantikine human sVEGF R1, R&D Systems).

RNA extraction, cDNA preparation and quantitative PCR

Total RNA from primary CTBs and HTR-8/SV Neo was prepared with the RNeasy Mini Kit (Qiagen, Valencia, CA). Equal amounts of RNA were reverse transcribed to generate cDNA with AffinityScript quantitative qPCR cDNA synthesis kit (Agilent Technologies, La Jolla, CA) with the following conditions: 25°C for 5 min for oligo (dT) and random primer annealing, 42°C for 45 min for cDNA synthesis, and 95°C for 5 min for termination.

Real-time qPCR was performed to measure *sFLT1*(*sFlt1-i13*), *sFlt1-e15a* (*sFlt1-14*), *FLT1*, *COX1*, *COX2* and 18S rRNA levels from CTBs. PCR primers for *FLT1*, *sFLT1-i13*, *sFlt1-e15a* and 18S have been published earlier.(4) Other primers used were as follows; *COX1_F* 5'-AAG TTC ATA CCT GAC CCC CAA GGC AC-3', *COX1_R* 5'-CCT TAA AGA GCC GCA GTT GAT ACT GAC G-3', *COX2_F* 5'-AGA AGA AAG TTC ATC CCT GAT CCC C-3' and *COX2_R* 5'-CCA TCC TTG AAA AGG CGC AGT TTA C-3'. Brilliant II SYBR Green qPCR master mix with Low ROX was used for the detection of amplicons in an Mx3000p Multiplex PCR system (Agilent Technologies). Data were calculated by C_t method with 18S rRNA as normalizer and results were reported as the relative mRNA fold change compared to treatment controls.

LDH cytotoxicity assay

LDH released into culture supernatants were measured using LDH cytotoxicity assay kits following manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL.). Briefly, 50µl cell culture supernatant (without serum) was transferred to individual wells on a 96-wells plate and 50µl reaction mixture was added and incubated for 30 minutes. After adding a Stop solution, absorbance at 490 nm and 680 nm was measured with a spectrophotometer. LDH activity was calculated by subtracting the absorbance at 680 nm from that at 490 nm.

Statistical analysis

Each experiment with CTBs was repeated with isolates derived from at least 3 different placentas, with each placenta used to obtain triplicate samples. There was some heterogeneity in response between different placentas and each sample was normalized to the mean of the triplicate controls within those placenta samples and then compared. All the data is presented as mean $\pm$ SE. The statistical analysis was performed using SigmaPlot® 12 (San Jose, CA). Student's t-test was used for single comparison of continuous variables. If the samples had a non-normal distribution, Mann Whitney rank sum test was used for comparing two samples. For multiple comparisons we used ANOVA. Differences were considered significant when $p < 0.05$.

Results

Lysates from the trophoblastic layer of normal placenta and from cultured primary CTBs were run alongside COS-7 cell lysates where tagged sFLT1-i13, sFLT1-e15a and FLT1 were overexpressed. The transferred gel was immunoblotted with AF-321, an antibody to the N-terminus of Flt1 that will detect N-terminal variants including sFLT1 (Figure 1A). sFlt1-i13 encoded a ~95–100 kDa protein, sFlt1-e15a encoded a 105–140 kDa product and a ~100–120 kDa product is cleaved from Flt1 and found in conditioned media. We demonstrated that placenta and CTBs express sFLT1 although the pattern was different likely indicating differences in glycosylation and/or the relative abundance of the sFLT1 isoforms, sFLT1-i13, sFLT1-e15a and cleaved FLT1 (3, 4). The CTB bands co-migrated with sFlt1-i13 and the lower band of sFlt1-e15a while placental bands co-migrated with sFlt1-i13 and both bands of sFlt1-e15a. In the absence of isoform specific antibodies, we cannot be certain that the placenta and CTBs express both sFlt1-i13 and sFlt1-e15a proteins although in earlier studies we have shown that both transcripts are abundantly expressed (4). CTBs and placental lysates did not appear to have a band corresponding to FLT1 that could be detected by immunoblotting.

We prepared placental lysates from women with preeclamptic pregnancies at different gestational ages as well as control samples from women without preeclampsia. The baseline characteristics of the patients are shown in Table 1 and included a total of 20 women. Although the patients with preeclampsia tended to be younger, with a lower gestational age (GA) and higher BMI, this was not statistically significant. There were 4 women with preterm delivery in the control group (mean GA 29.78 weeks) and 5 in the preeclampsia group (mean GA 29.23 weeks). The reason for preterm delivery in the control group was preterm premature rupture of membranes in 3 patients and spontaneous preterm labor in 1 patient. Preterm delivery was medically indicated in the patients with severe preeclampsia. We looked at expression of sFlt1 by immunoblotting and demonstrate that there was increased expression of sFLT1 in preeclamptic placenta compared to control placenta although there was considerable variability in expression (Figure 1B and 1C). These results correlate with studies by others who had demonstrated increased sFLT1 mRNA and/or protein expression in the placenta of preeclampsia (9, 33–35).

We isolated CTBs from normal placenta and examined the effect of hypoxia on sFLT1 expression after culturing them in 8% and 2% oxygen for 48 hr. Oxygen tension plays a key

role in placental development, where the pO_2 is 40–60 mmHg (5–8% oxygen) after 12 weeks gestation and continues at this level for the second and third trimesters (36, 37). We and others have used 8% O_2 as the normoxic control for CTBs and 2% O_2 to test the effect of hypoxia. sFLT1 was measured by immunoblotting in cell lysates, by ELISA in culture supernatants, and by qPCR (sFlt1-i13) from CTB RNA (Figure 2A–D). In each of these we found, as we and others have previously reported, that hypoxia increases sFlt1 expression (4, 20, 21).

We then examined the effect of aspirin on sFLT1 secretion in CTBs grown in 8% and 2% O_2 . We found that aspirin dose dependently reduces sFLT1 released into culture media from both oxygen concentrations (Figure 3A). We then measured the effect of aspirin on *sFLT1* mRNA and noted that, at the highest concentration, aspirin reduces *sFLT1*-i13 mRNA expression (Figure 3B). We then tested the highest dose of aspirin on sFlt1-e15a mRNA expression and saw a similar inhibitory effect on expression (Figure 3C). Aspirin at the highest dose used may have a transcriptional effect while at lower doses the effects on sFLT1 expression may be post transcriptional.

We asked if the effect of aspirin on sFlt1 would also be seen in other cell systems. HTR-8/SV Neo is an extravillous trophoblast cell line that has been used to study the regulation of sFlt1 gene expression. In our experiments we saw that 10 mM aspirin significantly reduces sFlt1-i13 mRNA expression at 24 hr (Figure 3D). We also tested HEK293 cells that secrete abundant sFlt1 when VEGFR2 was transiently overexpressed and examined the effect of aspirin (38). We demonstrate that aspirin at 1 and 4 mM dose dependently reduced sFlt1 secretion in these cells (Figure 3E).

To determine if the aspirin effect was mediated via inhibition of COX1 or COX2 activity we compared the effect of the COX2 inhibitor celecoxib, and the COX1 inhibitor sc-560, on sFLT1 levels in CTBs. We found that sc-560, but not celecoxib reduced sFLT1 secretion and *sFLT1*-i13 mRNA expression in CTBs subjected to both 2% and 8% O_2 (Figure 4A, B). At higher doses of celecoxib ($>10 \mu M$) we saw significant toxicity in cultured CTBs with morphological changes and increased cell detachment from culture dishes (data not shown). We conclude from these results that COX1 inhibition, but not COX2 recapitulated the effect of aspirin to reduce sFlt1 levels in CTBs.

We then examined the expression of FLT1, the full length receptor that is derived from the same gene as sFLT1 but the mRNA and protein differ in its 3' end and C-terminus respectively from sFLT1. We demonstrated that hypoxia increases *FLT1* mRNA expression and that aspirin and sc-560 reduced *FLT1* mRNA in CTBs (Figure 5A, B). We have reported that *sFLT1* was expressed, at least at the mRNA level, in CTBs, at about 1000 fold more than *FLT1*(4). We have also previously reported that, in addition to its transcriptional origin, sFLT1 can be derived by post-translational cleavage of FLT1(6). We were not able to detect FLT1 by immunoblotting in CTBs, presumably because of its low abundance (Figure 1A). We were also unable to consistently identify cleaved FLT1 in culture media of CTBs by immunoblotting (data not shown) and sFLT1 ELISA does not differentiate between the two transcriptionally derived forms of sFLT1 or from that which is cleaved from FLT1 (Figure

2C). We conclude that most if not all of the sFLT1 arising from CTBs are transcriptionally derived and not from cleavage of FLT1.

Aspirin increased total cellular RNA in CTBs grown in 2% and 8% O₂ suggesting that it may be cytoprotective (supplemental information). Aspirin also dose-dependently reduced LDH activity in conditioned media of CTBs exposed to 2% hypoxia, although sc-560 did not (Figure 5C). We measured the abundance of caspase 3 and cleaved PARP to determine if either hypoxia or aspirin affected apoptosis. We were unable to see any differences in cleaved PARP or caspase 3 with hypoxia or aspirin (supplemental information). Although the combination of increased total RNA and reduction in LDH in CTB with aspirin is best explained by an aspirin-induced reduction in cell death the mechanism does not appear to be via reduced apoptosis. However, it is possible that cells undergoing apoptosis after 48 hours of treatment could have lifted from the culture plate and been lost during the collection process and those left on the plate then would show less evidence of apoptosis.

Since we had seen an effect of aspirin on hypoxia-induced sFLT1 expression, we examined *COX1* and *COX2* levels and noted that hypoxia had no effect on *COX1* or *COX2* expression (Figure 6A). We found that aspirin, and sc-560 inhibited *COX1* expression and increased *COX2* expression (Figure 6B). Celecoxib had no statistically significant effect on *COX1* mRNA expression (Figure 6C). We then looked at COX-1 expression by immunoblotting and we did not see a reduction in COX-1 protein in CTBs exposed to aspirin in the presence of 8 or 2% O₂ (supplemental information). Thus although COX-1 mRNA was reduced with aspirin the abundance of COX-1 protein did not change and the aspirin effect on sFlt1 is presumably not through a change in COX-1 expression.

Discussion

In this report, we explore the effect of aspirin and COX1 on sFLT1 production in CTBs. We used primary cultured CTBs under hypoxia and demonstrate that aspirin can decrease sFLT1 production from CTBs and that the effect is dose dependent. We extended these observations by demonstrating that aspirin reduces sFlt1 in a trophoblast cell line HTR-8/SV Neo and in HEK293 cells. Our study also showed that the aspirin effect on sFLT1 production from CTBs is recapitulated by COX1 inhibition but not by COX2 inhibition.

Transgenic mice that overexpress STOX1 in the placenta develop a preeclampsia phenotype and aspirin reverses the hypertension, proteinuria and the glomerular abnormalities (39). Aspirin has been tested in a number of human studies and meta-analysis of those studies shows that aspirin can decrease the risk of preeclampsia recurrence in high risk patients (26, 27). The ACOG task force on hypertension in pregnancy has suggested daily low dose aspirin beginning in the late first trimester for patients with a history of early onset preeclampsia and preterm delivery (less than 34 weeks) to decrease the recurrence risk (40). Although the mechanism of aspirin action is not understood, it is hypothesized that low-dose aspirin could inhibit platelet thromboxane-mediated vasoconstriction and thereby protect against vasoconstriction and pathological blood coagulation in the placenta and prevent failure of physiological spiral artery transformation (26).

If sFLT1 elevation can contribute to the manifestations of preeclampsia then aspirin may exert its benefit at least partly via inhibition of sFLT1 expression. As can be seen from our data and that published by others the increase in sFLT1 seen in the placenta of preeclampsia is variable (see Figure 1B). In the clinical situation there may be heterogeneity in the sFLT1 response to aspirin and only some patients may be sensitive to the effects of low dose aspirin. *In vitro* in cultured CTB, we saw an effect of aspirin on sFlt1 production at doses as low as 3 mM (500 µg/ml). We also saw an effect of aspirin on sFlt1 in HTR-8/SV Neo, a transformed extravillous trophoblast cell line. Others, however, have reported that, in HTR-8/SV Neo cells, aspirin at 10 µg/ml had no effect on sFlt1 secretion by ELISA although no RNA or immunoblotting studies were done (41). *In vivo* a single oral immediate release or single IV dose of 50 mg of aspirin produced a maximum plasma concentration of 1.3 µg/ml and 6 µg/ml of aspirin respectively in normal human volunteers (42). In clinical practice, doses from 81 to 1000 mg are used although the recommended dose used for preeclampsia prevention is 60–80mg daily. There may be a number of other differences in the effects of aspirin *in vivo* after oral delivery compared to that in cell culture. Some of the differences relate to differences in deacetylation of aspirin in the liver *in vivo* with variations in deacetylation in cells in culture, the effect of plasma protein binding and the renal elimination of aspirin *in vivo* that cannot be mimicked in cell culture. Other differences come from the duration of cyclooxygenase inhibition which can last for several days in platelets but just a few hours in endothelial cells because of rapid re-synthesis of cyclooxygenase (43). It is difficult to know therefore if the effect of aspirin we saw *in vitro* could be seen with the doses currently used for preeclampsia prevention.

Aspirin inhibits the activity of COX1 and/or COX2 enzymes via acetylation to mediate its effects. Our study showed that aspirin probably inhibits the production of sFlt1 by the COX-1 pathway. Our study also showed that, hypoxia did not change *COX1* and *COX2* mRNA expression although some have reported that the hypoxia mimic, CoCl₂ increases COX2 but not COX1 in trophoblasts and others have reported that hypoxia reoxygenation increases COX-1 and COX-2 expression in murine placental explants (44, 45). We found that both aspirin and sc-560 decreased *COX1* mRNA expression while increasing *COX2* mRNA expression. The increased production of *COX2* mRNA may be secondary to inhibition of *COX1* as has been previously described (46, 47). The effect of aspirin on COX-1 expression was not verified by immunoblotting and the significance of the reduced COX-1 mRNA expression is unclear. We also did not verify whether the increase in COX2 mRNA with aspirin was accompanied by an increase in COX2 protein. It is interesting to note that oxidative stress increases with gestational age, is augmented in preeclamptic placentas and that oxidative stress induces COX1 and COX2 enzymes (44, 48). The expression of COX1 and COX2 has been studied in preeclamptic placentas and increased, reduced or unchanged expression have been described (49–51).

In summary we conclude that aspirin dose-dependently inhibits the production sFlt1 and expands our understanding of the mechanisms regulating sFlt1 expression in CTBs and provides a plausible explanation for the effect of aspirin to prevent preeclampsia. Further studies are needed to determine if modulation of sFlt1 in patients will have a definite impact on the incidence or severity of preeclampsia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *sFLT1* expression is increased in preeclamptic placentas compared to control placentas
- sFLT1 expression is increased in CTBs exposed to 2% O₂ compared to control CTBs.
- Aspirin and the *COX1* inhibitor sc-560 reduced *sFLT1* and *FLT1* mRNA in CTBs
- Aspirin reduced sFlt1 expression in HEK293 cells and in HTR-8/SV-Neo cells.
- This study suggests that aspirin may prevent preeclampsia by reducing sFLT1.

Figure 1. Expression of sFlt1 in human placenta

Panel A: Placental and CTB lysates resolved alongside COS-7 cell lysates where human Flt1, sFlt1 isoforms i-13 and e-15a (aka sFlt1-14) or control adenovirus were transduced and with conditioned media (CM) from Flt1 transduced cells. Samples were immunoblotted with AF321, which recognizes the N-terminal portion of Flt1. An arrowhead indicates a band in placenta and CTB that co-migrates with sFlt1-i13, the filled circle a band in placenta that co-migrates with sFlt1-e15a and the star indicates the position of a band in CTB and placenta that co-migrates with both sFlt1-i13 and e15a. A darker image of this blot is shown to the right. **Panel B:** A sample of placental lysates from women with preeclampsia or control pregnancy immunoblotted for AF321 and then reprobed with an anti-actin antibody. Both bands correspond to sFlt1. **Panel C:** Densitometric analysis for sFlt1 expression in placenta. Both bands of sFlt1 were analyzed, pooled and compared between control placenta and preeclampsia. sFlt1 expression in preeclamptic placenta expressed as fold change compared to control placenta. $P < 0.001$, $n=9$ control, 11 preeclampsia.

Figure 2. Expression of sFlt1 in cultured CTBs

Panel A: sFlt1 protein expression in CTB cell lysates following incubation in 2% or 8% O₂ for 48 hr. Immunoblotting was performed with AF321 and actin antibodies. **Panel B:** Densitometric analysis for sFlt1 expression in CTBs. Both bands of sFlt1 were analyzed, pooled and compared between 2% and 8% O₂. sFlt1 expression in 2% O₂ placenta expressed as fold change compared to 8% O₂ placenta. $P < 0.05$, $n=3$ in each group. **Panel C:** sFlt1 secretion into CTB conditioned media following incubation in 2% or 8% O₂ for 48 hr, expressed as fold change compared to 8% O₂. $**p < 0.001$ vs 8% O₂. **Panel D:** sFlt1-i13 mRNA by qPCR in CTB following incubation in 2% or 8% O₂ for 48 hr, expressed as fold change compared to 8% O₂. $**p < 0.001$ vs 8% O₂.

Figure 3. Effect of aspirin (ASA) on sFlt1 expression in CTBs exposed to 2% or 8% O₂ for 48 hr

Panel A: Effect of aspirin on secreted sFlt1 after correction for total RNA in sample, expressed as fold change compared to vehicle control at the same O₂ concentration. **Panel B:** sFlt1 mRNA by qPCR in CTB following incubation with indicated concentrations of aspirin, expressed as fold change compared to vehicle control at the same O₂ concentration. *p<0.05, **p<0.001 vs control. **Panel C:** Effect of aspirin on sFlt1-e15a mRNA by qPCR in CTB. Aspirin significantly reduces sFlt1-e15a expression at 24 hr. # p < 0.001. n=9 from 3 experiments. **Panel D:** Effect of aspirin on sFlt1 mRNA by qPCR in HTR-8/SVNeo. Aspirin significantly reduces sFlt1 expression at 24 hr. *p < 0.05; n=6 aspirin and 9 control from 3 experiments. **Panel E:** Effect of aspirin on sFlt1 secretion from HEK293 cells. Aspirin dose dependently reduces sFlt1 secretion at 24 hr. * p < 0.05, **p<0.001 compared to control; \$p<0.05 against 1 mM aspirin. N=6 from 2 experiments in triplicate.

Figure 4. Effect of sc-560 or celecoxib on sFlt1 expression in CTBs exposed to 2% or 8% O₂ for 48 hr

Panel A: Effect of sc-560 or celecoxib on secreted sFlt1 after correction for total RNA in sample, expressed as fold change compared to vehicle control at the same O₂ concentration.

Panel B: sFlt1 mRNA by qPCR in CTB following incubation with indicated concentrations of sc-560 or celecoxib, expressed as fold change compared to vehicle control at the same O₂ concentration. **p < 0.001 vs control.

Figure 5. Effect of hypoxia, aspirin (ASA) and sc-560 on Flt1 expression and LDH release in CTBs exposed to hypoxia for 48 hr

Panel A: Flt1 expression in CTB following incubation in 2% or 8% O₂ for 48 hr, expressed as fold change compared to 8% O₂. **p < 0.001 vs 8% O₂. **Panel B:** Effect of aspirin or sc-560 for the last 24 hr on Flt1 mRNA in CTB samples, expressed as fold change compared to vehicle control at the same O₂ concentration. *p < 0.05, **p < 0.001 vs control. **Panel C:** LDH release into conditioned media from CTB incubated in 2% O₂ in response to indicated concentrations of aspirin or sc-560. **p < 0.001 vs control.

Figure 6. Effect of hypoxia, aspirin (ASA), sc-560 or celecoxib on COX-1 and COX-2 mRNA expression in CTBs

Panel A: Effect of hypoxia on COX-1 and COX-2 mRNA expression in CTBs. **Panel B:** Effect of aspirin or sc-560 on COX-1 and COX-2 mRNA expression in CTBs grown in 2% or 8% O₂ for 48 hr. *p < 0.05, **p < 0.001 vs corresponding control. **Panel C:** Effect of celecoxib on COX-1 and COX-2 mRNA expression in CTBs grown in 2% or 8% O₂ for 48 hr.

Table 1

Baseline characteristics of patients.

	Control	Preeclampsia
Total No:	9	11
Preterm	4	5
Race		
Caucasian	6	10
African-American	3	0
Asian		1
Age:	28.44 ± 3.68	27 ± 6.21
BMI:	28.63 ± 5.85	31.09 ± 6.26
Gestational Age (GA)	34.83 ± 5.43	34.02 ± 5.03
Preterm GA	29.78 ± 3.89	29.23 ± 2.94
Average gravida	2.58 ± 1.44	2.35 ± 2.37
Hypertension	0	2
Diabetes	0	1