Hypoxia-Mediated Soluble Fms-Like Tyrosine Kinase 1 Increase Is Not Attenuated in Interleukin 6-Deficient Mice

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Abstract

The soluble fms-like tyrosine kinase 1 (sFlt-1), known to be increased in the serum of preeclamptic patients, is a relevant factor in causing maternal symptoms like hypertension and proteinuria. In this study, we aimed to reveal whether hypoxia is a cause of increased sFlt-1 levels and inflammation markers in vivo and whether these symptoms can be attenuated by interleukin 6 (IL-6) depletion. For this purpose, pregnant wild-type (wt) mice or IL-6^{-/-} mice on embryonic day 16 were placed under either normoxic (20.9% oxygen) or hypoxic (6% oxygen) conditions for 6 hours. This led to a rise of sFlt-1 levels in maternal serum, independent of the IL-6 status of the dam. Increased maternal sFlt-1 serum levels were, however, not due to an increase in sFlt-1 messenger RNA levels in the placenta. Moreover, there was no increase in inflammatory markers in neither wt mice nor IL-6^{-/-} mice. This suggests that hypoxia alone does not contribute to the induction of an inflammatory placenta. Also, the hypoxia-induced rise in sFlt-1 levels seems not to be mediated by IL-6 in vivo.

Keywords

hypoxia, placenta, sFlt-1

Introduction

Oxygen tension is an important regulator of the placentation process, since it affects trophoblast invasion, proliferation, and differentiation.¹⁻³ It is well accepted that O₂ levels are low in the developing human placenta with 17.9 mm Hg of partial oxygen pressure in the tissue up to week 8 to 10 of gestation. Around week 12 to 13, partial oxygen pressure rises to 39.6 mm Hg.⁴ Several complications in pregnancy result from a disturbed placentation, often resulting in a low oxygen state beyond the first 10 weeks of gestation. One of them is preeclampsia, a severe complication occurring in 4% to 8% of all pregnant women, which is still one of the main causes of maternal and neonatal morbidity.^{5,6} It is speculated that insufficient trophoblast invasion into maternal spiral arteries is one of the main causes for the disease,^{7,8} resulting in an impaired invasion and, thereby, remodeling of the spiral arteries into low-resistance arteries. This shallow invasion of the trophoblast causes hypoxia and, therefore, placental ischemia.⁹ As a consequence of the deregulated placentation process, several placental factors are upregulated and released into the maternal blood circulation. These factors include antiangiogenic factors like soluble fms-like tyrosine kinase 1 (sFlt-1), the soluble vascular endothelial growth factor (VEGF) receptor,¹⁰ but also proinflammatory

cytokines like interleukin 6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor (TNF) α .^{11,12} Moreover, anti-inflammatory factors like interleukin 10 (IL-10) are downregulated in the preeclamptic placenta.¹³ Investigations of isolated trophoblast cultures demonstrated that hypoxic conditions induce the release of the proinflammatory cytokines IL-6 and IL-8.¹⁴ These findings were supported by the detection of enhanced IL-6 synthesis in basal decidua cells during preeclampsia.¹⁵ However, the mechanism responsible for the expression of these proinflammatory mediators remains unclear.

To the best of our knowledge, it is not known so far whether hypoxic conditions in the placenta of patients with preeclampsia are source of the inflammatory response in the organ in vivo. There are studies demonstrating that low oxygen levels

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Abbreviations

IL-6	interleukin-6
wt	wildtype
sFlt-1	soluble fms-like tyrosine kinase-1

can stimulate the induction of cytokines; however, most of them have been performed *in vitro*, using human villous explants.¹⁶⁻¹⁹ Also, these studies were often performed under unphysiological conditions, using hyperoxic instead of normoxic conditions as control.¹⁹

sFlt-1 is produced, among others, by placental cells. It has been demonstrated that this antiangiogenic factor is increased in human and rat placental explants *in vitro* under low oxygen conditions^{20,21} and that the upregulation of sFlt-1 is mediated by the transcription factor hypoxia inducible factor (HIF) 1 α ,²² which is stabilized under hypoxic conditions.²³ *In vivo*, low oxygen conditions of 9.5% for 9.5 days are known to increase maternal circulating serum sFlt-1 levels, and this effect is dependent on IL-10.²⁴ Whether other cytokines are involved in the regulation of sFlt-1 production under hypoxic conditions *in vivo* has not been investigated so far. However, it is known that IL-6 inactivation in mice inhibits placental Flt-1 production induced by injection of human preeclamptic serum,²⁵ indicating a regulatory function of this cytokine on the placental sFlt-1 release.

In this study, we aimed to test the hypothesis that hypoxia is a cause of increased sFlt-1 levels and inflammation *in vivo*. Furthermore, we asked whether the inhibition of the proinflammatory cytokine IL-6 might dampen hypoxia-induced symptoms associated with preeclampsia.

Material and Method

Animal Procedures and Tissue Preparation

C57BL/6J (wild-type [wt]; Charles River, Germany) and B6.129S2-Il6tm1Kopf/J (interleukin-6 knockout [IL-6^{-/-}]; Jackson Laboratory, Maine) mice were bred at the animal facility of the University of Cologne (animal facilities of the Pharmaceutical Institute and the Center of Molecular Medicine Cologne) at 22°C on a 12-hour light, 12-hour dark cycle. Water was available *ad libitum* and the animals were fed a standard laboratory diet (#1310, Altromin, Germany). Female mice were mated at 8 to 12 weeks of age for 24 hours and assumed to be pregnant when a vaginal plug was expelled (g1). Pregnancy was validated by daily monitoring the body weight. At embryonic day 16 (ED 16), the animals were randomly subdivided into a normoxic and a hypoxic group. Mice from the hypoxic group were placed in a hypoxic chamber (O₂ control glove box for tissue culture; COY Laboratory Products Inc, Michigan) and exposed for 6 hours to a 6% oxygen atmosphere as described previously,²⁶ whereas normoxic mice were kept at regular atmosphere. After 6 hours, mice were sacrificed immediately without reoxygenation. Blood samples were taken by

cardiac puncture, incubated at room temperature for 30 minutes, and then centrifuged for 15 minutes at 4°C and 3000g to remove the clotted fraction. Serum was stored at -80°C. Placentas were removed and dissected to detach cord, amnion sac, and connective tissue. Tissue for total RNA and protein isolation was quick frozen on dry ice and tissue for histological analysis was fixated in phosphate-buffered formaldehyde (Roti-Histofix, Roth, Germany).

All procedures in this study were performed according to protocols approved by the German regulations and legal requirements and were also approved by the local government authorities (Bezirksregierung Koeln).

Total RNA Isolation and TaqMan Real-Time Polymerase Chain Reaction Analysis

For reverse transcriptase polymerase chain reaction (PCR), total RNA was prepared using the TRIzol reagent (Invitrogen, Germany) according to the manufacturer's recommendations. Total RNA of 1 μ g was reverse transcribed into complementary DNA (cDNA) with the MMLV Reverse Transcriptase (Promega, Germany) using Oligo-dT oligonucleotides and random hexamer oligonucleotides (Promega). To determine mRNA expression levels of the cytokine genes IL-6, IL-10, and TNF- α , we used a quantitative real-time PCR method. Expression was normalized to housekeeping genes GAPDH and β -actin. TaqMan PCR reagents (Platinum, Quantitative PCR SuperMix-UGD with ROX; Invitrogen) were applied according to the manufacturer's protocol. Complementary DNA of 2.5 μ L was used as template in a total reaction volume of 25 μ L. The thermocycler parameters were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C and 60°C for 1 minute, and the 7500 Real Time PCR System from Applied Biosystems (Germany) was used. For IL-10, we used 10 $\times$ real-time PCR buffer, ROX passive reference, dNTP mix, and enzymes Hot Star Gold and Uracil N-Glycosylase from Eurogentec (Germany). The following oligonucleotides and probes (MWG, Germany) were used: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for 5'-ATGTGTCGCTCGTGGATCTGA, rev 5'-TGCCTGCTTCACCACCTTCT, probe 5'-CCGCTG GAGAAACCTGCCAAGTATG; beta-actin for 5'-TGACAG-GATGCAGAAGGAGATTACT, rev 5'-GCCACCGATCCACACAGAGT, probe 5'-ATCAAGATCATTGCTCCTCCTGA GCGC²⁷; IL-6 for 5'-ACAAGTCGGAGGCTTAATTACACAT, rev 5'-AATCAGAATTGCCATTGCACAA, probe 5'-TCTTTTCTCATTTCACGATTTCCCAGAGAA,²⁸ IL-10 for 5'-GGCGCTGTCATCGATTCTC, rev 5'-CACCTTGCTC TTGGAGCTTATTA, probe 5'-CCTGTGAAAATAAGAGCAAG,²⁹ TNF- α for 5'-GGCTGCCCCGACTACGT, rev 5'-GACTTTCTCCTGGTATGAGATAGCAA, probe 5'-CCTCA CCCACACCGCTCAGCCG²⁸; Flt-1 for 5'-GGTGTCTGCTT CTCACAGGATATG, rev 5'-CGTGGGCAGAGACCATGAG T, probe 5'-AGCAGGCCAGACTCTCTTTCTCAAGTGCA; sFlt-1 for 5'-GGGAAGACATCCTTCGGAAGA, rev 5'-TCCG AGAGAAAATGGCCTTTT, probe 5'-CCGCAGTGCTCACC TCTAACGAGAACTTCT.³⁰ The probes were modified with

Figure 1. Hypoxia-inducible factor 1 α (HIF-1 α) levels are increased upon hypoxia in the placenta of wild-type (wt) and interleukin 6 (IL-6)^{-/-} mice. Pregnant mice at embryonic day 16 (ED 16) were exposed to either normoxic (20.9% O₂; nx) or hypoxic (6% O₂; hyx) conditions for 6 hours. Then, mice were sacrificed and placentas were dissected. Total protein was isolated and subsequently examined via an immunoblot. HIF-1 α was detected with a specific antibody, and α -tubulin was determined for normalization. A representative set of samples from wt mice (left) and from IL-6^{-/-} mice (right) is shown. For each mouse type (wt; IL-6^{-/-}), the samples derive from 2 independent experiments as indicated (exp 1 and exp 2).

6-carboxyfluorescein (FAM) at the 5' and tetramethylrhodamine (TAMRA) at the 3' end. The results of the real-time PCR analysis are calculated based on the $\Delta\Delta C_t$ method and expressed as fold induction of mRNA expression compared to the corresponding control group (1.0-fold induction).

Isolation and Immunoblot Analysis

For protein isolation, samples were mixed with protein extraction buffer (6.65 mol/L Urea, 10% glycerol, 1% sodium dodecyl sulfate [SDS], 10 mmol/L Tris-HCl pH 6.8, 5 mmol/L Dithiothreitol, and 0.5 mmol/L phenylmethanesulfonylfluoride) and homogenized. Concentration was measured using Bicinchoninic acid (BCA, Thermo Scientific). Total protein of 30 μ g was incubated with sample buffer (0.5 mol/L Tris, 20.6 % SDS, 25 % β -mercaptoethanol, 2.6 % bromophenolblue, and 10 % glycerol), incubated for 10 minutes at 70°C and then run on an SDS polyacrylamide gel electrophoresis. The gel was then transferred onto a nitrocellulose membrane (Whatman, Germany). After blocking the membrane with 5% milk and 2% bovine serum albumin in TBS-Tween (10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Triton-X 100; pH 7.5), it was incubated with the first antibodies diluted in TBS-Tween: the rabbit polyclonal anti-HIF-1 α antibody (#NB100-449, Novus Biologicals, Germany; 1:1000 dilution) and the mouse anti- α -tubulin antibody (clone DM1A, Sigma-Aldrich, Germany; 1:100 000 dilution) at 4°C overnight. The horseradish peroxidase (HPO)-linked secondary antibodies goat anti-mouse HPO and goat anti-rabbit HPO (Cell Signaling, Germany) were used at 1:2000 dilution at room temperature for 1 hour. After applying the ECL Prime Western Blotting Detection Reagent (GE Healthcare, United Kingdom) to the membrane, protein bands were acquired with the BIO-RAD Molecular Imager ChemiDOC XRS+ Imaging System (Bio-Rad Laboratories, Germany).

Quantification of Serum sFlt-1 Levels

Serum was analyzed using an enzyme-linked immunosorbent assay (ELISA) kit to determine sFlt-1 (DY471; R&D Systems, Germany) levels as suggested by the manufacturer. Serum was diluted 1:20 for sFlt-1 ELISA. A duplicate analysis was performed and absorption of samples was measured with a Nano Quant M200 (Tecan, Germany).

Papenheim Staining of Placenta Sections

Formaldehyde-fixed placentas were mounted in paraffin and sections of 10 μ m using a rotation microtome (Leica, Germany) were prepared. A Papenheim protocol was used for staining. The sections were first deparaffinated, then stained with a May-Grünwald staining solution (Roth, Germany), and counterstained with a Giemsa solution (VWR, Germany). Stained sections were analyzed using a microscope (Leica DM6000B; Leica) at 40 $\times$ magnification. For cell count, at least 3 randomized parts of the labyrinth section were chosen in each placenta and leukocytes were counted.

Statistical Analysis

Statistical analysis was performed with the GraphPad Prism Software (Version 5.03, GraphPad Software, Inc., California). All values in the text are mean $\pm$ standard error of the mean. Normality distribution was tested using the D'Agostino & Pearson test. Differences were evaluated either using a 2-tailed Student *t* test (for normally distributed data) or using a Mann-Whitney test (for nonnormally distributed data). Significant differences were taken at the *P* < .05 level.

Results

Levels of HIF-1 α are Increased Upon Hypoxia in the Placenta of wt and IL-6^{-/-} Mice

Preeclampsia is thought to evolve in the first trimester of pregnancy; however, symptoms like hypertension, proteinuria, and edema usually arise within the second and third trimester.^{31,32} Hence, we used mice at ED 16 in our experiments which corresponds to the last trimester of murine pregnancy. To ensure that the experimental setting induces hypoxic stress in the placenta, we performed immunoblot analyses to determine the level of HIF-1 α . This transcription factor is stabilized under hypoxic conditions²³ and is therefore often used as hypoxic marker. As shown in Figure 1, a 6-hour incubation of the pregnant wt animals under 6% O₂ atmosphere resulted in significant higher HIF-1 α levels compared to control animals kept under normoxic conditions. The same was true for IL-6^{-/-} mice. This confirms that our experimental setting results in severe hypoxia in the murine placenta as has been shown earlier.²⁶

Figure 2. Hypoxia does not induce an inflammatory response in the murine placenta. A, Placentas from mice exposed to either normoxic (20.9% O₂) or hypoxic (6% O₂) conditions for 6 hours were dissected on embryonic day 16 (ED 16) of pregnancy and total messenger RNA (mRNA) was isolated. A total of n = 10 normoxic (76 placentas) and n = 11 hypoxic (75 placentas) treated wild-type (wt) mice and a total of n = 10 normoxic (74 placentas) and n = 9 hypoxic (73 placentas) treated interleukin 6 (IL-6)^{-/-} mice were analyzed. The mRNA expression levels of IL-6 (white bar), IL-10 (gray bars), and TNF- α (black bars), normalized to β -actin, were measured and plotted as fold induction compared to normoxic mice (set as 1). ** P value < .005; *** P value < .0005; ns = not significant. Data for wt mice: left graph; data for IL-6^{-/-} mice: right graph. B, Some of the placentas collected from normoxic and hypoxic mice on ED 16 were analyzed by performing a Papanheim staining of the labyrinth part of placental sections. Leukocytes were counted under $\times 40$ magnification in at least 3 sections of the labyrinth of placentas from wt (left) and IL-6^{-/-} (right) mice, exposed to either normoxic (20.9% O₂) or hypoxic (6% O₂) conditions and plotted in the graph as (lc/mm)². Left graph: n = 4 normoxic wt mice; n = 4 hypoxic wt mice. Right graph: n = 4 normoxic IL-6^{-/-} mice; n = 3 hypoxic IL-6^{-/-} mice. ns indicates not significant.

Hypoxia Does Not Induce an Inflammatory Response in the Murine Placenta

One feature of the preeclamptic placenta is a proinflammatory state.^{11,12} In order to reveal a potential inflammatory response upon hypoxic stress in the placenta, we performed real-time PCR analyses and measured the expression levels of the proinflammatory cytokines IL-6 and TNF- α normalized to β -actin. In parallel, we also determined the mRNA expression rate of the antiangiogenic factor IL-10, which is known to be decreased in preeclamptic placentas.¹³ As can be seen in Figure 2A, there was no physiologically relevant variation in any cytokine measured, neither in wt (left panel) nor in IL-6^{-/-} (right panel) mice. We also normalized expression levels to GAPDH (data not shown) and found the same trends.

We quantified the amount of leukocytes in the labyrinth by performing a Papanheim staining of placenta sections, assuming that an increased rate of leukocytes in hypoxic compared to

normoxic placentas would indicate an inflammatory state. However, we saw no differences, both in wt (Figure 2B, left panel) and IL-6^{-/-} (Figure 2B, right panel) mice, between normoxic and hypoxic tissue. This further corroborates our findings from the real-time PCR analyses that hypoxic stress does not contribute to an inflammatory response in the murine placenta.

Maternal Circulating sFlt-1 Levels are Increased Upon Hypoxia

sFlt-1 is one of the best described factors that is upregulated in the human preeclamptic placenta and is then released into the maternal serum where it is responsible for inducing proteinuria and hypertension in the mother.³³ Release of sFlt-1 can be induced by hypoxia in vivo as has been shown previously.²⁴ Indeed, when we measured sFlt-1 levels in the maternal serum of normoxic and hypoxic-treated dams at ED 16, we saw a

Figure 3. Maternal circulating soluble fms-like tyrosine kinase 1 (sFlt-1) levels are increased upon hypoxia in both wild-type (wt) and interleukin 6 (IL-6)^{-/-} mice. A, sFlt-1 serum levels of normoxic or hypoxic-treated mice were determined by enzyme-linked immunosorbent assay (ELISA). A total of $n = 10$ normoxic and $n = 11$ hypoxic treated wt mice (left graph) and a total of $n = 10$ normoxic and $n = 9$ hypoxic-treated IL-6^{-/-} mice (right graph) were investigated. * P value $< .05$; ** P value $< .005$. B, Placentas of a total of $n = 10$ normoxic (76 placentas) and $n = 11$ hypoxic (75 placentas) treated wt mice (left graph) and placentas of a total of $n = 10$ normoxic (74 placentas) and $n = 9$ hypoxic (73 placentas) treated IL-6^{-/-} mice (right graph) were analyzed for Flt-1 (white columns) and sFlt-1 (gray columns) messenger RNA (mRNA) levels by real-time polymerase chain reaction (PCR) analysis. Expression levels in placentas of hypoxic-treated dams, normalized to β -actin, are plotted as fold induction compared to expression levels in placentas of normoxic mice (set as = 1). ns indicates not significant.

significant increase upon hypoxia in wt animals (see Figure 3A, left panel). However, IL-6 seems not to be involved in this regulatory process, since sFlt-1 release was also induced in IL-6^{-/-} mice under hypoxic conditions (see Figure 3A, right panel).

In order to find out whether the increased maternal serum sFlt-1 levels are due to an increased sFlt-1 production of the placenta, we analyzed both Flt-1 and sFlt-1 mRNA expression by real-time PCR. The quantification of total Flt-1 mRNA levels (both Flt-1 and sFlt-1 are detected in the assay) revealed no changes in placentas from hypoxic-treated dams compared to control dams, neither in wt nor in IL-6^{-/-} mice (Figure 3B, white columns). Also, when measuring sFlt-1 levels in a real-time PCR analysis, we saw no differences between hypoxic and normoxic wt or IL-6^{-/-} mice (Figure 3B, gray columns). We also normalized expression levels to GAPDH (data not shown) and found the same trends. Hence, maternal circulating serum sFlt-1 levels are upregulated under acute hypoxia, but the rise seems not to be due to an increased placental Flt-1 or sFlt-1 gene expression.

Discussion

In this study, we provide in vivo evidence that IL-6 does not contribute to the release of sFlt-1 upon hypoxia. Furthermore, we show that in vivo hypoxia alone does neither induce an increase in placental cytokine production nor leukocyte infiltration of the tissue.

In preeclampsia, the increase in placental sFlt-1 is not always accompanied by an increase in Flt-1.³⁴ This can be explained by alternative posttranscriptional processing of the full-length Flt-1 mRNA, meaning that there is a shift of Flt-1 to sFlt-1 mRNA levels.³⁵ However, other studies report an increase in both Flt-1 and sFlt-1 levels in preeclamptic placentas. This second scenario might be due to a general upregulation of Flt-1 expression, which was shown to be modulated by HIF-1 α .²² Besides that, placental sFlt-1 release can also be mediated by proteolytic cleavage of the transmembrane Flt-1 protein by proteases.³⁶ In vitro, it has been demonstrated that hypoxia upregulates sFlt-1 production in both human²⁰ and

rat²¹ villous explants. Also, in vivo data exist demonstrating that sFlt-1 is upregulated in both the uteroplacental tissue and the serum of pregnant mice exposed to low oxygen conditions from day g7.5 to day g17.²⁴ In our study, we were able to corroborate that sFlt-1 is upregulated in the maternal serum under low oxygen conditions in vivo, even after 6 hours. However, this upregulation is not due to increased placental Flt-1 or sFlt-1 mRNA levels. This means that the increased sFlt-1 in the maternal serum either derives from organs other than the placenta (e.g., from vascular endothelial cells,³⁷ from adipocytes,³⁸ or from monocytes³⁹) or that placental membrane-bound Flt-1 proteins are processed by proteases under hypoxia, thereby releasing sFlt-1 into the maternal serum. The cytokine IL-6 has been connected to the regulation of sFlt-1 production. In Human umbilical vein endothelial cells (HUVECs), cellular stress like hypoxia or IL-6 stimulation results in Gadd45a induction, p38 activation, and subsequently leads to the increase in sFlt-1 levels.⁴⁰ Also, when stimulating pregnant mice with serum from patients with preeclampsia, Zhou et al observed an increase in placental sFlt-1 expression that was blocked by pretreatment of the animals with an IL-6 neutralizing antibody.²⁵ This suggests a regulatory function of IL-6 in sFlt-1 production. In our study, we however saw no effect of IL-6 depletion on the production of sFlt-1 under low oxygen conditions. This might be due to the fact that the increased sFlt-1 levels upon a hypoxic stimulus in our mouse model do not arise from increased placental mRNA levels, and the blocking of sFlt-1 increase by IL-6 does only work in placental cells. On the other hand, different regulatory pathways for sFlt-1 release might exist, depending on the stimulus and hence, increased sFlt-1 production in response to treating mice with serum from patients with preeclampsia can be blocked by IL-6 inhibition but not increased sFlt-1 production in response to hypoxia.

In cell culture experiments using human placental explants, hypoxia has been shown to result in altered cytokine production. Several proinflammatory cytokines tend to be upregulated,^{16,18,19} while the anti-inflammatory cytokine IL-10 is downregulated under low oxygen conditions.^{18,19} Higher proinflammatory cytokine levels^{11,12} and lower IL-10 levels have also been observed in human preeclamptic placentas.¹³ However, the attempts to explain the regulatory in vivo mechanism are still incomplete. In this study, we aimed to test the hypothesis that low oxygen conditions participate in the induction of placental cytokine production and/or leukocyte infiltration of the tissue as an inflammatory response. We however did not see any differences in placental TNF- α , IL-6, and IL-10 levels under hypoxic conditions in wt mice. In IL-6^{-/-} mice, we also detected no changes in TNF- α or IL-10 expression upon hypoxia. For this mouse model, one can of course claim that, due to the knockout of IL-6, the other cytokines might not persist or function in this model. This assumption, however, is disproven by a study of van der Poll et al, demonstrating that intranasal inoculation with *Streptococcus pneumoniae* induces an increase in TNF- α , IL-1 β , interferon- γ , and IL-10 in the lung of IL-6^{-/-} mice.⁴¹ Probably, the discrepancy between the human in vitro data and our mouse in vivo data derive from species-specific

differences. We however rather assume that the placenta in vivo is able to compensate for changes in oxygen levels much better than isolated cells or isolated villous explants in vitro, hence not displaying deregulated cytokine levels under hypoxic conditions.

A limitation of this study is the use of a mouse model and we do not know at this point to what degree our findings can be translated to humans. However, the mouse placenta shares high similarities with the human placenta⁴² so that we believe that our results can provide novel insights into human placental function. Our study is also limited to the short but severe hypoxic treatment of the mice. Other studies often use milder hypoxic conditions for a longer time period. Since we aimed to examine initial changes in inflammatory signals that are directly related to the hypoxic stimulus, performing the analysis after 6 hours seemed to be more reasonable for this project. We feel that when looking at much later time points after the stimulus, the detected changes might be mainly due to secondary effects like regeneration or tissue repair, since the organism starts to adapt to low oxygen levels. Another limitation of this study is the lack of knowledge where the hypoxically induced sFlt-1 is coming from. Whether an increase in proteases in the placenta might lead to the increase in serum sFlt-1 levels or whether other tissues are responsible for the sFlt-1 increase in the maternal serum upon the acute and severe hypoxic stimulus needs further clarification.

In summary, we show that even after 6 hours of low oxygen conditions, sFlt-1 release into the maternal serum of pregnant mice is elevated and that the proinflammatory cytokine IL-6 is not involved in this pathway. Furthermore, we disprove the hypothesis that acute hypoxia alone is able to induce an inflammatory response in the placenta in vivo.

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Authors' Note

Sarah Appel and Eva-Maria Turnwald contributed equally to this work.

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