Pregnancy/Preeclampsia

A Critical Role of Interleukin-10 in Modulating Hypoxia-Induced Preeclampsia-Like Disease in Mice

Zhongbin Lai, Satyan Kalkunte, Surendra Sharma

Abstract—Hypoxia has been implicated in the pathogenesis of preeclampsia, a hypertensive disorder of pregnancy. However, in vivo evidence and mechanistic understanding remain elusive. Preeclampsia is associated with impaired placental angiogenesis. We have recently shown that interleukin (IL)-10 can support trophoblast-driven endovascular crosstalk. Accordingly, we hypothesize that pathological levels of oxygen coupled with IL-10 deficiency induce severe preeclampsia-like features coupled with elevated production of antiangiogenic factors, apoptotic pathways, and placental injury. Exposure of pregnant wild-type and IL-10 \(^{-/-}\) mice to 9.5% oxygen resulted in graded placental injury and systemic symptoms of renal pathology, proteinuria (wild-type 645.15 ± 115.73 versus 198.09 ± 93.45; IL-10 \(^{-/-}\) 819.31 ± 127.85 versus 221.45 ± 82.73 \(\mu\)g/mg/24 hours) and hypertension (wild-type 118.37 ± 14.45 versus 78.67 ± 14.07; IL-10 \(^{-/-}\) 136.03 ± 22.59 versus 83.97 ± 18.25 mm Hg). Recombinant IL-10 reversed hypoxia-induced features in pregnant IL-10 \(^{-/-}\) mice confirming the protective role of IL-10 in preeclampsia. Hypoxic exposure caused marked elevation of soluble fms-like tyrosine kinase 1 (110.8 ± 20.1 versus 44.7 ± 11.9 ng/mL) in IL-10 \(^{-/-}\) mice compared with their wild-type counterparts (81.6 ± 13.1 versus 41.2 ± 8.9 ng/mL), whereas soluble endoglin was induced to similar levels in both strains (approximately 380 ± 50 versus 180 ± 31 ng/mL). Hypoxia-induced elevation of p53 was associated with marked induction of proapoptotic protein Bax, downregulation of Bcl-2, and trophoblast-specific apoptosis in utero-placental tissue. Collectively, we conclude that severe preeclampsia pathology could be triggered under certain threshold oxygen levels coupled with intrinsic IL-10 deficiency, which lead to excessive activation of antiangiogenic and apoptotic pathways. (Hypertension. 2011;57:505-514.) ● Online Data Supplement

Key Words: angiogenesis ● apotosis ● hypertension ● hypoxia ● interleukins ● mouse

Preeclampsia is one of the most common complications of pregnancy. This condition, associated with hypertension, edema, and proteinuria, affects 5% to 10% of pregnancies worldwide and entails severe consequences for both the mother and the fetus.\(^1\)-\(^3\) Preeclampsia presents as either a mild or severe condition, distinguished most often by severity of hypertension and proteinuria symptoms. Although long-term postpartum effects are not well investigated in children born to preeclamptic mothers, intrauterine growth restriction (IUGR) is a relatively common comorbidity.\(^4\) Both preeclampsia and IUGR are thought to be associated with increased apoptosis in the placenta, altered trophoblast proliferation and differentiation, syncytiotrophoblasts, and circulating placental debris as well as random production of antiangiogenic factors such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng).\(^5\)-\(^11\) Importantly, placental bed biopsies from preeclampsia show signs of poor perfusion and ischemia at least in part due to defects in remodeling of spiral arteries and shallow trophoblast invasion.\(^12\)-\(^14\) It has been hypothesized that hypoxic/ischemic injury is the trigger for excessive placental cell death and expression of antiangiogenic factors.\(^15\)-\(^17\) However, in vivo evidence for hypoxia to induce preeclampsia-like syndrome in animals has not yet been reported.

During human gestation, early placentation develops in a relatively low oxygen (O\(_2\)) tension environment (17.9 mm Hg or 2.5% O\(_2\)).\(^18\) The developmental switch of trophoblasts from a proliferative to an invasive phenotype is controlled by the placental oxygen levels.\(^19\) Furthermore, disruption of regulatory genes controlling responses to hypoxia leads to failure in placentation and fetal death.\(^19\) However, studies on hypoxia have not yet resolved the controversial in vitro findings. Studies with trophoblast cell lines, first-trimester cytotrophoblasts, and placental explants suggest that low oxygen tension may induce trophoblast proliferation and/or apoptosis and thus impairs trophoblast invasion.\(^16\),\(^19\)-\(^21\) On the other hand, hypoxia-induced enhanced trophoblast invasion and differentiation to invasive phenotype have been suggested.\(^22\),\(^23\) To date it has not been possible to discern what oxygen levels impart in vivo pathological effects at the maternal–fetal interface and their role in the onset of preeclampsia and IUGR. Although maternal hypoxia (11%) was shown to increase vascularity and potentiate trophoblast invasion in pregnant rats,\(^24\) it is possible that this level of...
oxygen tension is not sufficient to cause pathological effects leading to preeclampsia-like symptoms. It is also plausible that intrauterine cytokine milieu and balance of vascular factors may modulate the effects of hypoxic levels of oxygen. Hypoxia affects the cytokine balance by reducing interleukin (IL)-10 production and inducing IL-6 and IL-8 in placental explants and trophoblasts. Thus, IL-10 deficiency coupled with hypoxia-induced inflammatory milieu may affect trophoblast functions and perturb the expression of apoptosis-associated pathways at the maternal–fetal interface. We hypothesize that hypoxia under certain threshold levels could lead to placental injury, apoptosis, and preeclampsia-like symptoms that are likely to be exacerbated by IL-10 deficiency.

Our studies demonstrate that exposure to 9.5% oxygen levels during gestational days (gd) 7.5 through 17 in IL-10−/− mice induces a full spectrum of features that closely mirror severe preeclampsia-like disease. Our results provide evidence for a pregnancy-specific constellation of events, including production of antiangiogenic factors, sFlt-1, and sEng, and activation of placental apoptotic pathways. Importantly, exogenous IL-10 was found to reverse hypoxia-induced preeclampsia-like features.

Materials and Methods

Syngeneic matings involving C57BL/6 (wild-type or IL-10−/−) mice (The Jackson Laboratory) were used for timed pregnancies. The day when the vaginal plug was detected was considered as gd 0. All protocols were approved by the Lifespan Animal Welfare Committee and conducted according to its guidelines. Mice (n=4 to 6 per group) were placed in the hypoxia chamber calibrated for varying (9.5% or 11%) levels of O2, 5% CO2, and 85.5% or 84% N2 from gd 7.5, 10, or 15 through gd 17 (see Data Supplement for details; available at http://hyper.ahajournals.org). To evaluate the protective role of IL-10 in hypoxia-induced preeclampsia, a subset of IL-10−/− mice were injected (intraperitoneally) with recombinant mouse IL-10.27 (R&D Systems; 500 ng/mouse, n=6) or saline daily from gd 8 through gd 16 and exposed to 9.5% hypoxia from gd 7.5 to gd 17. On gd 17, animals were euthanized, the fetuses separated from placenta, and weighed for monitoring IUGR. Before euthanizing the animals, blood pressure was monitored using a noninvasive computerized tail-cuff system (Digi-med, Inc. Louisville, KY). On gd 16, mice were transferred to the metabolic cage placed in the hypoxia chamber throughout the period of experiment for 24-hour urine collection. The metabolic chamber is designed to collect urine and fecal matter separately and minimizes their mixing and animals had access to food and water ad libitum. To assess proteinuria, urinary albumin and creatinine were measured using the Alburell and Creatinine Companion kits. For assessing the renal pathology, fixed kidney tissue was stained with hematoxylin/eosin and periodic acid Schiff reagent as described. Morphological changes were recorded using SPOT Advanced software at 10× and 100× magnification. For measuring circulating levels of mouse sFlt-1, sEng, and vascular endothelial growth factor by enzyme-linked immunosorbent assay, mice were euthanized on gd 17, and blood was collected by cardiac puncture. After 3 to 5 minutes at room temperature, blood was centrifuged at 500 g for 5 minutes and supernatant collected and stored at −80°C until further use (details are available in the Data Supplement). Similarly, nonpregnant mice (n=6) were subjected to normoxia or hypoxia treatment for 9.5 days and monitored for blood pressure, proteinuria, kidney pathology, serum sFlt-1, and sEng levels as described. Local hypoxia was detected using the marker EF-5, a gift from Dr Cameron Koch (University of Pennsylvania). EF-5 (10 µmol/L) was administered intraperitoneally 4 hours before harvesting the uteroplacental tissues on gd 13. The tissue was snap-frozen and 10-µm sections were processed and probed for EF-5-positive signal using ELK3-51 antibody (1:30) as described. Hypoxia-inducible factor-1α, p53, p21, Bax, Bcl-2, caspase 3, vascular endothelial growth factor A, vascular endothelial growth factor C, phospho-Stat3, total Stat3, and actin were monitored by Western blot in placental tissue collected from gd 17 (details are provided in the Data Supplement). Placental expression of sFlt-1 and sEng transcripts was monitored after normalizing to β2-microglobulin (β2 M) expression by real-time quantitative polymerase chain reaction. For detection of apoptotic cells, placental tissue was fixed in 10% paraformaldehyde, sectioned, and subjected to terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining. Uteroplacental tissue structural orientation was demarcated by hematoxylin & eosin staining. Sections from snap-frozen placental tissue were double-stained with fluorescent tagged antiapancytokerin antibody and TUNEL. Data were compared using the Student t test if the data were normally distributed. P<0.05 was considered significant. Data are presented as the mean±SEM from at least three independent experiments. Further methodological details are available in the Data Supplement.

Results

Hypoxia Induces a Full Spectrum of Preeclampsia-Like Features in Pregnant IL-10−/− Mice

We first assessed the effects of graded levels of hypoxia and duration of exposure on induction of preeclampsia-like features in pregnant wild-type and IL-10−/− mice. As shown in Supplemental Table I, exposure to 11% or 9.5% O2 from gd 10 or 15 through gd 17 did not induce any preeclampsia-like symptoms in wild-type mice. Similarly, no major changes were observed in IL-10−/− mice, except for a trend toward hypertension and proteinuria with 9.5% O2 from gd 10 to gd 17 (Supplemental Table I). Exposure to 8.5% O2 levels from gd 7.5 or gd 10 through gd 17 proved lethal to pregnant mice. However, pregnant mice, particularly IL-10−/− mice, when exposed to 9.5% O2 from gd 7.5 through gd 17, experienced a full spectrum of preeclampsia-like symptoms (Figure 1) and these conditions were used for further studying hypoxia-induced pathological effects.

As demonstrated in Figure 1A, exposure to 9.5% but not 11% O2 from gd 7.5 to gd 17 led to hypertension in wild-type and IL-10−/− mice, albeit much higher readings in IL-10−/− mice. Urinary albumin excretion (normalized to creatinine) was also significantly increased in 9.5% O2-exposed wild-type and IL-10−/− mice (Figure 1B). Surprisingly, the excreted protein content was significantly higher in IL-10−/− mice. In addition to hypertension and proteinuria, the other prominent clinical features of preeclampsia are fetal IUGR and maternal kidney pathology. Figure 1C demonstrates comparative size of fetuses on gd 17 from animals exposed to hypoxic (9.5% O2) conditions and those kept under normoxia (21% O2) conditions. Under 9.5% O2 conditions, we did not observe fetal resorption or preterm birth in pregnant animals as assessed by inspection of uterine horns at different stages of pregnancy or by allowing animals to deliver. Unlike exposure to 11% or 21% O2, it is evident that 9.5% O2 caused significant IUGR in both wild-type and IL-10−/− fetuses with the latter showing significantly more enhanced effects as indicated by the fetal weights from multiple experiments as shown in Figure 1D. Thus, these data indicate that
preeclampsia-like symptoms induced by hypoxia were exacerbated by IL-10 deficiency.

Figure 2 shows the hypoxia-induced renal pathological changes in both wild-type and IL-10−/− mice. Assessment of the renal tissue (10× magnification; Figure 2A) clearly shows disorganized kidney tissue. Atrophic tubules and interstitial edema with dispersed glomerular enlargement were observed. Extensive capillary occlusion with swollen cytoplasm was particularly evident at higher magnification (100× magnification; Figure 2B). Similar effects have been observed in sFlt-1-treated pregnant rats. Importantly, in contrast to pregnant mice, identical hypoxia (9.5% O2) treatment for 9.5 days in nonpregnant mice did not exhibit renal pathology. A representative histology of kidney section from nonpregnant mice subjected to hypoxia is shown in Figure 2C (10× magnification) and Figure 2D (100× magnification, a single glomerulus), suggesting pregnancy-specific impact of hypoxia on renal pathology. Interestingly, the murine preeclampsia models involving sFlt-1 or autoantibodies against angiotensin 1 receptor have shown some pathological effects in nonpregnant animals. However, in the current model of preeclampsia, our data demonstrate that hypoxic conditions (9.5% O2) used did not cause hypertension (Supplemental Figure IA), proteinuria (Supplemental Figure IB), or excess production of sFlt-1 (Supplemental Figure IC) in nonpregnant animals, suggesting that the model established here can be used to study pregnancy-specific onset of hypoxia-induced preeclampsia-like disease.

Hypoxia Induces Higher Production of sFlt-1 in Pregnant IL-10−/− Mice

The angiogenic factors sFlt-1 and sEng can cause preeclampsia-like symptoms in a rat model when administered at high doses. Here, we show that in response to 9.5% O2, serum levels of sFlt-1 were significantly elevated in pregnant wild-type and IL-10−/− mice (Figure 3A). sFlt-1 production was comparatively much higher in IL-10−/− mice. On the other hand, although sEng was also induced in response to hypoxic conditions, no significant differences were observed between wild-type and IL-10−/− mice (Figure 3B), suggesting specific effects of IL-10 deficiency only on sFlt-1 production. We also assessed sEng at the mRNA level (Supplemental Figure II) and the data corroborated those shown in Figure 3. Next, we assessed the serum and placental levels of proangiogenic factors vascular endothelial growth factor A and vascular endothelial growth factor C by enzyme-linked immunosorbent assay and Western blotting and found no significant effect of 9.5% O2 on overall levels of these growth factors (Supplemental Figure IIIA–C). Overall, these results point to a pregnancy-specific response to hypoxia.

Recombinant IL-10 Rescues Hypoxia-Induced Preeclampsia-Like Symptoms in Mice

To support our earlier observations that IL-10−/− mice are hypersensitive to hypoxia-induced effects, we hypothesized that their reconstitution with recombinant IL-10 should protect against hypoxia-induced preeclampsia-like features. Thus, daily administration of recombinant IL-10 from gd 8 to gd 16 was evaluated to reverse the hypoxia-induced effects. As shown in Figure 4, IUGR (Figure 4A) and fetal weight (Figure 4B), elevated systolic blood pressure (Figure 4C), and proteinuria (Figure 4D) were restored to normal values as compared with those under normoxia conditions. Furthermore, recombinant IL-10 significantly reduced hypoxia-induced excess production of sFlt-1 (Figure 4E). The dose...
and duration of administration of IL-10 were established based on pilot experiments. These data strongly point to the protective role of IL-10 against hypoxia-induced preeclampsia.

**Figure 2.** Maternal hypoxia induces renal pathology in pregnant mice. On gd 17, kidneys were harvested and renal tissue processed for hematoxylin & eosin staining from at least 3 animals per group. A representative image of renal tissue section from pregnant mice exposed to 21% (normoxia) or 9.5% (hypoxia) is shown. A, Lower magnification (10×) shows atrophic tubules and interstitial edema in hypoxia-exposed groups. Bold arrows show the increased interstitial space due to atrophy and (B) higher magnification (100×) shows glomerulus that is diffusely enlarged and hypertrophy of intracapillary cells on hypoxia exposure. A representative histology of kidney section from nonpregnant mice subjected to identical conditions of hypoxia is shown at low magnification (10×, C) and at higher magnification (100×, D). Unlike pregnant mice, identical hypoxia treatment in nonpregnant mice did not exhibit renal pathology.

Maternal Hypoxia Causes Uteroplacental Injury, Particularly in the Placental Junctional Zone

To directly correlate the data presented so far to hypoxia-induced placental injury, we next attempted to map hypoxic

**Figure 3.** Serum levels of sFlt-1 and sEng in pregnant wild-type and IL-10−/− mice exposed to normoxia or hypoxia. A, A significant increase was observed in the serum levels of sFlt-1 in response to hypoxia in pregnant mice with significantly higher levels in IL-10−/− mice (n=6). B, sEng levels were also elevated in response to hypoxia, although no differences were observed between wild-type and IL-10−/− mice (n=6). All values are shown as mean±SEM. **P<0.05 as compared with normoxia. #P<0.05 between wild-type and IL-10−/− mice in response to hypoxia.
regions in the uteroplacental tissue in vivo using the hypoxia sensor EF5. A representative experiment is shown in Figure 5A, EF5-specific staining was observed only in tissues from hypoxia-exposed mice with prominent effects in IL-10−/− mice. To map the hypoxic injury in the uteroplacental tissue, we oriented EF5 fluorescence with hematoxylin & eosin staining of the corresponding tissue to denote different regions (Figure 5A). Although some EF5 signals could be seen in the mesometrial and decidua basalis regions, a careful alignment of hypoxic regions suggested predominant EF5 signal in the placental junctional zone compared with normoxia controls (4× and 20× magnification). No significant EF5 signal was observed in the inner labyrinth layer. Wild-type mice exposed to hypoxia showed weaker EF5 staining. These observations are further supported by higher placental hypoxia-inducible factor-1α protein expression in response to hypoxia (Figure 5B). Unlike the enhanced preeclampsia pathology observed in IL-10−/− mice, no IL-10-dependent differences were observed for the induction of hypoxia-inducible factor-1α protein levels, suggesting that the extent of hypoxic injury is not dependent on the hypoxia-inducible factor-1α-mediated effects.

**IL-10 Deficiency Promotes Hypoxia-Induced Trophoblast Apoptosis in the Placenta**

As shown, chronic hypoxic conditions of 9.5% O₂ induced severe preeclampsia-like pathology in IL-10−/− mice with milder effects in wild-type mice. Next, we performed experiments to delineate whether maternal hypoxic exposure caused apoptosis in vivo. Although some TUNEL-positive areas were observed in uteroplacental tissue from normoxia-exposed mice or hypoxia-exposed wild-type mice, a large amount of TUNEL-positive cells was found in the gd 17 IL-10−/− placenta after exposure to hypoxia (data not shown).

To determine whether trophoblasts were undergoing apoptosis, the IL-10−/− placental sections from normoxic and hypoxic conditions were double-stained with TUNEL and an antibody for pancytokeratin for immunofluorescence analysis. As shown in Figure 6, no significant TUNEL-positive signal was detectable in tissue from mice housed under normoxic conditions. However, exposure to hypoxia resulted in TUNEL-positive staining and the overlay with the pancytokeratin staining demonstrated that TUNEL immunofluorescence was mainly present in cytotkeratin-positive cells in the junctional zone.

**Hypoxia Induces Robust Activation of p53-Bax-Bcl2-Caspase 3 Signaling Pathways in IL-10−/− Mice**

Because hypoxia was shown to induce cell death in trophoblasts, we next sought to investigate mechanistic pathways for hypoxia-induced apoptosis in the placenta. Hypoxia induces two major signaling pathways: p53 and Stat3 phos-
phorylation. First, Stat3 protein levels and phosphorylation in wild-type and IL-10/IL-10/IL-10 uteroplacental tissue were examined. No differences were observed for Stat3 phosphorylation and total protein levels after exposure to hypoxia (Supplemental Figure IV). In contrast, p53 was induced in response to hypoxia and its induction was especially prominent in the IL-10/IL-10/IL-10 tissue (Figure 7A). Consistent with this, the p53-dependent kinase inhibitor p21 was induced in a similar manner (Figure 7B).

To further dissect the p53-mediated apoptosis signaling pathways, modulation of its downstream targets, proapoptotic Bax, and antiapoptotic Bcl-2, was investigated. Bax was robustly increased by hypoxia in IL-10/IL-10 placental tissues compared with tissue from wild-type mice (Figure 7C). In contrast, Bcl-2 was concurrently decreased by hypoxia (Figure 7C). To induce active cell death, production of active caspase 3 is critical. The cleaved caspase 3 was elevated in response to hypoxic conditions. Significantly, the amount of cleaved caspase 3 in IL-10/IL-10 placental tissue was drastically higher compared with that seen in wild-type tissue (Figure 7D). These data corroborate enhanced apoptosis observed in the placenta of hypoxia-exposed IL-10/IL-10 mice. The blunting of apoptotic signaling proteins in wild-type placenta resulting in a milder phenotype clearly suggest the protective role of IL-10 in limiting the preeclampsia pathology.

Discussion

Although several reports have discussed the role of low oxygen tension in modulating trophoblast invasion and cell death, direct in vivo evidence linking pathological association of hypoxia with preeclampsia and mechanistic understanding of this pathology are still unsatisfactory. We provide compelling evidence for hypoxia-induced preeclampsia-like features in pregnant mice. As observed from the differential effects in wild-type and IL-10/IL-10 mice in this study, the severity of the disease can be programmed by predispositions such as that resulting from IL-10 deficiency. In this regard, several studies have reported that IL-10 deficiency is associated with adverse pregnancy outcomes such as recurrent preeclampsia.

Figure 5. Maternal hypoxia induces injury in the placenta. A, EF5 staining for normoxia- and hypoxia-exposed placentas in wild-type and IL-10/IL-10 mice. Upper panel shows hematoxylin & eosin sections mapped for mesometrium (M), decidua basalis (DB), junctional zone (JZ), and labyrinthine (LB) of uteroplacental units from animals from normoxia or hypoxia (4x). Middle panel shows EF5 staining of the uteroplacental section under low magnification (4x). Hypoxic injury was found localized to the junctional zone (JZ, indicated by the box). The lower panel shows the higher magnification (20x) of the junctional zone. IL-10/IL-10 mice exhibit more severe hypoxic injury in JZ. B, Immunoblot of hypoxia-inducible factor-1α (HIF-1α) protein in gd 13 uteroplacental tissue. HIF-1α protein in both wild-type and IL-10/IL-10 tissue. A representative of 3 independent experiments is shown.

Figure 6. Maternal hypoxia induces apoptosis in trophoblasts at the maternal–fetal interface. A representative immunofluorescence staining with pancytokeratin antibody (left panel) and TUNEL (middle panel) in IL-10/IL-10 placenta harvested on gd 17 is shown. Overlay (right panel) for both markers indicated trophoblasts undergoing apoptosis on exposure to hypoxia. The apoptotic region coincides with the junctional zone.
spontaneous abortion, preterm birth, and preeclampsia.\textsuperscript{34–37} To buttress this argument, we show here that use of recombinant IL-10 efficiently rescues hypoxia-induced hypertension, proteinuria, IUGR, and excess production of sFlt-1 in IL-10\textsuperscript{−/−} mice. This corroborates with the notion that IL-10 is a critical cytokine for maintenance of normal pregnancy, particularly in response to inflammatory triggers.\textsuperscript{38–41} Moreover, we have demonstrated that IL-10 rescues endovascular crosstalk between trophoblasts and endothelial cells and suppresses inflammation coupled with endothelial cell dysfunction.\textsuperscript{26,42}

Other published reports describing in vitro effects of low oxygen tension on placental development and trophoblast health have pointed to disparate consequences.\textsuperscript{19,20} These could very well be explained by nonpathologic and pathologic levels of O\textsubscript{2}. In our in vivo results confirm that 9.5%, not 11%, O\textsubscript{2} from gd 7.5 to gd 17, imparts pathological consequences and induces preeclampsia-like features, including hypertension, proteinuria, IUGR, production of sFlt-1 and sEng, and renal pathology. As our results demonstrate, the gestational age of initial hypoxia exposure is critical to the onset of preeclampsia symptoms because exposure from either gd 10 or gd 15 to gd 17 did not result in preeclampsia-like features. Importantly, these features occurred strictly in a pregnancy-specific manner because no pathology was seen in nonpregnant mice. In addition, hypoxia-exposed mice, when allowed to recover, experienced a normal second pregnancy cycle with the exception of mild IUGR (data not shown). Our data concur with the notion that low oxygen levels under certain threshold can adversely affect pregnancy and can contribute to the onset of the preeclampsia disease.\textsuperscript{43}

It is well documented that O\textsubscript{2} levels fluctuate during the early stages of pregnancy and regulate trophoblast differentiation and invasion.\textsuperscript{44,45} This process could also be significantly affected by maternal hypoxic conditions and has been shown to be defective in preeclampsia, suggesting that the O\textsubscript{2} levels might have altered and probably reached severe hypoxia levels,\textsuperscript{20} at least in a significant proportion of preeclampsia patients. Recently, attempts have been made in the mouse and rat models to map the in vivo effects of hypoxia.\textsuperscript{24,46} In the rat model, exposure to 11% O\textsubscript{2} levels between gd 6.5 and 13.5 caused more extensive opening of uterine blood vessels and increased invasion by trophoblasts.\textsuperscript{24} In this report, it was not clear if there was a localized hypoxic injury in the placenta. Schaffer et al\textsuperscript{47} have reported on the hypoxic injury in the mouse placenta exposed to acute hypoxia (6% to 7% O\textsubscript{2}) for 24 hours. Under these conditions, hypoxia-inducible factor-1\(\alpha\) was robustly induced in the periphery but not the labyrinth of the placenta. We report a similar pattern of hypoxic injury as detected by intense EF5 staining particularly in IL-10\textsuperscript{−/−} mice. Our data are intriguing in that exposure to 9.5% O\textsubscript{2} between gd 7.5 and gd 17 caused placental hypoxic injury, which could be mapped primarily to the junctional zone bordering the decidua. Hypoxia in wild-type mice resulted only in a weaker EF5 signal that corroborates with the milder activation of apoptotic pathways and systemic effects, implying the moderating role of IL-10. It is noteworthy that hypoxia is reported to inhibit IL-10 production in placental tissues,\textsuperscript{25} which could possibly explain the onset of milder preeclampsia-like symptoms in wild-type mice exposed to 9.5% O\textsubscript{2}. Recent studies in mice that are deficient in natural killer cells suggest that impaired spiral artery remodeling may not cause hypoxic injury and hypertension.\textsuperscript{48,49} Because these mice are IL-10-proficient, we speculate that IL-10 minimizes placental hypoxic injury and cell death pathways as suggested by our current work.

Our data for the first time demonstrate that maternal hypoxia induces placenta-specific overproduction of sFlt-1 and sEng because nonpregnant mice under identical conditions did not exacerbate sFlt-1 or sEng levels. Although the trigger for increased production of these antiangiogenic factors is not clear, studies clearly demonstrate that reduced uterine placental perfusion,\textsuperscript{36,38} placent al hypoxia,\textsuperscript{15,51,52} and other upstream factors\textsuperscript{53} can lead to excess production of sFlt-1 and sEng. However, it is not known whether maternal hypoxia can cause placental injury and cause excessive production of sFlt-1 and sEng. In this regard, our results are noteworthy in that maternal hypoxia induced significant

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**Figure 7.** Maternal hypoxia induces p53-Bax-caspase 3 pathway for placental cell death. A, p53 protein expression in tissue harvested on gd 17. Hypoxia induces significant induction of p53 compared with normoxia tissue, and the induction was more robust in IL-10\textsuperscript{−/−} tissue when compared with wild-type mice. B, A representative immunoblot of p21 protein. p21 exhibits a parallel expression profile as that seen for p53. C, Bax and Bcl-2 expression in normoxic and hypoxic tissue. Bax is induced significantly, particularly in IL-10\textsuperscript{−/−} tissue, whereas Bcl-2 expression decreased significantly. D, Procaspase 3 and cleaved caspase 3 expression in gd 17 uteroplacental tissue. Active caspase 3 bands are uniquely prominent in hypoxic tissue. Data represent at least 3 independent experiments for each protein.
production of these antiangiogenic factors, albeit at a much higher rate in IL-10−/− mice. Moreover, IL-10 deficiency seems to regulate the severity of hypertension, proteinuria, IUGR, hypoxic injury, and placental apoptosis as observed in IL-10−/− mice. Because sFlt-1 and sEng are randomly elevated in patients with preeclampsia, it is plausible that other concurrent pathways contribute equally to hypoxia-induced pathology.

Several in vitro studies have suggested that hypoxic conditions cause apoptosis in a subpopulation of trophoblasts and that the p53 and Bcl-2 pathways may be involved.16,45,54 In agreement with these findings, our studies provide direct in vivo evidence that exposure to hypoxia beyond early pregnancy stages induces apoptotic machinery in the placenta with pronounced effects in IL-10−/− mice. Our data support a model (Figure 8) whereby hypoxia activates the p53 signaling pathway resulting in excessive production of Bax protein, downregulation of Bcl-2, and hyperactivation of caspase 3. Concurrently, hypoxia also triggers increased placental expression and release of sFlt-1 and sEng. These proteins have been postulated to contribute to systemic complications, including hypertension and proteinuria. These signaling pathways are partially blocked by endogenous IL-10 in wild-type mice and by administration of recombinant IL-10 in IL-10−/− mice, suggesting that deficiency in this cytokine may trigger severe pathology.

Using the IL-10−/− mouse model described here, we were able to investigate the precise role of hypoxia in the pathogenesis of preeclampsia. Our studies provide evidence that hypoxia caused localized injury in the uteroplacental tissue, disrupted the placental and systemic angiogenic balance, and contributed to the preeclampsia pathology. Absence of IL-10 further exacerbated this imbalance and contributed to severe preeclampsia. Thus, our model provides potential opportunities to study the influence of IL-10, hypoxia, and immune cell interactions, particularly uterine natural killer cells and regulatory T cells, and their contribution to preeclampsia.

Perspectives
We provide in vivo evidence for hypoxia-induced preeclampsia-like features. We demonstrate that O2 beyond a threshold is capable of inducing severe preeclampsia-like features in pregnant IL-10−/− mice. The pathogenesis in this in vivo model for preeclampsia implicates placental injury, elevation of soluble antiangiogenic factors, and activation of the p53 signaling cascade resulting in placental apoptosis. Wild-type mice exhibit mild features of preeclampsia, and rescue of preeclampsia-like features by recombinant IL-10 suggests a regulatory role of IL-10 in programming the severity of preeclampsia. The in vivo model described here can be used to characterize trophoblast functions, immune regulation, and signaling pathways leading to preeclampsia-like disease in response to graded O2 levels.

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Disclosures
None.

References


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A critical role of IL-10 in modulating hypoxia-induced preeclampsia-like disease in mice

Short Title: In Vivo Model of Hypoxia-induced Preeclampsia

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**Detailed Materials and Methods**

**Animals**

The mice and mating procedures used in this study have been previously described.\(^1,2\) Briefly, C57BL/6 (wild type) and C57BL/6 IL-10\(^{-/-}\) (IL-10\(^{-/-}\)) were obtained from the Jackson Laboratory. Unless otherwise stated, experimental and control animals were strain- and age-matched. For timed pregnancies, syngeneic matings were set up, and the day when the vaginal plug was detected was considered as day 0. All mice were housed in a specific pathogen-free facility supervised by the Central Research Department of Rhode Island Hospital. All protocols were approved by the Lifespan Animal Welfare Committee and conducted according to its guidelines.

**Exposure to hypoxic environment**

Normobaric conditions were employed to avoid potential harmful consequences of rapid pressure variations. To achieve this, mice were placed in the hypoxia chamber (Coy Laboratory Products, Inc., Grass Lake, MI) calibrated for varying (9.5%, or 11%) levels of O\(_2\), 5% CO\(_2\), and 85.5% N\(_2\) from either gestational day (gd) 7.5 or gd 10 or gd 15 through gd 17. The oxygen levels were decreased 2% every 10 minutes until they reached the desired levels. To evaluate the protective role of IL-10 in hypoxia-induced preeclampsia, a subset of IL-10\(^{-/-}\) mice were injected with recombinant mouse IL-10 (500 ng/ mouse, R&D Systems, MN, USA) or saline from gd 8 to gd 16 and exposed to 9.5% hypoxia as described above. The dose of IL-10 was selected based on our previous rescue experiments.\(^1,2\) Similarly non-pregnant animals were exposed to hypoxia for 9.5 days and monitored for blood pressure, proteinuria, kidney pathology and serum levels of anti-angiogenic factors as described below.

**Blood pressure measurement**

Blood pressure was recorded by an established tail-cuff method which utilizes a programmed sphygmanometer.\(^3\) A noninvasive computerized tail-cuff system (Digi-med, Inc., Louisville, KY) was utilized for measuring blood pressure in mice. The procedure used for this instrument was performed according to the manufacturer's instruction manual. For 5–7 days, mice were trained to acclimatize to restraint and tail-cuff procedure. The restraint platform was maintained at ~37–39°C. At least 8 readings were taken, the highest and lowest readings were discarded, and the remaining readings were averaged for a single session value. At least three independent experiments for each group were performed and a representative was shown.

**24-hour urine collection and urine protein assay**

On pregnancy day 16, mice were transferred to the metabolic cage placed in hypoxic chamber for the duration of experiment for 24-hour urine collection. The metabolic chamber is designed to collect urine and fecal matter separately and minimizes their mixing and animals had access to food and water *ad libitum*. Urine was then centrifuged at 500 g at 4°C for 5 minutes to pellet cellular debris. Albumin and creatinine levels were measured using the Albuwell (Exocell, Inc., Philadelphia, PA) and Creatinine Companion (Exocell, Inc., Philadelphia, PA) kits according to the manufacturer's protocols. Urinary albumin was normalized to creatinine excretion and is presented as micrograms of albumin per milligram of creatinine.
Assessment of renal pathology
Kidney tissue were harvested from gd 17 mice, fixed in 10% buffered formalin and were stained with hematoxylin/eosin and periodic acid Schiff for histopathological examination as described. Non-pregnant mice exposed to normoxia or hypoxia for similar period were processed and stained. A number of randomly selected glomeruli were assessed by at least two pathologists. Morphological changes were recorded using SPOT™ Advanced software (Diagnostic Instruments Inc, MI, USA) at 100x magnification (Nikon Eclipse 80i microscope).

Serum collection and ELISA for sFlt-1 and sEng
On gd 17, mice were sacrificed and blood was collected by cardiac puncture. After 3-5 minutes at room temperature, blood was centrifuged at 500 g for 5 minutes and supernatant collected and stored at -80°C until further use. Free sFlt-1 and VEGF in serum was measured according to the manufacturer’s instructions (Quantikine® mouse sVEGF R1, and VEGF ELISA kits, R&D Systems Inc., Minneapolis, MN). Free sEng in serum was also measured by ELISA (Mouse Endoglin/CD105 DuoSet, R&D Systems Inc., Minneapolis, MN) according to the manufacturer’s instructions. Samples were measured in duplicate and the values are expressed as mean± SEM.

In vivo detection of tissue hypoxia
Local hypoxia was detected by the marker EF-5, a gift from Dr. Cameron Koch (University of Pennsylvania). EF-5 is biochemically reduced in response to low oxygen levels and forms intracellular adducts with cellular macromolecules in viable hypoxic cells, thereby demarcating regions of hypoxia. Briefly, EF-5 (10 µM) was administered intraperitoneally four hours before harvesting the utero-placental tissues on gd 13. The tissue was snap-frozen and 10-µm sections were processed and probed for EF-5-positive signal using ELK3–51 antibody (1:30) as described. For orientation placental morphology was monitored by harvesting uteroplacental units on gd 13, fixed and paraffin embedded, sectioned and stained with hematoxylin and eosin (H&E) staining as described. We then acquired the images with a Nikon Eclipse 80i microscope (Nikon) at 4x magnification.

Real-time quantitative PCR
Placental tissue was first homogenized and total RNA was isolated using the Qiagen RNeasy kit (Valencia, CA) according to the manufacturer’s protocol. After DNase (Valencia, CA) treatment, 2 µg of total RNA was reverse transcribed into cDNA using Superscript III and Random Hexamers (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Real-time quantitative PCR amplification was performed on a cycler (Mastercycler ep realplex, Eppendorf) using SYBR Green I PCR Core Reagents (PE Applied Biosystems, Foster City, CA). To quantify the amount of cDNA for an individual transcript, SYBR Green I fluorescence was measured at the end of each cycle. All samples and standards were run in triplicate for any given experiment. The values of individual gene were normalized to β2-microglobulin (β2M) by dividing the average copy number of the respective transcript by the average copy number of β2M in the respective sample. The primer sets used here are: β2M, sense 5'-
AATCCAGTTTCTAATATGCTA-3', antisense 5’- TATTGCTCAGCTATCTAGG-3’; sFlt-1, sense 5’-CAGCACATCATGCAAGCAG-3’ antisense 5’-TGCAGAATTGTTGCCATT-3’; sEng, sense 5’-AGCCTCCAGCCCCACAAGT-3’, antisense 5’-CCTTCGAGACCTGGCTAGTG-3’. Specific products were verified by melt-curve analysis and gel electrophoresis.

**Western Blot analysis**
Placental tissue was collected and stored at -80°C. After homogenizing and lysis with T-per buffer (Pierce, Rockford, IL), 50 µg of protein was resolved on a SDS-PAGE gel, then transferred to a nitrocellulose membrane (Millipore, Bedford, MA), blocked with 5% non-fat milk in Tris-Buffered Saline Tween-20 (TBST), and blotted with an indicated antibody in 1% BSA. Following incubation with an HRP-conjugated secondary antibody, membranes were developed by Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The following antibodies were used for the studies: anti-p53, anti-p21, anti-Bax, anti-Bcl-2, anti-caspase 3, anti-VEGF A, anti-VEGF C, anti-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-Stat3, anti-phospho-Stat3 (Cell Signaling, Beverly, MA), and anti-HIF-1α was purchased from Novus (Littleton, CO).

**In situ detection of apoptotic cells**
Placental tissue was fixed in 10% paraformaldehyde and sectioned. The tissue sections were then subjected to TUNEL staining for the presence of apoptotic cells using the ApopTag Peroxidase Kit (Chemicon, Rosemont, IL) according to manufacturer’s recommendations. Uteroplacental tissue structural orientation was demarcated by H & E staining.

**Immunofluorescence**
Frozen placental sections were air-dried for 30 minutes prior to fixation and then fixed for 10 minutes in ice cold acetone. Air dried sections were blocked with 10% horse serum overnight at 4°C and incubated with anti-pancytokeratin (Santa Cruz, CA) overnight at 4°C following Alexa Fluor 594 IgG (Invitrogen, Carlsbad, CA) incubation for 2 hours at room temperature. Then the section was permeabilized with 1% Triton 100 and subjected to TUNEL staining (**In Situ** cell death detection kit, Fluorescein, Roche).

**Statistics**
Data were compared using the Student’s t-test if the data were normally distributed. A p-value less than 0.05 were considered significant. Data are presented as the mean ± SEM from at least three independent experiments.
Supplemental References


Supplementary Table
Table S1: Evaluation of hypoxic conditions for assessment of preeclampsia-like features in pregnant mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild type</th>
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<tbody>
<tr>
<td>Oxygen levels</td>
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<td>21.0% 9.5% 11.0%</td>
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<tr>
<td>Blood Pressure on gd17 (mmHg)</td>
<td>±8.9 ±10.4 ±8.9</td>
<td>±8.3 ±9.9</td>
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<tr>
<td>Proteinuria† on gd17 (µg/mg)</td>
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<tr>
<td>Fetal Weight on gd17 (g)</td>
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<td>±0.5 ±0.2</td>
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<tr>
<td>sFlt-1 on gd17 (ng/ml)</td>
<td>±13.6 ±20.1 ±17.6</td>
<td>±17.7 ±19.7</td>
</tr>
</tbody>
</table>

All data were obtained from pregnant mice (n = 3–4 per group) subjected to normoxia or hypoxia from the indicated gestational day to gd 17 and are expressed as Mean ± SD. *Exposure to 8.5% O₂ from gd 7.5 to gd 16 turned out to be lethal to pregnant mice. †Expressed as albumin/creatinine ratio.
Figure S1. Hypoxia failed to induce preeclampsia-like symptoms in non-pregnant mice. Non-pregnant mice (wild type and IL-10−/−) were subjected to normoxia (21% O₂) or hypoxia (9.5% O₂) from day 7.5 to day 17. (A) Systolic blood pressure remains unchanged in response to hypoxia treatment. No differences were observed between wild type and IL-10−/− mice after treatment (n=6). (B) No significant differences were observed in proteinuria between normoxia and hypoxia exposure in wild type and IL-10−/− mice (n=6). (C) Measurement of serum sFlt-1 levels by ELISA showed no significant differences between normoxia and hypoxia conditions. All values are shown as Mean ± SEM.
Figure S2. mRNA expression of sFlt-1 and sEng in uteroplacental tissue from wild type and IL-10⁻/⁻ mice. Mice (wild type or IL-10⁻/⁻) were exposed to normoxia (21% O₂) or hypoxia (9.5% oxygen) from gd 7.5 to gd 17 as described in Methods. Total RNA was isolated and sFlt-1 and sEng transcripts were quantified by real-time PCR and normalized to β2 microglobulin (β2M). Panel (A) shows sFlt-1 mRNA. A significant induction in mRNA of sFlt-1 is observed in response to hypoxia with significantly higher signal in IL-10⁻/⁻ mice (n=6). (B) Shows sEng mRNA quantification. A significant increase in mRNA of sEng was observed in response to hypoxia in wild type and IL-10⁻/⁻ mice (n=6). All values are shown in mean ± SEM. * p<0.05 significance between normoxia and hypoxia group, ** p<0.05 significance between wild type and IL-10⁻/⁻ mice under hypoxia.
Figure S3. VEGF A and VEGF C protein measurement in uteroplacental tissue. (A and B) and in serum (C). Uteroplacental tissue was harvested on gd 17, protein lysates were prepared and subjected to western blotting with specific antibodies for VEGF-A (Panel A), VEGF-C (Panel B), and actin. This data shows a representative immunoblot from three independent experiments. Panel C shows serum levels of total VEGF as measured using mouse VEGF-specific ELISA. No significant differences were observed in placental VEGFs or in serum levels of VEGF in response to hypoxia (9.5% O₂) exposure either in wild type or IL-10⁻/⁻ mice as compared to normoxia (21% O₂).
Figure S4. Immunoblot analysis of Stat3 and its phosphorylated forms (phospho-Stat3 Y705 and Ser727). Uteroplacental tissue was harvested on gd 17, protein lysates were prepared and subjected to western blotting with specific antibodies for native Stat3, its phosphorylated forms, and actin. This data shows a representative immunoblot from three independent experiments. No differences were observed either after hypoxia (9.5% O₂) exposure or between wild type and IL-10⁻/⁻ mice.