Hypoxia-Independent Upregulation of Placental Hypoxia Inducible Factor-1α Gene Expression Contributes to the Pathogenesis of Preeclampsia

Takayuki Iriyama, Wei Wang, Nicholas F. Parchim, Anren Song, Sean C. Blackwell, Baha M. Sibai, Rodney E. Kellems, Yang Xia

Abstract—Accumulation of hypoxia inducible factor-1α (HIF-1α) is commonly an acute and beneficial response to hypoxia, whereas chronically elevated HIF-1α is associated with multiple disease conditions, including preeclampsia, a serious hypertensive disease of pregnancy. However, the molecular basis underlying the persistent elevation of placental HIF-1α in preeclampsia and its role in the pathogenesis of preeclampsia are poorly understood. Here we report that HIF-1α mRNA and HIF-1α protein were elevated in the placentas of pregnant mice infused with angiotensin II type I receptor agonistic autoantibody, a pathogenic factor in preeclampsia. Knockdown of placental HIF-1α mRNA by specific siRNA significantly attenuated hallmark features of preeclampsia induced by angiotensin II type I receptor agonistic autoantibody in pregnant mice, including hypertension, proteinuria, kidney damage, impaired placental vasculature, and elevated maternal circulating soluble fms-like tyrosine kinase-1 levels. Next, we discovered that HIF-1α mRNA levels and HIF-1α protein levels were induced in an independent preeclampsia model with infusion of the inflammatory cytokine tumor necrosis factor superfamily member 14 (LIGHT). SiRNA knockdown experiments also demonstrated that elevated HIF-1α contributed to LIGHT-induced preeclampsia features. Translational studies with human placentas showed that angiotensin II type I receptor agonistic autoantibody or LIGHT is capable of inducing HIF-1α in a hypoxia-independent manner. Moreover, increased HIF-1α was found to be responsible for angiotensin II type I receptor agonistic autoantibody or LIGHT-induced elevation of Flt-1 gene expression and production of soluble fms-like tyrosine kinase-1 in human villous explants. Overall, we demonstrated that hypoxia-independent stimulation of HIF-1α gene expression in the placenta is a common pathogenic mechanism promoting disease progression. Our findings reveal new insight to preeclampsia and highlight novel therapeutic possibilities for the disease. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.05314.)

Key words: hypoxia inducible factor-1α ■ preeclampsia ■ tumor necrosis factor superfamily member 14

Preeclampsia is a life-threatening hypertensive complication of pregnancy and is a leading cause of maternal and neonatal morbidity and mortality.1,2 Despite intensive research efforts and several large clinical trials, current strategies for managing preeclampsia remain inadequate and are limited to symptomatic therapy or the termination of pregnancy. Thus, uncovering novel factors and signaling pathways that contribute to the pathogenesis of preeclampsia are needed for the establishment of mechanism-based preventative and therapeutic strategies to improve the prognosis of the disease.

Hypoxia inducible factor-1 (HIF-1) is a key transcription factor that plays a central role in the cellular response to low oxygen tension under physiological and pathological conditions.3,4 HIF-1 is a heterodimer consisting of 2 subunits, α and β. Although HIF-1β is constitutively expressed, HIF-1α levels are precisely regulated by post-translational modification depending on oxygen tension. HIF-1α is rapidly degraded under normoxic conditions, but quickly stabilized when oxygen availability is reduced. Thus, hypoxia-induced HIF-1α is usually transient and brief at the protein level.4 A variety of studies have shown that women with preeclampsia are characterized by persistently elevated placental HIF-1α levels that promote enhanced transcription of genes encoding soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng), and endothelin-1 (ET-1), all known to contribute to preeclampsia.5-7 However, the molecular basis underlying prolonged elevated placental HIF-1α in preeclampsia and the pathological role of
sustained elevated HIF-1α in preeclampsia are largely unknown.

Numerous recent studies have shown that HIF-1α levels can be regulated by means that are independent of hypoxia. For example, angiotensin II and the inflammatory cytokines tumor necrosis factor (TNF) and interleukin (IL)-6 induce HIF-1α gene expression in vascular smooth muscle cells, kidney cells, and hepatocytes, respectively. Multiple studies have revealed that inflammatory cytokines and autoantibodies are elevated in preeclampsia patients and contribute to pathophysiology preeclampsia. For example, earlier studies showed that injection or infusion of pathogenic autoantibodies, such as the angiotensin II type 1 receptor agonistic autoantibody (AT1-AA) or the inflammatory cytokine tumor necrosis factor superfamily member 14 (LIGHT), into pregnant mice results in features of preeclampsia, including hypertension, proteinuria, placental abnormalities, and increased circulating sFlt-1, soluble endoglin, and endothelin-1. Thus, we hypothesized that the pathogenic autoantibody, AT1-AA, and the inflammatory cytokine, LIGHT, stimulate placental HIF-1α production and in this way contribute to features of preeclampsia. Here we conducted both mouse and human studies to assess these hypotheses.

Methods

For detailed descriptions, refer to the Methods section in the online-only Data Supplement.

Results

Increased Placental HIF-1α Contributes to the Development of Preeclampsia Features in an Autoantibody-Injection Model of Preeclampsia in Pregnant Mice

To examine a potential role of elevated HIF-1α in preeclampsia, we took advantage of an experimental model of preeclampsia in mice induced by the injection of patient-derived-IgG (PE-IgG) known to contain the pathogenic autoantibodies, AT1-AA. We found that Hif-1α gene expression was induced significantly in the placentas of mice injected with PE-IgG compared with the pregnant mice injected with IgG from normotensive pregnant women (NT-IgG; Figure 1A). In contrast, no significant difference was observed in the kidneys between PE-IgG- and NT-IgG-injected pregnant mice (Figure S1 in the online-only Data Supplement). We also confirmed that PE-IgG induced the elevation of placental HIF-1α expression at the protein level by immunoblotting (Figure 1B). Additionally, immunohistochemical analysis revealed that the PE-IgG-induced elevation of HIF-1α protein expression throughout the placenta (Figure 1C and 1D). In addition, the PE-IgG-induced elevation of placental HIF-1α expression was almost completely inhibited when PE-IgG was coinjected with losartan, an angiotensin II type 1 receptor (AT1) blocker, or with the autoantibody-neutralizing 7 amino acid epitope peptide (Figure 1A–1D). These results indicate that the elevation of placental HIF-1α resulting from injection of PE-IgG was because of the activation of AT1Rs by AT1-AA.

Global HIF-1α-deficient mice die in midgestation from cardiac and vascular malformation. This embryonic lethality makes it difficult to examine the in vivo role of HIF-1α. To determine the pathophysiologic significance for PE-IgG-induced placental HIF-1α expression, we conducted siRNA-induced in vivo knockdown of Hif-1α mRNA. Briefly, siRNA-encapsulated nanoparticles were injected into pregnant mice on embryonic days 13.5 and 14.5, together with PE-IgG to specifically knockdown Hif-1α mRNA levels. As shown in Figure 2A, placental Hif-1α mRNA levels were successfully downregulated in Hif-1α siRNA-injected mice compared with control scrambled siRNA-injected mice. We also confirmed the reduction of HIF-1α protein expression levels in the placentas of Hif-1α siRNA-injected mice by immunoblotting (Figure S2). As a result of in vivo knockdown of Hif-1α mRNA, we found that PE-IgG-induced diagnostic features of preeclampsia, hypertension and proteinuria, were significantly reduced compared with control siRNA-injected mice (Figure 2B and 2C). Histological analysis of mouse kidneys revealed that PE-IgG-induced pathological changes seen in the glomeruli of control siRNA-injected mice (ie, swollen glomeruli with narrowed capillary and Bowman’s spaces) were attenuated in the kidneys of Hif-1α siRNA-injected mice (Figure 2D). Additionally, we found that placentas of Hif-1α siRNA-injected mice displayed significantly less tissue damage, including placental calcifications, a hallmark of placental distress observed in placentas of preeclampsia patients, as compared with those of control siRNA-injected mice (Figure S3). Moreover, we examined placental vasculature using CD31 staining. As a result, PE-IgG-induced disorganized and impaired vasculature in the labyrinthine zone of control siRNA-injected mice (low density of CD31-positive vessels and narrowed capillary spaces) was attenuated in the placentas of Hif-1α siRNA-injected mice (Figure 2E).

The Flt-1 gene is a direct transcriptional target of HIF-1α. A splice variant encodes sFlt-1, an antiangiogenic factor secreted by the placenta into the maternal circulation, that is believed to contribute to the development of systemic endothelial dysfunction, hypertension, and multiorgan damage, including the kidneys in preeclampsia patients. As such, we also found that Hif-1α mRNA knockdown in vivo suppressed the PE-IgG-induced elevation of Flt-1 mRNA in the placenta, as well as circulating sFlt-1 levels (Figure 2F and 2G). These results provide in vivo evidence that the induction of HIF-1α in the placenta contributes to the development of pathogenic autoantibody-induced features of preeclampsia and is also involved in the increased sFlt-1 production.

Elevated HIF-1α Contributes to the Development of LIGHT-Induced Preeclampsia Features

Emerging evidence indicates that an increased inflammatory response is involved in preeclampsia. Supporting this concept, a recent study showed that a member of the TNF superfamily, LIGHT, is elevated in the circulation and placentas of preeclampsia patients and that the injection of LIGHT into pregnant mice induces features of preeclampsia, including the overproduction of sFlt-1. The following experiments were conducted to determine whether elevated HIF-1α contributes to LIGHT-induced preeclampsia features...
in pregnant mice. We found that LIGHT injection into pregnant mice resulted in increased levels of Hif-1α mRNA in placentas (Figure 3A) but not in kidneys (Figure S1). Next, we found that LIGHT-induced placental Hif-1α mRNA levels were significantly reduced by neutralizing antibodies specific for LIGHT receptors: lymphotoxin β receptor and herpes virus entry mediator (Figure 3A). These results indicated that LIGHT signaling via its receptors induced placental Hif-1α gene expression.

To assess whether elevated HIF-1α in the placenta plays a detrimental role in the LIGHT-induced preeclampsia development as it does in PE-IgG-injected mice, we conducted in vivo knockdown of Hif-1α mRNA by injecting Hif-1α siRNA-encapsulated nanoparticles, together with LIGHT, into pregnant mice. Hif-1α siRNA injection successfully reduced the levels of placental Hif-1α mRNA compared with those of control siRNA-injected mice (Figure 3A). Moreover, preeclamptic features induced by LIGHT injection (hypertension and proteinuria)
were attenuated significantly in Hif-1α siRNA-injected pregnant mice compared with control siRNA-injected pregnant mice (Figure 3B and 3C). Additionally, we found that Hif-1α mRNA knockdown attenuated the LIGHT-induced elevation of Flt-1 mRNA levels, as well as circulating sFlt-1 protein (Figure 3D and 3E). These findings indicate that placental elevated HIF-1α contributes to the development of preeclampsia features in the LIGHT-induced experimental model of preeclampsia.
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HIF-1α Is Elevated in Placentas of Preeclampsia Patients and AT1-AA or LIGHT Directly Induces HIF-1α Expression in Cultured Human Placental Villous Explants Independent of Hypoxia

To extend our mouse findings to humans, we determined that the expression of HIF-1α was elevated in placentas of preeclampsia patients at both mRNA and protein levels compared with those of normotensive pregnant women (Figure 4A–4C).

To determine whether AT1-AA or LIGHT can directly induce HIF-1α gene expression in the human placenta independent of hypoxia, we used primary human placental villous explants isolated from normotensive pregnant women. We cultured human villous explants treated with PE-IgG, NT-IgG, or LIGHT under ambient oxygen levels. We found that PE-IgG significantly induced HIF-1α mRNA levels compared with the NT-IgG-treated human villous explants, and the induction was significantly reduced by cotreatment with losartan to inhibit AT1R activation or 7 amino acid epitope peptide to neutralize AT1-AA (Figure 4D). Similarly, we found that treatment of cultured villous explants with LIGHT resulted in increased HIF-1α mRNA levels compared with the controls (Figure 4E). Additionally, we also confirmed that PE-IgG or LIGHT induced the elevation of HIF-1α protein (Figure 4F).

Thus, these results indicate that AT1-AA and LIGHT are capable of directly inducing HIF-1α gene expression in cultured human villous explants independent of hypoxia.

AT1-AA- or LIGHT-Induced HIF-1α Promotes Flt-1 Gene Expression and Subsequent sFlt-1 Secretion in Human Villous Explants Independent of Hypoxia

We examined whether PE-IgG- or LIGHT-induced HIF-1α is capable of promoting Flt-1 gene expression and subsequent sFlt-1 production in human placental explants independent of hypoxia. To test this possibility, we treated human villous explants under ambient oxygen levels with PE-IgG or LIGHT in the presence or absence of CAY10585, a specific HIF-1α inhibitor. First, we confirmed that the elevation of HIF-1α induced by PE-IgG or LIGHT was suppressed by the treatment of CAY10585 (Figure 5A). We found that Flt-1 mRNA levels and the amount of secreted sFlt-1 were increased by the treatment of human villous explants with PE-IgG or LIGHT (Figure 5B and 5C). In contrast, treatment with a HIF-1α inhibitor, CAY10585, significantly reduced PE-IgG- or LIGHT-induced Flt-1 gene expression and sFlt-1 production.

Figure 3. Specific knockdown of Hif-1α mRNA in vivo suppresses the features of preeclampsia induced by tumor necrosis factor superfamily member 14 (LIGHT). Pregnant mice were injected with saline or LIGHT with or without neutralizing antibodies against lymphotoxin β receptor (anti-LTβR) or herpes virus entry mediator (anti-HVEM) on embryonic days 13.5 and 14.5. Additionally, in vivo siRNA-mediated Hif-1α mRNA knockdown was conducted. Samples were collected on E18.5. A, Placental Hif-1α mRNA levels were quantified using real-time RT-PCR (n=4–7 mice per group; **P<0.01 vs saline, *P<0.05 vs LIGHT, †P<0.01 vs LIGHT+control siRNA). B, Blood pressure was measured by tail-cuff plethysmography on a daily base (n=5 or 6 mice per group; *P<0.05, **P<0.01 vs saline, *P<0.01 vs LIGHT+control siRNA at the same time points). C, Proteinuria was determined as urine albumin to creatinine ratio by ELISA (n=5 or 6 mice per group; *P<0.05 vs saline, **P<0.01 vs LIGHT+control siRNA). D, Placental Flt-1 mRNA levels were quantified using real-time RT-PCR (n=5 or 6 mice per group; **P<0.01 vs saline, *P<0.05 vs LIGHT+control siRNA). E, The levels of circulating soluble fms-like tyrosine kinase-1 (sFlt-1) in mouse plasma were determined by ELISA (n=6 or 7 mice per group; **P<0.01 vs saline, *P<0.05 vs LIGHT+control siRNA). HIF-1α indicates hypoxia inducible factor-1α.
Here we report that α Hif-1 expression in a HIF-1-dependent manner in cultured human α gene induction and subsequent sFlt-1 production by nonhypoxic stimuli in various cell types. For example, studies in vascular smooth muscle cells showed that angiotensin II stimulates HIF-1α protein production by a protein kinase C–mediated transcriptional activation of the Hif-1α gene expression and by a reactive oxygen species–dependent mechanism, leading to enhanced translation of Hif-1α mRNA.10,21 In this case, angiotensin II induces HIF-1α protein, leading to an increase in vascular endothelial growth factor gene expression. Other studies have shown that angiotensin II induces Hif-1α mRNA production in renal glomerular cells.25 Women with preeclampsia harbor autoantibodies (AT1-μ) that mimic the action of AT1R. These pathogenic autoantibodies may serve as hypoxia-inducible factor-1α, capable of directly inducing HIF-1α gene expression in human villous explants independent of hypoxia. We also showed that specific siRNA knockdown of Hif-1α mRNA attenuated hallmark features of preeclampsia, including hypertension, proteinuria, kidney damage, impaired placental vasculature, and maternal elevated circulating sFlt-1 in both preeclampsia mouse models. These results indicate that increased HIF-1α gene expression is a common pathogenic factor contributing to preeclampsia. Extending animal studies to humans, we confirmed that HIF-1α mRNA and HIF-1α protein levels were elevated in the placentas of preeclampsia patients. Using human villous explant cultures under nonhypoxic conditions, we showed that AT1-AA or LIGHT induced HIF-1α mRNA and HIF-1α protein levels, resulting in increased sFlt-1 secretion in a hypoxia-independent manner. Overall, we provide both in vivo animal studies and in vitro human evidence showing the pathogenic role of elevated HIF-1α gene expression in preeclampsia and hypoxia-independent mechanisms underlying its elevation in the placentas.

Numerous early studies showed that HIF-1α can be induced by nonhypoxic stimuli in various cell types. For example, studies in vascular smooth muscle cells showed that angiotensin II stimulates HIF-1α production by a protein kinase C–mediated transcriptional activation of the Hif-1α gene expression and by a reactive oxygen species–dependent mechanism, leading to enhanced translation of Hif-1α mRNA.10,21 In this case, angiotensin II induces HIF-1α protein, leading to an increase in vascular endothelial growth factor gene expression. Other studies have shown that angiotensin II induces Hif-1α mRNA production in renal glomerular cells.25 Women with preeclampsia harbor autoantibodies (AT1-μ) that mimic the action of angiotensin II and activate the major angiotensin receptor, AT1R. These pathogenic autoantibodies may serve as hypoxia-inducible factor-1α, capable of directly inducing HIF-1α gene expression in human villous explants independent of hypoxia. We also showed that specific siRNA knockdown of Hif-1α mRNA attenuated hallmark features of preeclampsia, including hypertension, proteinuria, kidney damage, impaired placental vasculature, and maternal elevated circulating sFlt-1 in both preeclampsia mouse models. These results indicate that increased HIF-1α gene expression is a common pathogenic factor contributing to preeclampsia. Extending animal studies to humans, we confirmed that HIF-1α mRNA and HIF-1α protein levels were elevated in the placentas of preeclampsia patients. Using human villous explant cultures under nonhypoxic conditions, we showed that AT1-AA or LIGHT induced HIF-1α mRNA and HIF-1α protein levels, resulting in increased sFlt-1 secretion in a hypoxia-independent manner. Overall, we provide both in vivo animal studies and in vitro human evidence showing the pathogenic role of elevated HIF-1α gene expression in preeclampsia and hypoxia-independent mechanisms underlying its elevation in the placentas.

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Figure 5. HIF-1α is responsible for angiotensin II type I receptor agonistic autoantibody (AT1-AA)- or tumor necrosis factor superfamily member 14 (LIGHT)-induced elevation of Flt-1 gene expression and increased production of soluble fms-like tyrosine kinase-1 (sFlt-1) in human villous explants independent of hypoxia. A, HIF-1α protein levels in human villous explants detected by immunoblotting. Human villous explants were pretreated with or without 10 μM CAY10585 for 15 minutes and then treated with 100 μg/mL NT-IgG or PE-IgG or 100pg/mL LIGHT for 24 hours. Explants were also treated with 10 μM dimethyloxaloylglycine (DMOG), a prolyl hydroxylase (PHD) inhibitor, for 24 hours as a positive control. B, Flt-1 mRNA levels were quantified using real-time RT-PCR (n=4 independent experiments; **P<0.01 vs NT-IgG or PBS-treated group, #P<0.05, ##P<0.01 vs PE-IgG or LIGHT-treated group). C, Secreted sFlt-1 protein levels in the culture media were determined by ELISA (n=5 independent experiments; **P<0.01 vs NT-IgG or PBS-treated group, #P<0.01 vs PE-IgG or LIGHT-treated group). D, Knockdown of HIF-1α in human villous explants. The explants were treated with nanoparticle-encapsulated control (con) -or HIF-1α-siRNA for 24 hours and then were treated with NT-IgG or PE-IgG (100 μg/mL) or LIGHT (100 pg/mL) for 24 hours. HIF-1α protein was assessed by immunoblotting. E, Flt-1 mRNA levels of human villous explants were quantified using real-time RT-PCR (n=4 independent experiments; Continued
Figure 5 Continued. **P<0.01 vs NT-IgG or PBS+control siRNA, *P<0.05, **P<0.01 vs PE-IgG or LIGHT+ control siRNA group). F, Secreted sFlt-1 protein levels in the culture media were determined by ELISA (n=4 independent experiments; **P<0.01 vs NT-IgG or PBS+control siRNA, ***P<0.01 vs PE-IgG or LIGHT+ control siRNA group). G, Working model of our study. AT1-AA or LIGHT-induced HIF-1α production in placental trophoblasts is followed by HIF-1α-mediated induction of Flt-1 gene expression and subsequent sFlt-1 protein production independent of hypoxia. Persistent elevation of HIF-1α causes chronic overproduction of sFlt-1 and contributes to impaired placental vascular development, maternal endothelial dysfunction, and disease progression. HIF-1α indicates hypoxia inducible factor-1α; NS, nonspecific bands; NT-IgG, pregnant mice injected with purified IgG from normotensive pregnant women; and PE-IgG, pregnant mice injected with purified IgG from preeclampsia patients.

Supporting our hypothesis, we have provided human in vitro studies showing that purified PE-IgG, which contains AT1-AA, activates AT1Rs, resulting in the induction of HIF-1α gene expression under nonhypoxic conditions. Additionally, we demonstrated that Hif-1α expression is induced significantly in the placentas of mice injected with PE-IgG. We realize that the PE-IgG used in our experiment is a complex mixture of immunoglobulins that is likely to contain other autoantibodies. However, we have shown in prior publications that the same effects are seen when AT1-AA is specifically purified by affinity chromatography.17 Furthermore, the PE-IgG or affinity-purified AT1-AA-induced preeclampsia features are blocked by losartan or the 7aa epitope peptide, indicating that the effects are mediated by interaction with the specific epitope on the AT1 receptor. Our results are also in good agreement with those of Wenzel et al, who showed that pregnant rats infused with a rabbit antibody that activates AT1Rs resulted in elevated HIF-1α in rat placentas.21 Our current study has provided human and mouse evidence that the activation of AT1Rs by AT1-AA induces HIF-1α gene expression in the placenta, thereby contributing to the development of preeclampsia.

A growing body of studies indicate that preeclampsia is characterized by increased circulating levels of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-17, and LIGHT.12,13,15 A pathological role for these cytokines is supported by experimental evidence showing that infusion of these cytokines into pregnant rodents produces features of preeclampsia, including the production of AT1-AA.15,16 These results suggest that the elevation of inflammatory cytokines is an early event in the development of preeclampsia and functions upstream of AT1-AA production. Additional evidence shows that inflammatory cytokines induce HIF-1α under nonhypoxic conditions.4 For example, TNF-α and IL-1β activate HIF-1α gene expression in human hepatoma cells,24 TNF-α increases HIF-1α mRNA levels, as well as the expression of HIF-1 target genes glucose transporter type 1 and type 3 in human embryonic kidney cells.9 IL-6 induces both HIF-1α mRNA and HIF-1α protein levels and the expression of the HIF-1α target gene, erythropoietin, in hepatocytes.11 IL-1β induces HIF-1α-mediated VEGF secretion in trophoblast cells.25 Thus, a pathogenic role for HIF-1α in response to elevated inflammatory cytokines associated with preeclampsia may be an important contributor to disease pathogenesis. However, a role for HIF-1α in increased inflammatory cytokine-induced features in preeclampsia was not recognized before the results of our experiments reported here with the inflammatory cytokine, LIGHT. As a member of the TNF superfamily, LIGHT is known to be elevated and contributes to preeclampsia features in pregnant mice.15 As with AT1-AA, we have shown here that infusion of LIGHT stimulates production of HIF-1α in placentas of pregnant mice and in human villous explants independent of hypoxia. The increased levels of HIF-1α stimulate the transcriptional activation of the Flt-1 gene, thereby providing for excessive sFlt-1 production, leading to the features of preeclampsia. Our data represent the first in vivo animal evidence showing the pathological role of HIF-1α in inflammatory cytokine-induced preeclampsia pathophysiology.

Hypoxia is known to induce HIF-1α transiently, largely as a result of protein stabilization.26 However, how HIF-1α remains persistently elevated in the placenta in the setting of preeclampsia was previously unknown, and the role of HIF-1α in preeclampsia remained unclear. Here we provide both in vivo animal and in vitro human evidence that AT1-AA and LIGHT are 2 hypoxia-independent factors that stimulate HIF-1α gene expression, resulting in subsequent HIF-1α-mediated activation of Flt-1 gene expression. Our studies support a working model (Figure 5G) in which AT1-AA or LIGHT-induced HIF-1α expression in placental trophoblasts is followed by HIF-1α-mediated induction of Flt-1 gene expression and subsequent sFlt-1 production independent of hypoxia. Considerable evidence now indicates that elevated production of sFlt-1 plays a pathogenic role in preeclampsia.20 Thus, interfering with persistently elevated HIF-1α is likely to reduce the overproduction of sFlt-1 and slow the progression of the disease (Figure 5G).

Perspectives

In conclusion, our current studies have added significant new insight to the pathogenesis of preeclampsia by identifying the detrimental role of chronic elevated placental HIF-1α initially triggered by hypoxia-independent factors, a pathogenic autoantibody and an inflammatory cytokine. Chronically elevated placental HIF-1α promotes excessive sFlt-1 production and disease progression. Supporting this working model, we demonstrated that reducing elevated HIF-1α by siRNA-induced mRNA knockdown successfully halted HIF-1α-induced sFlt-1 production and prevented disease development in 2 independent mouse models of preeclampsia in vivo. Altogether, these findings suggest that HIF-1α suppression may serve as a target for pharmacological intervention for preeclampsia.

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Disclosures

None.

References


Novelty and Significance

What Is New?

- Hypoxia inducible factor-1α (HIF-1α) gene expression and protein levels were induced in the placentas of 2 independent animal models of preeclampsia infused with angiotensin II type 1 receptor agonistic autotibody or tumor necrosis factor superfamily member 14 (LIGHT).

- In vivo knockdown of HIF-1α gene expression using siRNA attenuated hallmark features of preeclampsia, including hypertension, proteinuria, kidney damage, impaired placental vasculature, and maternal elevated circulating soluble fms-like tyrosine kinase-1 in both preeclampsia mouse models.

- Using human villous explant culture, we found that angiotensin II type 1 receptor agonistic autotibody or LIGHT directly induced HIF-1α gene expression and upregulated HIF-1α was responsible for angiotensin II type 1 receptor agonistic autotibody or LIGHT-induced elevation of Fit-1 gene expression and the production of soluble fms-like tyrosine kinase-1 independent of hypoxia.

What Is Relevant?

- Our current studies have provided new insight to the pathogenesis of preeclampsia by identifying the detrimental role of chronically elevated placental HIF-1α initially triggered by hypoxia-independent factors. Additionally, our discoveries indicate therapeutic possibilities targeting HIF-1α.

Summary

We have provided both mouse and human evidence that increased HIF-1α in the placenta plays a general pathological role in the pathogenesis of preeclampsia induced by a pathogenic autotibody or an inflammatory cytokine. Our findings highlight novel therapeutic possibilities for preeclampsia.
Hypoxia-Independent Upregulation of Placental Hypoxia Inducible Factor-1α Gene Expression Contributes to the Pathogenesis of Preeclampsia
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Hypoxia-independent up-regulation of placental HIF-1α gene expression contributes to the pathogenesis of preeclampsia

By

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Methods

Animals

Wild type (WT) 8 to 10 week-old timed pregnant C57BL/6 mice (mated with syngeneic males) were obtained from Harlan Laboratories (Indianapolis, IN). The mice were housed in the animal care facility of the University of Texas Health Science Center Houston and had access to food and water ad libitum. All protocols involving animal studies were reviewed and approved by the Institutional Animal Welfare Committee.

Introduction of human autoantibody (AT1-AA) or LIGHT into pregnant mice

PE mouse models induced by AT1-AA or LIGHT were conducted. Briefly, purified IgGs were isolated from preeclamptic (PE) or normotensive (NT) pregnant patient sera (PE-IgG, NT-IgG respectively) as previously described \(^1\). Pregnant mice were treated with NT-IgG or PE-IgG (0.8mg) on E13.5 and E14.5 by retro-orbital sinus injection. For the neutralization experiments, either losartan (8 mg/kg), an angiotensin II receptor antagonist, or 7-amino acid epitope peptides (AFHYESQ), were premixed with PE-IgG and coinjected with PE-IgG on E13.5 and E14.5. For LIGHT injection experiments, recombinant mouse LIGHT (2 ng; R&D Systems) or the same volume of saline was introduced into pregnant mice by retro-orbital sinus injection on E13.5 and E14.5 as
previously reported ². For neutralization experiments, either lymphotoxin β receptor (LTβR) monoclonal Antibody (100 μg) or herpes virus entry mediator (HVEM) monoclonal antibody (100 μg) was simultaneously coinjected with LIGHT. All mice were sacrificed on E18.5 prior to delivery, and their blood and organs were collected.

**In vivo siRNA-induced knockdown of Hif-1α in pregnant mice**

To knockdown Hif-1α mRNA levels in PE-IgG or LIGHT-injected pregnant mice, a siRNA knockdown method (Altogen Biosystems) using nanoparticles to surround siRNA constructs was used as previously reported ³. Briefly, scrambled siRNA control or HIF-1α siRNA (Sigma) encapsulated in nanoparticles was prepared according to instructions from the company (Altogen Biosystems) and was administered on E13.5 and E14.5 retro-orbital sinus injection together with PE-IgG or LIGHT.

**The measurement of blood pressure and proteinuria**

The systolic blood pressure of all mice was measured at the same time daily by tail cuff plethysmography using a carotid catheter-calibrated system (CODA, Kent Scientific). The mice were kept warm using a warming pad (AD Instruments Co). For the measurement of proteinuria, urine was collected for analysis using metabolic cages (Nalgene). Total microalbumin and creatinine in the urine were determined by ELISA
(Exocell) and then the ratio of urinary albumin to creatinine was calculated as an index of proteinuria as previously described \(^1\).

**Real-time RT-PCR analysis**

RNA isolation and real-time RT-PCR were conducted as previously described \(^2\). Syber green was used for the analysis of all transcripts measured using the following primers:

Mouse HIF-1\(\alpha\): forward; 5'-GAAATGGCCCAGTAGAGAAAA-3’ and reverse; 5’-CTTCCAVTGTTGACTTAGTTGA-3’;

Mouse Flt-1: forward; 5’-CCACCTCTCTATCCGCTGG-3’ and reverse; 5’-ACCAATGTGCTAACGTCTTA-3’;

Mouse GAPDH: forward; 5’-CTTCCCATTCTCGGCCTTTA-3’ and reverse; 5’-CTTCCCACTCTCGGCCTTT-3’;

Human HIF-1\(\alpha\): forward; 5’-TGACCTCAACTACATGGTCTACA-3’ and reverse; 5’-CTTCCCATTCTCGGCCTTT-3’;

Human Flt-1: forward; 5’-TGTCATCAGTTGCCCCTTC-3’ and reverse; 5’-TTTGCCTGAAATGGTGAGTAAGG-3’;

Human GAPDH: forward; 5’-TTTGCTGAAATGGTGAGTAAGG-3’ and reverse; 5’-ACAGTCTTCTGGGTCGAGTG-3’.
**Immunohistochemistry**

Formalin fixed tissue blocks were cut into 4-μm thick sections and subjected to immunohistochemistry. Briefly, endogenous peroxidase activity was quenched by 10 min of incubation in a 3% hydrogen peroxide/methanol buffer. Antigen retrieval was conducted by incubating slides in sodium citrate buffer (pH 6.0) at 89°C for 15 min. After blocking with the normal goat serum, the slides were then incubated with antibody against mouse CD31 (1:200, ab124432, Abcam) or human/mouse HIF-1α (1:100, LS-B2823, Lifespan Biosciences) in a humidified chamber at 4°C overnight. After the primary antibody incubation, ABC staining system kit (VEACTASTAIN, VECTOR LAB) was used according to the manufacturer’s suggested protocol. Antigen-antibody reactions were visualized with a dual alkaline phosphatase / fluorescence development system (VECTOR Red Substrate Kit, VECTORLAB). The slides were counterstained with Mayer's hematoxylin. The positive staining for HIF-1α was quantified by Image J software. The intensity of red positive staining was obtained from 6 fields under X100 magnification and averaged to get mean values.

**Immunoblotting**
Placental explants were lysed with RIPA lysis buffer (Santa Cruz) in the presence of proteinase inhibitor cocktail (Roche Diagonistics). Lysates were resolved on SDS–PAGE and electroblotted onto polyvinylidene difluoride membranes. After blocking with Odyssey Blocking Buffer (LI-COR), the membranes were probed with antibody against human / mouse HIF-1α (1:1000, LS-B2823, Lifespan Biosciences), and then probed with secondary antibodies labeled with IRDye fluorophores (LI-COR). The antibody/antigen complexes were scanned and detected using the ODYSSEY infrared imaging system and software (LI-COR).

Patients

Patients who were admitted to Memorial Hermann Hospital were identified by the obstetrical faculty of the University of Texas Medical School at Houston. Preeclamptic patients were diagnosed with severe disease based on the definition set by the National High Blood Pressure Education Program Working Group Report 4. The criteria of inclusion, including no previous history of hypertension, were reported previously 1. Control pregnant women were selected on the basis of having an uncomplicated, normotensive (NT) pregnancy, with an expected normal-term delivery. Human subject data utilized in current study were summarized and indicated in Table S1. The research
protocol was approved by the Institutional Committee for the Protection of Human Subjects.

**Human Placental Villous Explant Culture**

Human placentas were obtained from preeclamptic or normotensive patients who delivered vaginally at term at Memorial Hermann Hospital in Houston. The explant culture system was conducted as described previously. On delivery, the placentas were immediately placed on ice and submerged in phenol red–free DMEM containing 10% BSA and antibiotics. Villous explant fragments weighing 50mg were dissected from the placenta and transferred to 24-well plates at 37°C under 5% CO₂. The explants were incubated for 24 hours and then pretreated with or without HIF-1α inhibitor (10μM CAY10585) (Santa Cruz) for 15 min and then treated with NT- or PE-IgG (100μg /ml) or recombinant human LIGHT (100 pg/mL) (R&D Systems) or dimethyloxaloylglycine (DMOG) (10μM), a prolyl hydroxylase (PHD) inhibitor that results in the stabilization and accumulation of HIF-1α (Santa Cruz) for 24 hours. The explants were also treated with nanoparticle encapsulated control-or HIF-1α-siRNA for 24 hours as previously described in *in vivo* knockdown method section and then were treated with NT- or PE-IgG (100μg /ml) or recombinant human LIGHT (100 pg/mL) for 24 hours.
**Statistical analysis**

All data are expressed as the mean ± SEM. Mann-Whitney’s $U$ test was applied in two-group analysis in Figure 4A, 4C, and 4E. Comparison of the data obtained at different time points from multiple groups as repeated measurements in Figures 2B and 3B were analyzed by two-way repeated measures analysis of variance, followed by the Bonferroni post hoc test. Differences among the means of multiple groups in every analysis except for ones described above were compared by the one-way analysis of variance (ANOVA), followed by a Tukey’s post hoc test. Categorical variables in Table S1 were analyzed by the Fisher’s exact test. Statistical significance was set as $P < 0.05$.

Statistical programs were run by GraphPad Prism 5 statistical software (GraphPad).

**Supplementary References**


Table S1 Clinical characteristics for human subjects

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>NT (n=10)</th>
<th>PE (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>26.5±2.7</td>
<td>28.1±1.6</td>
</tr>
<tr>
<td>Primigravida - no. (%)</td>
<td>4 (40%)</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>Race - no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>3 (30%)</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>African American</td>
<td>4 (40%)</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1 (10%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Other or unknown</td>
<td>2 (20%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Body-mass index</td>
<td>31.8±1.8</td>
<td>34.3±2.2*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>119.3±3.4</td>
<td>168.8±5.9**</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>70.8±1.9</td>
<td>107.2±4.1**</td>
</tr>
<tr>
<td>Proteinuria (mg/24h)</td>
<td>N/A</td>
<td>1546±290</td>
</tr>
<tr>
<td>Gestational age at delivery (week)</td>
<td>37.9±2.1</td>
<td>36.3±1.9</td>
</tr>
<tr>
<td>Infant’s birth weight (g)</td>
<td>3032±153</td>
<td>2321±342**</td>
</tr>
<tr>
<td>Small-for gestational age infant - no. (%)</td>
<td>0 (0%)</td>
<td>3 (30%)*</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01 vs NT
S1. Renal Hif-1α mRNA expression assessed by real-time RT-PCR.

Hif-1α mRNA expression levels in kidneys of mice injected with NT- or PE-IgG (n=4 mice per group) or LIGHT (n=3 mice per group) were determined by real-time RT-PCR. mRNA expression level was determined as a relative value to Gapdh, and each value was expressed as fold induction relative to placentas of NT-IgG- or PBS-treated mice. No significant difference was observed between groups.
S2. The effect of *in vivo* siRNA-induced knockdown of *Hif-1α* in pregnant mice.

Expression of HIF-1α protein in mouse placentas detected by immunoblotting. Placental HIF-1α protein expression was successfully reduced in *Hif-1α* siRNA-injected mice compared with control scrambled siRNA-injected mice.
S3. Placental histology assessed by H&E staining. PE-IgG-induced placental pathologic changes seen in the labyrinth zone of control siRNA-injected mice (calcification; arrows, the disorganization of tissue resulting in abnormal blood pooling; *) was reduced in the placenta of mice injected with Hif-1α siRNA. Scale bar, 200 µm. The number of calcification per field obtained under X100 magnification is quantified. (5 fields per placenta; 4 mice per group). (**P<0.01 vs NT-IgG, #P<0.05 vs PE-IgG+con-si)