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■ H Y P O T H E S I S

Toll-like receptor 9 activation: a novel mechanism linking placenta-derived mitochondrial DNA and vascular dysfunction in pre-eclampsia

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A B S T R A C T

Emerging evidence suggests that in addition to being the ‘power houses’ of our cells, mitochondria facilitate effector responses of the immune system. Cell death and injury result in the release of mtDNA (mitochondrial DNA) that acts via TLR9 (Toll-like receptor 9), a pattern recognition receptor of the immune system which detects bacterial and viral DNA but not vertebrate DNA. The ability of mtDNA to activate TLR9 in a similar fashion to bacterial DNA stems from evolutionarily conserved similarities between bacteria and mitochondria. mtDNA may be the trigger of systemic inflammation in pathologies associated with abnormal cell death. PE (pre-eclampsia) is a hypertensive disorder of pregnancy with devastating maternal and fetal consequences. The aetiology of PE is unknown and removal of the placenta is the only effective cure. Placentas from women with PE show exaggerated necrosis of trophoblast cells, and circulating levels of mtDNA are higher in pregnancies with PE. Accordingly, we propose the hypothesis that exaggerated necrosis of trophoblast cells results in the release of mtDNA, which stimulates TLR9 to mount an immune response and to produce systemic maternal inflammation and vascular dysfunction that lead to hypertension and IUGR (intra-uterine growth restriction). The proposed hypothesis implicates mtDNA in the development of PE via activation of the immune system and may have important preventative and therapeutic implications, because circulating mtDNA may be potential markers of early detection of PE, and anti-TLR9 treatments may be promising in the management of the disease.

INTRODUCTION

PE (pre-eclampsia) is a pregnancy syndrome that is defined by the onset of hypertension and proteinuria after 20 weeks of gestation [1]. It affects every maternal

organ and fetal development, is an important cause of pre-term delivery in developed countries, and a leading cause of maternal and fetal morbidity and mortality in developing countries. One of the main characteristics of the syndrome is an inability of the trophoblasts to invade

Key words: mitochondrial DNA, placenta, pre-eclampsia, pregnancy, Toll-like receptor 9, vascular dysfunction.

Abbreviations: BP, blood pressure; ERK, extracellular-signal-regulated kinase; IL, interleukin; IUGR, intra-uterine growth restriction; LPS, lipopolysaccharide; MAPK, mitogen-activated kinase; mtDNA, mitochondrial DNA; ODN, oligodeoxynucleotide; PAMP, pathogen-associated molecular pattern; PE, pre-eclampsia; SBP, systolic BP; TLR, Toll-like receptor.

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the decidual arteries, causing defective placentation, and reduced placental perfusion and nutrient supply [2]. Other features of the disease include placental and systemic oxidative stress, and dysfunction of the maternal vasculature [3–5]. These are also associated with reduced placental perfusion. As PE progresses to a clinical stage, the mother presents with symptoms such as hypertension, proteinuria, coagulopathy and/or hepatic dysfunction [6]. In most cases, removal of the placenta alleviates the clinical symptoms of the disease, indicating that placenta-derived factors are probably responsible for the pathogenesis and/or manifestation of PE.

Components of the immune system have been detected at the maternal–fetal interface [7,8] and their function in pregnancy has recently become an emerging field of investigation in an effort to understand the role of the immune system in defending the fetus and the mother from infections. Bacterial and viral infections are often responsible for pregnancy complications such as pre-term labour and PE [9,10]. Consequently, several investigations have addressed the question of how exogenous (viral and bacterial) products induce poor pregnancy outcomes. In this Hypothesis article, we address the question of how endogenous molecules released by the placenta induce clinical symptoms of PE, such as maternal vascular dysfunction and hypertension, as well as insufficient fetal growth.

TLRs (Toll-like receptors) are cellular components of the immune system that detect conserved sequences known as PAMPs (pathogen-associated molecular patterns) [11]. Our main knowledge regarding the role of TLR signalling in pregnancy derives from studies in placental explants and trophoblast cells. The human placenta expresses transcripts for TLR1–TLR10 [7–12], and placentas from patients with PE show greater expression of TLR2, TLR3, TLR4 and TLR9 compared with controls [7,13], indicating that TLR signalling may be involved in the development of placental deficiencies and the pathogenesis of PE.

PE is characterized by exaggerated trophoblast apoptosis and necrosis [14,15], and increased expression of TLR9 in placental [13] and dendritic cells [16]. Furthermore, pregnancies complicated with IUGR (intra-uterine growth restriction), a common feature of PE, show elevated levels of circulating mtDNA (mitochondrial DNA) [17]. Interestingly, the highest mtDNA levels were found in the more severe IUGR subsets that were complicated with maternal PE [17]. On the basis of recent evidence that mtDNA induces an immune response via activation of TLR9 signalling pathway [18], we propose the hypothesis that abnormal trophoblast cell death (i.e. exaggerated necrosis) results in the release of mitochondrial products, including mtDNA, which stimulate TLR9 to mount an immune response

and produce systemic maternal inflammation, vascular dysfunction and IUGR.

TLR SIGNALLING

TLRs are type I integral membrane glycoproteins that contain leucine-rich repeats in their extracellular domain and a cytoplasmic TIR [Toll/IL (interleukin)-1 receptor] signalling domain [19]. These receptors recognize PAMPs associated with bacteria and viruses, and induce signals which are critical for eliciting innate and adaptive immune responses to invading micro-organisms [11]. In addition to detecting molecular structures of microbial origin, TLRs respond to endogenous molecular structures known as DAMPs (damage-associated molecular patterns), which are released due to cell death and injury [18]. At least 11 TLRs have been reported in mammals (TLR1–11). TLRs that recognize constituents of bacterial and fungal cell wall are localized on the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6), whereas those that recognize pathogen-specific nucleic acids (TLR3, TLR7, TLR8 and TLR9) are localized to intracellular membranes and bind their ligands in phagosomes or endosomes [20–23].

TLR9 recognizes bacterial DNA containing the dinucleotide CG where the C is unmethylated (CpG-containing DNA) [24]. TLR9 resides in the endoplasmic reticulum and, upon cell activation with CpG DNA, the distribution of TLR9 changes, with a portion of the total protein translocating first into early endosomes and later into lysosomal compartments, where signal transduction is initiated [21]. Following CpG DNA binding, TLR9 associates with the intracellular adapter protein MyD88 (myeloid differentiation factor 88) [25] to activate signal transducing proteins, such as members of the IRAK (IL-1-receptor-associated kinase) family, MAPKs (mitogen-activated kinases) or IRFs (interferon regulatory factors) [26]. These events initiate the synthesis and release of inflammatory cytokines and antimicrobial products, and regulate co-stimulatory molecules [25].

The ability of TLR9 to discriminate between foreign and self-DNA is due to the higher frequency and presence of unmethylated CpG dinucleotides in bacterial and viral compared with mammalian DNA [27]. Mitochondria, however, evolved from saprophytic bacteria to become intracellular organelles [28] and, therefore, mtDNA is structurally similar to bacterial DNA and shares unmethylated CpG DNA repeats [29]. Consequently, mtDNA is a ligand for TLR9 [18]. In this Hypothesis article, we propose that mtDNA released by necrotic trophoblasts induces a maternal immune response via TLR9 signalling activation, leading to the development of PE and its associated clinical symptoms.

TLR ACTIVATION AND CLINICAL SYMPTOMS OF PE

Intrauterine infections are associated with PE in humans [10,30], and viral and bacterial ligands have been often used to induce PE-like symptoms in animals. For instance, pregnant rats infused with low concentrations of endotoxin (a TLR4 ligand) [31] or treated with the viral mimetic poly(I:C) (a TLR3 ligand) [32] developed maternal hypertension, vascular dysfunction and proteinuria. Endotoxin or poly(I:C) treatment had no effect in non-pregnant rats [32,33]. Thus activation of TLR3 and TLR4 causes PE-like symptoms in rats, providing compelling evidence that viral or bacterial infection may contribute to the development of the disease through TLR signalling.

During a systemic or intra-uterine infection in pregnancy, invading micro-organisms and their breakdown products provide an increased pathogenic load to the maternal–fetal environment. Hypomethylated CpG motifs presented by infectious agents may therefore overstimulate TLR9, mediating maternal immune activation [34]. Previous studies have examined the role of the CpG/TLR9 axis in animal models of pregnancy, focusing on pregnancy outcomes such as pup survival and development [34,35]. High doses of a synthetic CpG ODN (oligodeoxynucleotide) during mouse pregnancy stimulated Th1-cytokine release and induced fetal resorptions, craniofacial and limb defects, placental cell necrosis, calcification and inflammation, suggesting that activation of TLR9 signalling may have adverse pregnancy outcomes [35]. Thaxton et al. [34] confirmed these findings and also showed that anti-inflammatory cytokine proficiency protects against CpG-induced pregnancy complications. These previous studies focused on pup survival and growth, but did not examine maternal physiological functions, which may be compromised in the presence of an inflammatory environment.

Preliminary observations in our laboratory suggest that activation of TLR9 via a synthetic CpG oligonucleotide elicits PE-like symptoms in pregnant rats. Figure 1(A) shows that continuous activation of TLR9 by exogenous synthetic oligonucleotides increases SBP [systolic BP (blood pressure)] by ~20 mmHg in pregnant, but not in non-pregnant, rats. Furthermore, treatment with the TLR9 agonist did not affect the number of pups/litter (13.3 ± 1.3 compared with 12.7 ± 0.3 pups in treated compared with untreated rats respectively), but reduced fetal weights (Figure 1B). These findings suggest that activation of TLR9 during pregnancy not only affects fetal development as reported previously [34,35], but it also induces maternal hypertension, which is a main feature of pregnancies with PE. In contrast with pregnant rats, non-pregnant rats did not have a hypertensive response to TLR9 activation.

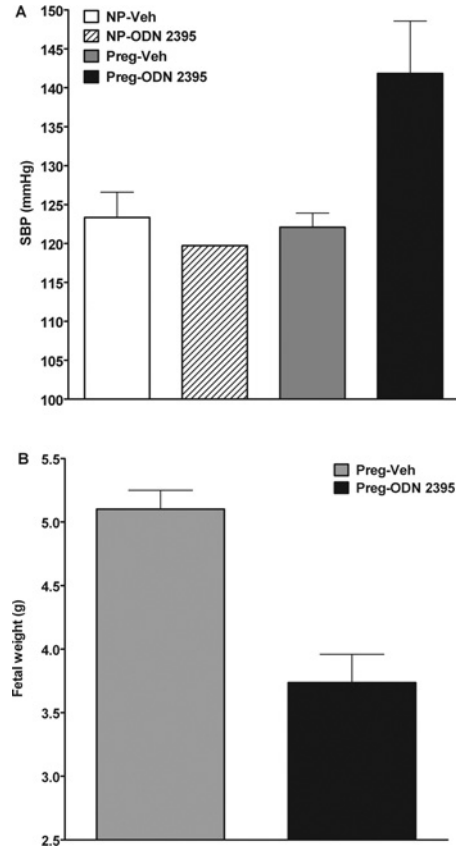


Figure 1 Effect of a synthetic TLR9 ligand on blood pressure and fetal weight in pregnant rats

A synthetic TLR9 ligand (ODN 2395; InvivoGen) was administered by intraperitoneal injection ($0.1 \mu\text{g}/\text{rat}$) on days 14, 17, and 19 of gestation (term = 21 days) in pregnant rats or on the corresponding days in non-pregnant rats. BP was measured via the tail cuff method on gestational day 20 and rats were killed on day 21. (A) SBP of non-pregnant (NP) and late pregnant rats (Preg) treated with ODN 2395 (NP-ODN 2395, $n = 1$; Preg-ODN 2395, $n = 2$) or vehicle (NP-Veh, $n = 3$; Preg-Veh, $n = 2$). (B) Fetal weights from pregnant rats treated with ODN 2395 or vehicle. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education and in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Previous studies have shown that the intracellular localization of TLR9 determines the access of the receptor to different sources of DNA [20]. It is unknown, however, whether pregnancy affects TLR9 localization. An increase in TLR9 expression with gestation could also explain the differential responses to TLR9 in non-pregnant and pregnant rats. Accordingly, normal and complicated pregnancies may determine TLR9 responses to endogenous and/or exogenous threats by modifying TLR9 localization and expression. The effects of pregnancy on TLR9 localization and protein

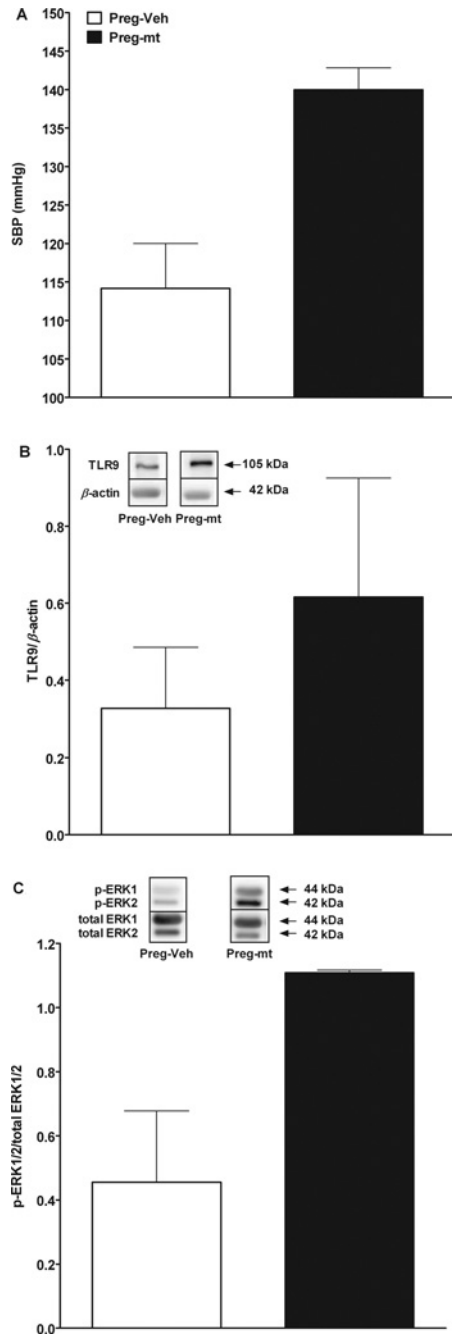


Figure 2 Effect of mitochondria on blood pressure, TLR9 protein expression and ERK1/2 phosphorylation when injected into pregnant rats

Mitochondria were isolated from rat liver (Mitochondria Isolation Kit; Pierce Biotechnology) and their integrity was disrupted by sonication. Mitochondria solution (4 mg of tissue/rat, diluted in saline) was injected into pregnant rats on gestational day 15. BP was measured via the tail cuff method on gestational day 18 and rats were killed on day 19. (A) SBP of pregnant rats injected with mitochondria (Preg-mt, $n = 2$) or Vehicle (Preg-Veh, $n = 2$). (B) Densitometric intensity and representative Western blots for TLR9 protein, in relation to β -actin, in second-order mesenteric arteries from pregnant rats injected with mitochondria (Preg-mt, $n = 2$) or Vehicle (Preg-Veh, $n = 2$). (C) Densitometric intensity and representative Western blots for phospho-ERK1/2, in

expression in different cell types are currently under investigation.

Both bacterial and mtDNA are ligands for TLR9 [18,24]. mtDNA is released from necrotic cells [18] and circulating levels of mtDNA are elevated in pregnancies complicated with maternal PE and IUGR [17]. Furthermore, pregnancies with PE are characterized by exaggerated trophoblast cell apoptosis and necrosis [14,15], which may be the source of mtDNA released in the maternal circulation. Interestingly, smoking is associated with reduced risk of PE [36] and, although the exact mechanism of this protection is unknown, it may relate to the fact that maternal smoking depletes mtDNA in the placenta [37]. To examine the effects of TLR9 activation by mitochondrial products on maternal cardiovascular responses, we injected mitochondria isolated from rat liver in pregnant rats. Dams injected with 'damaged' mitochondria developed high BP (change in SBP = 26 mmHg) compared with controls (Figure 2A).

Collectively, previous studies and our preliminary observations suggest that activation of TLR3, TLR4 and TLR9 lead to the development of PE-like symptoms in pregnant animals. Given that TLR9 can be activated by both bacterial and mtDNA, we suggest that both endogenous and exogenous DNA leads to PE via TLR9 signalling, and we propose that the source of the endogenous DNA is mitochondria released by necrotic trophoblasts.

TLR9 ACTIVATION AND MATERNAL VASCULAR FUNCTION

Maternal vascular dysfunction is a hallmark of PE that may significantly contribute to the manifestations of the disease, such as maternal hypertension and IUGR [38]. Furthermore, PE poses a risk of future maternal cardiovascular disease [39], which may stem from alterations in the function of the vasculature during pregnancy. The exact mechanisms, by which placenta-derived factors cause maternal vascular dysfunction, are currently unknown.

Sustained maternal immune system activation via a TLR3 ligand [poly(I:C)] during rat pregnancy resulted in reduced endothelium-dependent conduit artery dilation [32], indicating that TLR signalling plays a role in the development of maternal vascular dysfunction in

relation to total ERK1/2, in mesenteric arteries from pregnant rats injected with mitochondria (Preg-mt, $n = 2$) or vehicle (Preg-Veh, $n = 2$). All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education and in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

pregnancies with PE. A recent study showed that infusion of LPS (lipopolysaccharide; a TLR4 ligand) on gestational day 15 decreased myogenic tone and increased wall thickness of posterior cerebral arteries in pregnant, but not in non-pregnant, rats [33]. These investigators did not examine the role of TLR4 signalling in LPS-mediated vascular effects, but previous studies have provided compelling evidence that LPS-induced signal transduction is mediated by TLR4 [40]. In addition, injection with poly(I:C) (a TLR3 ligand) in pregnant rats [32] and mice [41], and infusion of LPS in pregnant rats [33] resulted in increased serum concentrations and vascular mRNA levels of pro-inflammatory cytokines respectively. Furthermore, it has been reported that exogenous IL (interleukin)-10 treatment in pregnant mice injected with poly(I:C) prevented maternal endothelial dysfunction [41]. These findings show that the effects of TLR signalling on maternal vascular function are probably mediated by the induction of pro-inflammatory cytokines and can be regulated by anti-inflammatory cytokines, such as IL-10.

TLRs are expressed on immune [42] and trophoblast cells [13], but have been also detected in the vascular endothelial [43] and smooth muscle [44] cells. Our laboratory has shown recently that treatment with an anti-TLR4 antibody reduced BP and small vessel contractility via a COX (cyclo-oxygenase)-dependent mechanism in a rat model of hypertension [45], implicating TLR signalling in hypertension-associated vascular dysfunction. Studies on TLR signalling in pregnancy suggest that the vascular effects of TLR activation are due to an increase in pro-inflammatory cytokines [32,33,41]. There is a possibility, however, that exogenous and endogenous ligands directly act on TLRs in the vascular wall, inducing a cytokine-independent signalling pathway that leads to vascular dysfunction.

In immune cells, activation of TLR9 via mtDNA and bacterial DNA induces an immune and inflammatory response via activation of p38 MAPK [18]. Other studies suggest the involvement of other MAPKs, such as ERK (extracellular-signal-regulated kinase) and JNK (c-Jun N-terminal kinase), in the downstream TLR9 signalling pathway [46,47]. To examine the effects of TLR9 activation on the activity of ERK1/2 in maternal resistance vessels, we measured protein expression of TLR9 and phospho-ERK1/2 in mesenteric arteries from pregnant rats treated with mitochondria isolated from rat liver and from rats treated with vehicle. Expression of TLR9 and phospho-ERK1/2 were greater in mitochondria-treated pregnant rats compared with controls (Figures 2B and 2C). Given that ERK1/2 plays a significant role in vascular responses to constrictor stimuli (i.e. phenylephrine and thromboxane mimetics) [48,49], we speculate that ERK1/2 is a downstream effector of the CpG/TLR9 axis in maternal vascular tissue, mediating a direct effect of TLR9 ligation by mtDNA on maternal

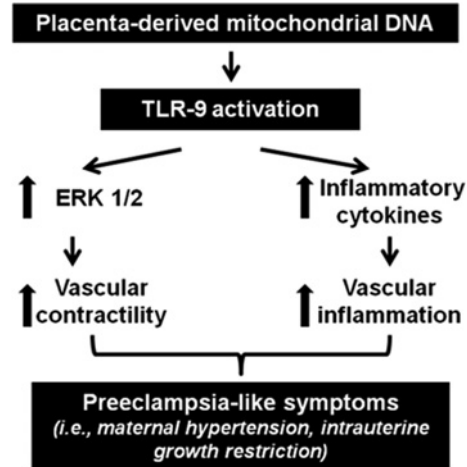


Figure 3 Overview of the hypothesis that placenta-derived mtDNA induces PE-like symptoms

During pregnancy, placenta-derived mtDNA, through TLR9 activation, increases vascular reactivity to constrictor stimuli via an ERK1/2-dependent signalling pathway and potentiates the release of pro-inflammatory cytokines, contributing to PE-like symptoms (i.e. maternal hypertension and IUGR).

vascular function. Indeed, there are reports to suggest that activation of TLR9 leads to ERK1/2 activation in various cells [50].

According to our preliminary results, we propose that during pregnancy activation of the CpG/TLR9 axis via mtDNA released by necrotic placental cells increases the activation of ERK1/2, contributing to increased maternal vascular reactivity to constrictor stimuli, increased peripheral vascular resistance, maternal hypertension and insufficient uterine blood flow (Figure 3). This mechanism may be independent of the effects of pro-inflammatory cytokines released upon TLR9 activation on the maternal vasculature, but this speculation warrants further investigation. Integrative approaches including physiological, pharmacological, biochemical, molecular and cellular techniques, as well as translational studies, are required to test the proposed hypothesis and to investigate the role of the innate immune system in maternal vascular inflammation and dysfunction, and its contribution to the development of maternal hypertension and IUGR. Studies of maternal vascular reactivity, BP responses, uterine blood flow, levels of maternal proteinuria and fetal development in pregnant animals treated with mtDNA isolated from placentas and use of *Tlr9*-knockout mice can establish a causal relationship between mtDNA and PE-like symptoms. Furthermore, cell culture studies can provide information regarding the direct effects of mtDNA on TLR9 signalling in vascular smooth muscle and endothelial cells. In addition, assessment of circulating mtDNA content in blood from women with PE is necessary to verify the relevance of the proposed hypothesis to pregnancies with PE.

PERSPECTIVES AND CLINICAL IMPLICATIONS

mtDNA is released by necrotic cells inducing a systemic inflammatory response via activation of TLR9 [18]. Furthermore, under certain pathophysiological conditions, the ability of TLR9 to discriminate between self and foreign DNA can be circumvented and this leads to immune pathologies and chronic inflammation [51]. In the present article, we propose the hypothesis that mtDNA is a placenta-derived factor with immunostimulatory properties that is secreted in the maternal circulation as a result of exaggerated trophoblast necrosis, activating the maternal immune system via TLR9 signalling. These events lead to systemic maternal inflammation and vascular dysfunction, hypertension and IUGR. The proposed hypothesis implicates mtDNA in the development of PE via activation of the immune system and may have important preventative and therapeutic implications. For instance, circulating mtDNA may be potential markers of early detection of PE, and anti-TLR9 treatments may be promising in the management of the disease.

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