**Pregnancy-specific expression of protease-activated receptor 1: a therapeutic target for prevention and treatment of preeclampsia?**

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Neutrophils extensively infiltrate maternal blood vessels in preeclampsia. This could explain why multiple organs are affected in this enigmatic disorder. Lipid peroxides produced by the placenta are probably the first factors that activate neutrophils as they circulate through the intervillous space, but then a second factor specific to pregnancy comes into play, protease-activated receptor 1. The only time neutrophils express protease-activated receptor 1 is during pregnancy. This means that neutrophils can be activated by a mechanism specific to pregnancy, that is, by proteases. Two proteases that are elevated in preeclampsia and activate protease-activated receptor 1 are matrix metalloproteinase-1 and neutrophil elastase. There is an 8-fold increase in vascular protease-activated receptor 1 expression in women with preeclampsia, and protease-activated receptor 1 is also expressed on the placenta, a pregnancy-specific tissue. The question arises if the pregnancy-specific expression of protease-activated receptor 1 is essential to the pathophysiology of preeclampsia. Protease activation of protease-activated receptor 1 in neutrophils of women with normal pregnancies causes activation of RhoA kinase. RhoA kinase phosphorylates nuclear factor-kappa B causing its translocation from the cytosol into the nucleus, increasing the expression of inflammatory genes. This signaling pathway is blocked by inhibition of either protease-activated receptor 1 or RhoA kinase activity. In contrast, neutrophils obtained from preeclamptic women are already activated, with nuclear factor-kappa B localized in the nucleus. Surprisingly, inhibition of either protease-activated receptor 1 or RhoA kinase results in an efflux of nuclear factor-kappa B from the nucleus back into the cytoplasm. Cyclooxygenase-2 seems to be a downstream mediator between protease-activated receptor 1 and RhoA kinase because aspirin inhibits the nuclear translocation of nuclear factor-kappa B and inhibits neutrophil production of superoxide, thromboxane, and tumor necrosis factor alpha. Currently, low-dose aspirin is the standard of care to prevent preeclampsia in high-risk women. Generally, the actions of low-dose aspirin are attributed to selective inhibition of maternal platelet thromboxane production. However, a recent study showed that beneficial effects extend to the placenta, where aspirin corrected the imbalance of increased thromboxane and reduced prostacyclin and oxidative stress. Selective inhibition of placental thromboxane is possible because thromboxane and prostacyclin are compartmentalized. Thromboxane is produced by trophoblast cells and prostacyclin by endothelial cells, so as aspirin crosses the placenta, its levels decline, sparing prostacyclin. Placental oxidative stress is attenuated because cyclooxygenase-2 inhibition decreases the generation of reactive oxygen species to decrease the formation of isoprostanes. The clinical manifestations of preeclampsia can be explained by protease activation of protease-activated receptor 1 in different tissues. In neutrophils, it can account for their activation and inflammatory response. In vascular tissue, protease-activated receptor 1 activation leads to enhanced vascular reactivity to angiotensin II to cause hypertension. In the placenta, it leads to oxidative stress, increased soluble fms-like tyrosine kinase, and thromboxane production. Activation of protease-activated receptor 1 on endothelial cells causes contraction, leading to edema and proteinuria, and activation on platelets leads to coagulation abnormalities. As proteases that activate protease-activated receptor 1 are elevated in the circulation of women with preeclampsia, consideration should be given to the inhibition of protease-activated receptor 1 as a treatment. Recently, The Food and Drug Administration (FDA) approved a protease-activated receptor 1 inhibitor, creating an opportunity to test whether protease-activated receptor 1 inhibition can prevent and/or treat preeclampsia, but a standard dose of aspirin might be just as effective by blocking its downstream actions.

**Key words:** aspirin, cyclooxygenase, elastase, isoprostanes, matrix metalloproteinase-1, neutrophils, nuclear factor-kappa B, placenta, preeclampsia, pregnancy, prostacyclin, protease-activated receptor 1, proteases, RhoA kinase, thromboxane
Maternal Vascular Infiltration of Neutrophils in Preeclampsia

Normal pregnancy (NP) is characterized by leukocytosis caused by the proliferation of neutrophils in the maternal circulation in the second and third trimesters of pregnancy. The number of neutrophils increases 2.5-fold by 30 weeks' gestation in women with NP, but the number increases further in women with preeclampsia (PE). Neutrophils are part of the innate immune system and the first line of defense against infection, but accumulating evidence indicates that they also play a role in “sterile” inflammatory diseases, that is, an inflammatory response in the absence of an infection.

Neutrophils need to infiltrate tissue to manifest their inflammatory effects, and in women with PE, even though they are not infected, there is extensive neutrophil infiltration into the maternal systemic blood vessels (Figure 1). In women with PE, 80% to 90% of vessels are infiltrated, and although all classes of leukocytes are activated, vascular infiltration is restricted to neutrophils. Neutrophil infiltration is associated with significant increases in inflammatory markers in omental and subcutaneous vascular tissue, such as nuclear factor-kappa B (NF-κB), cyclooxygenase-2 (COX-2), thromboxane synthase (TBXAS1), intercellular adhesion molecule-1 (ICAM-1), myeloperoxidase, and the potent neutrophil chemokine, interleukin 8 (IL-8). Figure 2 demonstrates the extensive vascular infiltration of neutrophils evidenced by staining for TBXAS1, but it also reveals vascular inflammation, as endothelial cells and vascular smooth muscle cells are also stained. The extensive infiltration of neutrophils into blood vessels of women with PE could explain the systemic vascular inflammation and why multiple organs are affected.

How are neutrophils activated in PE? The placenta would seem to be a source for the activator because preeclampsia only occurs in the presence of placental tissue. Lipid peroxides are potent activators of leukocytes, and the human placenta produces lipid peroxides and secretes them into the maternal circulation. In women with PE, placental production of lipid peroxides is significantly higher than in women with NP. Therefore, it is plausible that activation occurs as neutrophils circulate through the intervillous space and are exposed to lipid peroxides released by the placenta.

Pregnancy-Specific Expression of Protease-Activated Receptor 1 On Neutrophils

Protease-activated receptor 1 (PAR-1) is constitutively expressed in many cell types, including endothelial cells, smooth muscle cells, neurons, platelets, and leukocytes, but interestingly, it is not expressed on neutrophils except in pregnant women, so the neutrophil expression of PAR-1 is specific to pregnancy. This is important because it means neutrophils can be activated by a mechanism that is specific to pregnancy, that is, by proteases. Specific neutrophil gene activation might explain why the clinical symptoms of PE are unique to pregnancy.

PAR-1 is activated by serine proteases, such as thrombin and neutrophil elastase. It was originally known as the thrombin receptor, but it was recently reported that matrix metalloprotease-1 (MMP-1) also activates PAR-1, so the name was changed to reflect a more general role. Activation leads to downstream signaling that includes the RhoA kinase (ROCK) phosphorylation pathway. ROCK is in the protein kinase C family of phosphorylating enzymes and has previously been shown to activate NF-κB to increase the expression of inflammatory genes. ROCK is a recognized mediator of enhanced vascular reactivity, so neutrophil infiltration could play a role in the hypertension of preeclampsia.

Women with PE have an 8-fold increase in gene and protein expression of PAR-1 in their blood vessels compared with women with NP. Figure 3 shows omental fat vessels of women with PE and women with NP immunostained for PAR-1. In PE, PAR-1 is darkly stained in neutrophils present in the lumen of the vessel, endothelial cells, and vascular smooth muscle. Neutrophils that adhere...
to the endothelium and infiltrate the vessel are also darkly stained. In NP, weak staining is present in the endothelium and neutrophils in the vessel lumen.

The concentrations of MMP-1 and neutrophil elastase are significantly elevated in the circulation of women with PE, and both activate neutrophils in pregnancy via PAR-1. MMP-1 is significantly elevated 10 weeks before clinical symptoms of PE at a time when the women are still considered to have an NP (Figure 4). Neutrophil elastase is not significantly elevated until after the appearance of clinical symptoms. This suggests that MMP-1 may be responsible for the initial neutrophil activation, but once started, neutrophil activation becomes a feed-forward process accelerated by both MMP-1 and neutrophil elastase as more neutrophils become activated. Such a scenario fits with the progressive worsening of clinical symptoms in women with PE.

Figure 5 shows confocal images of the p65 subunit of NF-κB for neutrophils obtained from a woman with NP. In untreated neutrophils, the p65 subunit is localized to the cytosol, but on treatment with MMP-1, p65 moves from the cytosol into the nucleus. The nuclear movement of p65 was associated with a significant increase in IL-8. The protease-activating mechanisms are mediated via PAR-1 and ROCK because either PAR-1 inhibition or ROCK inhibition prevents the nuclear translocation of p65.

Furthermore, Figure 5 shows confocal images of neutrophils obtained from a woman with PE. Neutrophils of women with PE were already activated with the p65 subunit of NF-κB localized to the nucleus. Surprisingly, inhibition of either PAR-1 or ROCK resulted in the emptying of p65 from the nucleus and its movement back into the cytoplasm. This finding has important implications. First, it means that the movement of p65 between the cytoplasm and nucleus is reversible. Secondly, this has important therapeutic implications for the treatment of PE based on inhibition of PAR-1 because several lines of

**FIGURE 2**
Thromboxane synthase in an omental fat vessel of a woman with preeclampsia

The dark brown staining for thromboxane synthase was present in leukocytes, which were mainly neutrophils, not only in the vessel lumen but also in the endothelium and vascular smooth muscle. Adapted from Mousa et al.10


**FIGURE 3**
Increased expression of PAR-1 in PE as compared to NP

8-fold increased in PE vs. NP

In women with PE, the dark brown staining for PAR-1 was present in neutrophils in the VL, and the neutrophils adhered to the endothelium and infiltrated into the VSM. Furthermore, the dark brown staining for PAR-1 was present in the endothelium and VSM in women with PE. In women with NP, the staining for PAR-1 was very light in the endothelium and neutrophils in the VL. Women with PE had an 8-fold increase in their gene and protein expression of PAR-1 compared with women with NP. Adapted from Walsh et al.33

EC, endothelial cells; NP, normal pregnancy; PAR-1, protease-activated receptor 1; PE, preeclampsia; VL, vessel lumen; VSM, vascular smooth muscle.

Evidence indicates that protease activation of PAR-1 may play a central role in the pathology of PE as discussed below.

Pregnancy-Specific Expression of Protease-Activated Receptor 1 in the Placenta

PAR-1 is expressed in the placenta, which is tissue specific to pregnancy and dysfunctional in PE. Figure 6 shows staining for PAR-1 in a placental villus. PAR-1 is expressed in the syncytiotrophoblast and macrophages of the villous core.

There is evidence that PAR-1 mediates placental dysfunction in PE. Because PAR-1 is expressed in the syncytiotrophoblast, which is bathed by maternal blood, elevated levels of proteases in the intervillous space could activate PAR-1, leading to placental dysfunction. Protease stimulation of trophoblast PAR-1 causes increased release of the angiogenic factor, soluble fms-like tyrosine kinase (sFlt), by activation of placental nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to generate reactive oxygen species (ROS). Activation of NADPH oxidase via PAR-1 could be responsible for placental oxidative stress, which drives the imbalance of increased thromboxane and decreased prostacyclin production.

Beneficial Effects of Low-Dose Aspirin in the Placenta

Currently, low-dose aspirin is the standard of care for the prevention of PE in high-risk populations. Generally, the actions of low-dose aspirin are attributed to selective inhibition of maternal platelet thromboxane formation. However, beneficial effects must extend to other cells and tissues that play important roles in the pathology of PE. One of these tissues is the placenta, which is a major source of eicosanoids, and is characterized by an imbalance of increased thromboxane, decreased prostacyclin, and oxidative stress. Evidence that aspirin affects the placenta is provided in a recent study that found that placental thromboxane was not increased and prostacyclin was not decreased in women prescribed low-dose aspirin, so the imbalance was not present.

Correction of the placental imbalance between thromboxane and prostacyclin is possible because these eicosanoids are compartmentalized within the placenta (Figure 7). The trophoblast cells on the maternal side of the placenta are the main source of thromboxane, whereas the placental vasculature on the fetal side is the main source of prostacyclin. This allows for selective inhibition because as aspirin crosses the placenta from the maternal intervillous space, its concentrations are the highest in the trophoblast cells, where it selectively inhibits COX-2 associated with thromboxane formation. As aspirin crosses from the maternal to fetal side, its concentration gradually declines according to Fick’s second law of diffusion, sparing prostacyclin production by the endothelial cells of the placental vasculature. Only 34% of maternal aspirin crosses to the fetal side. In vitro studies demonstrated that low-dose aspirin preferentially inhibits placental thromboxane while sparing prostacyclin.

Other evidence that maternal ingestion of aspirin affects the placenta is that placental oxidative stress is attenuated in

Maternal circulating levels of MMP-1 and neutrophil elastase

MMP-1 was significantly increased in the mother’s circulation 10 weeks before clinical diagnosis of PE compared with women who went on to have NPs. MMP-1 remained significantly elevated in women with PE after diagnosis. Neutrophil elastase was significantly elevated in women with PE, but only after clinical diagnosis. Single asterisk denotes $P<.05$, double asterisk denotes $P<.01$, and triple asterisk denotes $P<.001$. Adapted from Walsh et al.33

MMP-1, matrix metalloproteinase-1; NP, normal pregnancy; PE, preeclampsia.

women prescribed low-dose aspirin.\textsuperscript{41} Two of the most abundant isoprostanes, 8-isoprostane and 5-isoprostane, which are significantly elevated in placentas of preeclamptic women,\textsuperscript{15} were not elevated in women on aspirin therapy. Isoprostanes are reliable markers of endogenous lipid peroxidation and oxidative stress. They are prostaglandin-like products formed in vivo by free radical–catalyzed nonenzymatic peroxidation of arachidonic acid.\textsuperscript{47}–\textsuperscript{49} The attenuation of oxidative stress may explain why the imbalance between thromboxane and prostacyclin is not present because the imbalance is driven by oxidative stress.\textsuperscript{21,39}

The fact that placental isoprostanes did not increase in women taking low-dose aspirin could also be owing to an indirect effect of cyclooxygenase inhibition. Cyclooxygenase generates ROS,\textsuperscript{50} so inhibition of cyclooxygenase could have removed the source of free radicals to generate isoprostanes from arachidonic acid (Figure 8). This could explain why low-dose aspirin inhibits lipid peroxides and thromboxane in the maternal circulation and the placenta.\textsuperscript{46,51}

**Beneficial Effects of Low-Dose Aspirin in Neutrophils**

Maternal platelets and placental trophoblasts may not be the only aspirin targets. Neutrophils are also a target because they express cyclooxygenase, produce thromboxane, and generate ROS. The expression of COX-2 is increased in neutrophils of women with preeclampsia,\textsuperscript{7,52} and aspirin inhibits neutrophil production of thromboxane and TNFα and the generation of ROS mediated by COX-2.\textsuperscript{13,14} Furthermore, aspirin inhibits the nuclear translocation of NF-κB induced by protease activation of PAR-1 in neutrophils in pregnancy (unpublished observations). Neutrophils could be a major source of thromboxane and oxidative stress because of the marked increase in their numbers during pregnancy. The inhibition of neutrophil activation by aspirin would be beneficial because unactivated neutrophils will not infiltrate the mother’s blood vessels.

**Central Role of Protease-Activated Receptor 1 in the Clinical Manifestations of Preeclampsia**

Protease activation of PAR-1 may play a central role in the pathology of PE (Figure 9). Protease activation of PAR-1 promotes an inflammatory response in neutrophils in pregnancy and MMP-1 causes vasoconstriction and enhances vascular reactivity to angiotensin II, which is prevented by PAR-1 inhibition.\textsuperscript{53} Similarly, ROS released by
PAR-1 is present in the syncytiotrophoblast cells of the placenta, which are directly bathed by maternal blood in the intervillous space. Therefore, elevated levels of MMP-1 and neutrophil elastase in the blood of women with PE would activate placental PAR-1. According to the literature, activation of PAR-1 causes several placental abnormalities related to PE, so protease activation of PAR-1 could be responsible for placental dysfunction in PE. Furthermore, staining for PAR-1 is present in macrophages within the placental villus. Adapted from Walsh and Strauss.40

**FIGURE 6**

**PAR-1 in the placenta**

PAR-1 is present in the syncytiotrophoblast cells of the placenta, which are directly bathed by maternal blood in the intervillous space. Therefore, elevated levels of MMP-1 and neutrophil elastase in the blood of women with PE would activate placental PAR-1. According to the literature, activation of PAR-1 causes several placental abnormalities related to PE, so protease activation of PAR-1 could be responsible for placental dysfunction in PE. Furthermore, staining for PAR-1 is present in macrophages within the placental villus. Adapted from Walsh and Strauss.40

Blood vessel; MMP-1, matrix metalloproteinase-1; PAR-1, protease-activated receptor 1; PE, preeclampsia.


Activated neutrophils enhance vascular reactivity to angiotensin II via ROCK, the downstream mediator of PAR-1.6 These findings implicated protease activation of PAR-1 in vascular inflammation and hypertension of PE. Activation of PAR-1 may explain other features of preeclampsia as well. For example, PAR-1 mediates coagulation abnormalities, platelet aggregation, and thromboxane generation. In addition, protease activation of endothelial PAR-1 activates NF-κB, upregulates cell adhesion molecules (ICAM-1), triggers the production of neutrophil chemokines (IL-8), and increases endothelial permeability to trigger edema formation and proteinuria.22,23,54–58 Furthermore, PAR-1 may contribute to the elevation in soluble flt-1 (sFlt-1) because trophoblast and decidual production of sFlt-1 is stimulated by protease activation of PAR-1.35,58 Elevated levels of proteases in the maternal circulation could activate placental PAR-1 as they pass through the intervillous space. Placental oxidative stress may be a consequence of protease stimulation of trophoblast PAR-1, because PAR-1 activates NADPH oxidase to generate ROS, resulting in the release of sFlt.34 Activation of NADPH oxidase could explain the placental imbalance of increased thromboxane and decreased prostacyclin because oxidative stress drives this imbalance.29

Neutrophil activation could explain other features of PE. For example, as neutrophils have a limited life span of about 8 days, their rapid turnover would explain why maternal symptoms clear shortly after delivery because new neutrophils not expressing PAR-1 enter the circulation. Some women develop preeclampsia in the immediate postpartum period. Labor is recognized to be an inflammatory process, and even in normal term labor, there is extensive infiltration of neutrophils into maternal systemic vasculature.19 Women who develop postpartum PE might have been on the verge of developing preeclampsia and then neutrophil infiltration that occurs with labor pushes them over the edge. Therefore, the inhibition of PAR-1 may have multiple beneficial effects.

**FIGURE 7**

**Compartmentalization of thromboxane and prostacyclin in the human placenta**

The compartmentalization of thromboxane and prostacyclin in the placenta allows for selective inhibition of thromboxane. Thromboxane is primarily synthesized in the trophoblast cells, which are directly bathed by maternal blood, whereas prostacyclin is synthesized by the endothelial cells of the placental vasculature on the fetal side. According to Fick’s second law of diffusion, the concentration of a substance declines the further away it is from the source. Therefore, as aspirin diffuses across the placenta from its source on the maternal side, its concentration is the greatest in the trophoblast cells to inhibit thromboxane, but as it continues to cross the placenta, its concentration declines, so prostacyclin production by the placental vasculature on the fetal side is minimally affected. In fact, only 34% of aspirin from the maternal side of the placenta reaches the fetal circulation.

ASA, aspirin; COX-2, cyclooxygenase-2; TX, thromboxane; PGI2, prostacyclin.

Treatment of women with PE based on inhibition of PAR-1 could be translated to the clinical setting because the FDA has approved a PAR-1 inhibitor for the reduction of thrombotic cardiovascular events, and the pathology of PE includes thrombotic events, such as platelet aggregation, platelet consumption, and disseminated intravascular coagulation. The drug seems to be safe with the main side effect of an increase in bleeding with prolonged use.

Consideration should be given to the use of aspirin in treating women with PE. Many years ago, aspirin was declared to be contraindicated for use in pregnancy because of concern that it might reduce the amniotic fluid volume or cause closure of the ductus arteriosus. However, this concern may be unwarranted because only 30% of an aspirin dose crosses from the maternal to the fetal side of the placenta, and more importantly, the safety of aspirin was demonstrated by the Collaborative Perinatal Project in the 1970s. This project involved more than 40,000 pregnant women and their offspring, more than 24,000 of whom took aspirin during their pregnancy, 1500 of whom were heavily exposed. The Collaborative Perinatal Project found no harmful effect of aspirin use on neonates. The treatment of PE would likely require a standard dose of aspirin and would be best coupled with a calcium carbonate antacid supplement to prevent stomach upset. Calcium is an important nutrient for pregnant women, and calcium carbonate is a good source of calcium. In 1978, Goodlin et al. successfully treated a woman with hemolysis, elevated liver enzymes, and low platelet count using a standard dose of aspirin. Clinical symptoms disappeared during aspirin treatment but reappeared when aspirin was briefly stopped before starting aspirin again. A healthy neonate was born. Bleeding is a known risk factor for aspirin, so patients on aspirin therapy should be carefully monitored both before and after delivery. The treatment with aspirin would have the advantage of being inexpensive and readily available for treatment worldwide.

Figure 10 summarizes a mechanism whereby aspirin could shut down the neutrophil inflammatory response in preeclampsia because of their pregnancy-specific expression of PAR-1. As neutrophils circulate through the intervillous space, they are activated by increased placental secretion of lipid peroxides that induce the expression of COX-2. As the neutrophils return to the mother’s circulation, they are further activated via PAR-1 by elevated levels of inflammatory cytokines. The clinical symptoms of PE can be explained by protease activation of PAR-1 in neutrophils, platelets, placenta, and vascular tissue. Maternal circulating levels of MMP-1 and neutrophil elastase are elevated in PE and can activate PAR-1 in many cells and tissues, which could explain why so many organs are affected. For example, activation of placental PAR-1 causes placental dysfunction by inducing oxidative stress, which drives increased release of sFlt and the imbalance of increased thromboxane and decreased prostacyclin. Protease stimulation of PAR-1 on neutrophils in pregnancy activates them. The extensive vascular infiltration of neutrophils in women with PE is associated with inflammation, dysfunction, and enhanced vascular reactivity to angiotensin II, which could explain hypertension. Protease activation of endothelial PAR-1 causes contraction of the endothelial cells. This would lead to edema in the maternal systemic circulation and proteinuria in the kidney. Finally, PAR-1 activation on platelets triggers platelet aggregation and coagulation abnormalities. Adapted from Walsh and Strauss.

**FIGURE 8**
Mechanism whereby aspirin can inhibit the formation of isoprostanes

Isoprostanes are formed in vivo by free radical—catalyzed nonenzymatic peroxidation of arachidonic acid. They are accurate markers of oxidative stress. Cyclooxygenase generates ROS when activated. By inhibiting cyclooxygenase, aspirin prevents the source of ROS to generate isoprostanes from arachidonic acid.

ASA, aspirin; ROS, reactive oxygen species; COX-2, cyclooxygenase-2.


**FIGURE 9**
Clinical manifestations of PE

The clinical symptoms of PE can be explained by protease activation of PAR-1 in neutrophils, platelets, placenta, and vascular tissue. Maternal circulating levels of MMP-1 and neutrophil elastase are elevated in PE and can activate PAR-1 in many cells and tissues, which could explain why so many organs are affected. For example, activation of placental PAR-1 causes placental dysfunction by inducing oxidative stress, which drives increased release of sFlt and the imbalance of increased thromboxane and decreased prostacyclin. Protease stimulation of PAR-1 on neutrophils in pregnancy activates them. The extensive vascular infiltration of neutrophils in women with PE is associated with inflammation, dysfunction, and enhanced vascular reactivity to angiotensin II, which could explain hypertension. Protease activation of endothelial PAR-1 causes contraction of the endothelial cells. This would lead to edema in the maternal systemic circulation and proteinuria in the kidney. Finally, PAR-1 activation on platelets triggers platelet aggregation and coagulation abnormalities. Adapted from Walsh and Strauss.

As neutrophils circulate through the intervillous space, they are activated by increased placental secretion of lipid peroxides that induce the expression of COX-2. As the neutrophils return to the mother’s circulation, they are further activated via PAR-1 by elevated levels of proteases in the mother’s circulation. PAR-1 then activates COX-2, which activates ROCK, causing phosphorylation of NF-κB and its translocation from the cytosol into the nucleus to increase expression of inflammatory genes. Aspirin by inhibiting COX-2 shuts down this pregnancy-specific inflammatory response.

**REFERENCES**