Published in final edited form as:

Reprod Sci. 2020 November ; 27(11): 2115–2127. doi:10.1007/s43032-020-00232-4.

Proteases Activate Pregnancy Neutrophils by a Protease-Activated Receptor 1 Pathway: Epigenetic Implications for Preeclampsia

Scott W. Walsh, Ph.D.^{1,2}, William H. Nugent, Ph.D.¹, Marwah Al Dulaimi, M.B.Ch.B.¹, Sonya L. Washington, M.S., R.N.¹, Phoebe Dacha, M.D.¹, Jerome F. Strauss III, M.D., Ph.D.¹

¹Department of Obstetrics and Gynecology, School of Medicine, Virginia Commonwealth University, Richmond, VA, 23298-0034, USA

²Department of Physiology and Biophysics, School of Medicine, Virginia Commonwealth University, Richmond, VA, 23298-0034, USA

Abstract

We tested a novel hypothesis that elevated levels of proteases in the maternal circulation of preeclamptic women activate neutrophils due to their pregnancy-specific expression of protease-activated receptor 1 (PAR-1). Plasma was collected longitudinally from normal pregnant and preeclamptic women and analyzed for MMP-1 and neutrophil elastase. Neutrophils were isolated for culture and confocal microscopy. Omental fat was collected for immunohistochemistry. Circulating proteases were significantly elevated in preeclampsia. Confocal microscopy revealed that tet methylcytosine dioxygenase 2 (TET2), a DNA de-methylase, and p65 subunit of NF- κ B were strongly localized to the nucleus of untreated neutrophils of preeclamptic women, but in untreated neutrophils of normal pregnant women they were restricted to the cytosol. Treatment of normal pregnancy neutrophils with proteases activated PAR-1, leading to activation of RhoA kinase (ROCK), which triggered translocation of TET2 and p65 from the cytosol into the nucleus, mimicking the nuclear localization in neutrophils of preeclamptic women. IL-8, an NF- κ B gene, increased in association with TET2 and p65 nuclear localization. Co-treatment with inhibitors of

Terms of use and reuse: academic research for non-commercial purposes, see here for full terms. <http://www.springer.com/gb/open-access/authors-rights/aam-terms-v1>

Corresponding Author: Scott W. Walsh, Ph.D., Virginia Commonwealth University Medical Center, Department of Obstetrics and Gynecology, P.O. Box 980034, Richmond, VA 23298-0034, (804) 828-1772 – Work, (804) 828-0573 – Fax, scott.walsh@vcuhealth.org.

Publisher's Disclaimer: This Author Accepted Manuscript is a PDF file of a an unedited peer-reviewed manuscript that has been accepted for publication but has not been copyedited or corrected. The official version of record that is published in the journal is kept up to date and so may therefore differ from this version.

Conflicts of Interest: The authors declared they have no conflict of interest.

Ethics approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the Office of Research Subjects Protection of Virginia Commonwealth University (HM20009145, HM20005160).

Consent to participate: Each patient from whom samples were obtained signed a consent form indicating they consented to participate in this research study.

Consent for publication: In the consent form, patients were informed that their data will be shared in summarized form grouped with other patients in published papers.

Availability of data and material: Not applicable

Code availability: Not applicable

PAR-1 or ROCK prevented nuclear translocation and IL-8 did not increase. Treatment of preeclamptic pregnancy neutrophils with inhibitors emptied the nucleus of TET2 and p65, mimicking the cytosolic localization of normal pregnancy neutrophils. Expression of PAR-1 and TET2 were markedly increased in omental fat vessels and neutrophils of preeclamptic women. We conclude that elevated levels of circulating proteases in preeclamptic women activate neutrophils due to their pregnancy specific expression of PAR-1 and speculate that TET2 DNA de-methylation plays a role in the inflammatory response.

Keywords

Preeclampsia; neutrophils; matrix metalloprotease 1; neutrophil elastase; protease activated receptor 1; RhoA kinase; tet methylcytosine dioxygenase 2; NF- κ B; interleukin-8

Introduction

Preeclampsia is a hypertensive disorder of pregnancy that affects multiple maternal organs, placental function and fetal well-being. It occurs in 5-7% of all pregnancies, and is a leading cause of maternal and fetal morbidity and mortality [1]. Although the cause of preeclampsia is not known, the pregnancy-specific expression of protease-activated receptor 1 (PAR-1) on neutrophils may shed light on the origins of the disease and the underlying causes of maternal organ dysfunction. The extensive infiltration of activated neutrophils into blood vessels of women with preeclampsia [2–4] could explain why multiple maternal organs are affected.

PAR-1 is expressed on neutrophils, but only during pregnancy [5]. PAR-1 is not expressed on neutrophils of non-pregnant subjects [6], so neutrophil expression of PAR-1 is specific to pregnancy. This makes its expression unique to pregnancy and different from the expression of other receptors. PAR-1, originally known as thrombin receptor, is activated by serine proteases, such as thrombin and neutrophil elastase, but it was recently reported that MMP-1 also activates PAR-1 [7–9]. Activation leads to downstream signaling mechanisms that include the RhoA kinase (ROCK) phosphorylation pathway [10, 11]. ROCK is a recognized mediator of enhanced vascular reactivity and also regulates the shape and movement of cells [12, 13].

In normal pregnancy, adverse outcomes are prevented, in part, by the silencing of inflammatory genes. One possible silencing mechanism is DNA methylation, which masks binding sites for transcription factors implicated in the inflammatory response, such as NF- κ B. Conversely, if methylation marks are erased, the sites open, leading to increased gene expression. One mechanism for erasing methylation marks involves the TET proteins (Ten-Eleven Translocation proteins, aka tet methylcytosine dioxygenases). TET proteins regulate gene expression by enzymatic de-methylation of DNA [14–17] [18]. They catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, which is further oxidized and then removed by DNA base excision repair enzymes and replaced with unmodified cytosine. They were first discovered in 2009 [16], but little is known about their regulation or role in disease. TET2 is the main TET protein expressed in leukocytes, and its activation has been shown to play an essential role in regulating hematopoietic differentiation, which proceeds

in mature cells without cell division, normally during emigration from the circulation into tissue [19–21].

We were interested in MMP-1 and neutrophil elastase as PAR-1 activating proteases because both the *MMP-1* and *ELANE* genes are epigenetically regulated during pregnancy [22–24], and both proteases have been reported to be increased in women with preeclampsia [25–27]. With regard to MMP-1, immunohistochemical studies revealed intense staining in blood vessels and neutrophils of preeclamptic women as compared to normal pregnant women, and its gene and protein expression were significantly increased in omental arteries [25]. Furthermore, plasma levels of active MMP-1 were significantly elevated in women with preeclampsia, as opposed to negligible levels in women with normal pregnancy [25].

Based on the information described above, we propose a novel hypothesis that elevated levels of proteases in the maternal circulation of preeclamptic women activate neutrophils due to their pregnancy-specific expression of PAR-1. The activation of PAR-1 leads to activation of ROCK, which triggers translocation of TET2 and NF- κ B from the cytosol into the nucleus, resulting in increased expression of genes involved in inflammation.

Materials and Methods

Study Subjects

Omental fat biopsies (approximately 2 cm x 4 cm x 2 cm) were collected for immunohistochemistry from normal pregnant and preeclamptic women undergoing Cesarean section at MCV Hospitals, Virginia Commonwealth University Medical Center. Omental fat was studied because it is richly vascularized, and it is an accessible vascular bed representative of the maternal systemic circulation. Plasma samples were collected for MMP-1 and neutrophil elastase analysis longitudinally during pregnancy from a separate group of women. Neutrophils for confocal studies were isolated from whole blood from a third group of preeclamptic and normal pregnant women. Normal pregnant women had maternal blood pressures $\leq 110/70$ mmHg, no proteinuria and no other complications. Preeclamptic women had blood pressures $\geq 140/90$ mmHg on 2 occasions at least 4 hours apart after 20 weeks' gestation and proteinuria (0.3 gm/24 hours, protein/creatinine ratio 0.3, or $\geq 1+$ urine dipstick). The Office of Research Subjects Protection of Virginia Commonwealth University approved this study (HM20009145, HM20005160). All subjects gave informed consent, and the procedures followed were in accordance with institutional guidelines. Clinical characteristics of the patient groups are given in Table 1.

Immunohistochemistry

Omental fat samples were formalin-fixed, paraffin embedded and cut into 7 μ m sections with a microtome. Immunohistochemistry was performed as previously described [2, 28, 4]. To quench endogenous tissue peroxidase activity, slides were incubated in 3% hydrogen peroxide in methanol for 30 minutes. For antigen retrieval, slides were heat treated in 10 mM citrate buffer for 5 minutes with a pressure cooker. Slides were blocked for 1 hour in 10% goat serum. Tissues were immunostained with rabbit polyclonal antibody to TET2 (1:50, Proteintech, Rosemont, IL, Cat. #21207-1-AP), rabbit polyclonal antibody to PAR-1

(1:50, Proteintech, Cat. #15607-1-AP) or rabbit IgG isotype negative control pre-diluted in phosphate-buffered saline (Invitrogen, Cat. #086199) using Vector ABC Kit (Thermo Fisher Scientific, Waltham, MA, Cat. #PK-6101) and ImmPACT DAB (Thermo Fisher Scientific, Cat. #SK-4105). Slides were counterstained with 1:5 dilution of Hematoxylin QS (Vector Laboratories, Burlingame, CA). The staining protocol was the same for all samples with regard to processing, incubation times and temperature. Slides were analyzed with an Olympus BH-2 microscope (Olympus, Center Valley, PA) attached to a digital camera (Olympus QColor5) using image analysis software (cellSens, Olympus).

Analysis of Maternal Plasma MMP-1 and Neutrophil Elastase Levels

Maternal plasma MMP-1 levels were analyzed by a specific MMP-1 Human Biotrak ELISA System (Cat. #RPN2610, GE Healthcare Life Sciences, Marlborough, MA). Maternal plasma neutrophil elastase levels were analyzed by a specific Human PMN-Elastase ELISA Kit (Cat. #BMS269, Invitrogen, ThermoFisher Scientific, Grand Island, NY).

Confocal Immunofluorescent Experiments

Activation of TET2 and NF- κ B were assessed by their translocation from the cytosol into the nucleus detected by immunofluorescence staining and confocal microscopy. Two 10 ml heparin tubes of blood were collected from normal pregnant women and preeclamptic women. Lymphocytes/monocytes were separated from granulocytes (96% of which are neutrophils) by Histopaque (1077/1119) density gradient centrifugation according to the manufacture's protocol (Sigma Aldrich, St. Louis, MO) and as previously described [29, 30, 25, 3, 31]. Neutrophils were seeded at 200,000 cells per ml in Falcon 4-well cell culture slides (#354104) and cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum and 1% antibiotics and antimycotics (100 U/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B) at 37°C in a humidified 5% CO₂ atmosphere. We performed time course experiments for MMP-1 (15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours) and dose response experiments for MMP-1 (activated MMP-1: 1, 5, 25 ng/ml) and elastase (0.0033, 0.033, 0.33 U/ml). The middle and higher doses were used for experiments with a 1-hour incubation time to correlate with the maximal nuclear localization reported for NF- κ B [32].

Neutrophils from normal pregnant women were used for protease treatment experiments. Treatments were run in duplicate and added for 1 hour: 1) Control media; 2) MMP-1 (5 ng/ml, 25 ng/ml, Calbiochem, San Diego, CA) activated with p-amino phenylmercuric acetate (APMA) [25]; and 3) elastase (0.033, 0.33 U/ml, Sigma Aldrich). The MMP-1 doses are equivalent to those of active MMP-1 in the circulation of preeclamptic women [25]. For inhibitor treatments, they were added before activating treatments: 1) specific PAR-1 inhibitor (2 μ M, SCH-79797, Tocris, Minneapolis, MN); 2) specific ROCK inhibitor (20 μ M Y-27632, Tocris). Media were collected, cells washed with PBS and fixed with 4% formalin for 1 hour. Cells were washed, then blocked and permeabilized for 1 hour in 10% goat serum +3% BSA+0.25% Triton X-100. Cells were incubated overnight at 4°C with primary rabbit antibody specific for TET2 (1:250, Proteintech) or the p65 subunit of NF- κ B (1:50, Proteintech). The next morning, cells were washed and incubated with anti-rabbit IgG secondary green fluorescence dye (Alexa Fluor 488, 1:3000, Jackson ImmunoResearch,

Burlingame, CA) for TET2 or red fluorescence dye (Cy3, 1:2000, Jackson ImmunoResearch) for p65 at room temperature for 1 hour. Cells were washed and mounted with VectaMount medium containing DAPI (Vector Laboratories) for nuclear DNA staining. Images were taken using a confocal microscope (Zeiss LSM 700). Percentages of cells with nuclear localization of TET2 or p65 (i.e., negative vs. positive) were determined in 1-2 fields (x63 lens) for each treatment, and intensity of fluorescence in cytosol vs. nucleus was quantified using Image J software (NIH) by two independent evaluators using the Freehand tool and Integrated Density measurement. The density of the nucleus was taken as a percentage of the density for the whole cell. An average of 12 cells were analyzed per well.

Neutrophils from women with preeclampsia were evaluated for nuclear localization of TET2 and p65. Neutrophils were incubated as above for 1 hour in quadruplicate with the following treatments: 1) Control media; 2) PAR-1 inhibitor; 3) ROCK inhibitor. An average of 13 cells were analyzed for nuclear translocation of TET2 and p65 per well.

Cell Culture for Interleukin-8 (IL-8)

Cell culture for IL-8, an NF- κ B regulated gene, was done as above except neutrophils were seeded at a density of 1,000,000 cells per ml in 24-well culture dishes and incubated for 4 hours. Media was collected and frozen at -20°C until assay. Media concentrations of IL-8 were measured by specific ELISA (R&D Systems, Minneapolis, MN).

MMP-1 Activation

Pro-MMP-1 (Calbiochem) was activated using the organomercurial protocol described in the manufacturer's product data sheet. Pro-MMP-1 (400 ng/100 μl) was incubated in tris-triton-calcium buffer containing 1 mM p-amino phenylmercuric acetate (APMA, Calbiochem) for 2 hours at 37°C . The product was followed by ultra-filtration at 4°C to remove the APMA using a Microcon Ultracel Filter device YM-10 (Millipore, MW 10000). To prevent sticking and allow for maximum MMP-1 recovery, the flow-through filter was first treated with 100 μL of 1 mg/mL bovine serum albumin for 30 minutes at 37°C .

Data Analysis

Demographic data are presented as means $\pm$ SD and experimental data are presented as means $\pm$ SE. Data were analyzed as appropriate by t-test or one-way ANOVA with Bonferroni multiple comparisons test using a statistical software program (Prism 4, GraphPad software, San Diego, CA). If variances were not equal, non-parametric Mann-Whitney test or Kruskal-Wallis test were used. Treatment replicates were averaged for statistical analysis. Power analysis indicated sufficient sample size for each experiment to achieve 0.80 power to detect an α of 0.05. A $P < 0.05$ was considered statistically significant.

Results

Vascular Expression of PAR-1 and TET2 in Normal Pregnancy and Preeclampsia

Figure 1 shows representative images of omental fat vessels of preeclamptic and normal pregnant women immunostained for PAR-1 and TET2. In normal pregnancy, weak staining

was present for PAR-1 in the endothelium and for neutrophils in the vessel lumen (Panel A). In preeclampsia, PAR-1 expression was markedly increased with dark staining in endothelial cells, vascular smooth muscle, and neutrophils flattened and adhered to the endothelium, infiltrated into the vessel, and present in the lumen of the vessel (Panel B). In preeclampsia, $78 \pm 2\%$ of vessels stained for PAR-1 as compared to only $12 \pm 1\%$ of vessels in normal pregnancy ($n = 8$ for each group, $P < 0.0001$). Immunostaining for TET2 mirrored the staining for PAR-1 (Panels C and D) with significantly more staining in omental vessels of preeclamptic than normal pregnant women. In preeclampsia, $88 \pm 2\%$ of vessels stained for TET2 with neutrophils infiltrated into the vessel wall, as compared to normal pregnancy of only $16 \pm 5\%$ of vessels with staining ($n=8$ for each group, $P<0.0001$). When neutrophils were present in normal vessels, they were usually in the lumen of the vessel. The polymorphonuclear nucleus of neutrophils was clearly discernible by dark staining for TET2 in preeclampsia (Panel F), as opposed to diffuse staining of neutrophils in normal pregnancy (Panel E).

Maternal Plasma Levels of MMP-1 and Neutrophil Elastase in Normal Pregnant and Preeclamptic Women

Figure 2 shows maternal plasma levels of MMP-1 and neutrophil elastase. For analysis, samples were grouped according to gestational age at the time of sample collection and according to whether the samples were collected before or after clinical diagnosis of preeclampsia. MMP-1 levels were significantly elevated in the second trimester (13 – 28 weeks) and third trimester (29 – 40 weeks) and neutrophil elastase was significantly elevated in the third trimester in women who developed preeclampsia as compared to women who had normal pregnancies. Because samples were collected longitudinally throughout pregnancy, many of the preeclamptic samples were collected while the subjects were not yet diagnosed with preeclampsia. For this reason, we also evaluated the levels before and after diagnosis of preeclampsia. MMP-1 was significantly elevated an average of 10 weeks before the diagnosis of preeclampsia, as well as after diagnosis, whereas neutrophil elastase was significantly elevated only after diagnosis of preeclampsia.

Cellular Localization of TET2 and NF- κ B in Neutrophils of Women with Preeclampsia and Women with Normal Pregnancies

The clear demarcation of the polymorphonuclear nucleus of neutrophils present in preeclamptic women by TET2 immunostaining (Fig. 1) suggested that TET2 was activated in preeclampsia. To confirm this, we used confocal microscopy. As shown in Figures 3 and 4, TET2 (green) and the p65 subunit of NF- κ B (red) were strongly localized to the nucleus of untreated neutrophils of preeclamptic women, but not in untreated neutrophils of normal pregnant women where they were localized to the cytosol. This indicated that TET2 and NF- κ B were indeed activated in neutrophils of preeclamptic women, but not in neutrophils of normal pregnant women. When neutrophils of normal pregnant women were treated with MMP-1, MMP-1 caused the translocation of TET2 and p65 from the cytosol to the nucleus, mimicking the nuclear localization present in neutrophils of preeclamptic women. In time-course experiments, TET2 started moving into the nucleus as early as 15 minutes after MMP-1 treatment, which is consistent for proteins containing a nuclear localization signal

(prosite.expasy.org). Nuclear localization was maximal at 1 hour, the same as that reported for NF- κ B [32], so these two mediators appear to be linked.

Protease Activation of TET2 and NF- κ B Via a PAR-1, ROCK Pathway

To determine the protease activating pathway, we treated neutrophils of normal pregnant women with MMP-1 and specific inhibitors of PAR-1 or ROCK. Figures 3 and 4 show that the intense localization of TET2 and p65 into the nucleus induced by MMP-1 was prevented by co-treatment with either PAR-1 inhibitor or ROCK inhibitor. TET2 and p65 remained in the cytosol indicating nuclear translocation was inhibited.

We wondered if the movement of TET2 and p65 between the cytosol and the nucleus was a fluid process, and if so, if it could be reversed by inhibition of PAR-1 or ROCK. To test this, we treated neutrophils of preeclamptic women with inhibitors. Remarkably, treatment with either PAR-1 inhibitor or ROCK inhibitor for just one hour resulted in a significant decrease in the nuclear localization TET2 and p65 with their brightest staining now back in the cytosol (Figs. 3 and 4).

Figure 5 presents the summary of results for % nuclear localization of TET2 and p65 in response to neutrophils from normal pregnant women treated with MMP-1 (Panels A and B). MMP-1 significantly increased the nuclear localization of TET2 and p65. Co-treatment with inhibitors of PAR-1 or ROCK prevented the translocation of TET2 and p65 from the cytosol to the nucleus. Similar results to MMP-1 were obtained when the protease treatment was elastase (Panel C). Figure 6 shows the summarized results for preeclamptic pregnancy neutrophils. The % nuclear localization of TET2 and p65 in untreated neutrophils of preeclamptic women was equivalent to that of MMP-1 treated neutrophils of normal pregnant women. Treatment with PAR-1 or ROCK inhibitors significantly decreased the amount of TET2 or p65 localized to the nucleus of preeclamptic neutrophils.

To further confirm protease activation of PAR-1 in the inflammatory response of pregnancy neutrophils, we measured media concentrations of IL-8, an NF- κ B regulated gene, in response to treatments. Media concentrations of IL-8 were significantly increased by treatment with MMP-1 or elastase (Fig. 7), which correlated with the movement of TET2 and p65 from the cytosol to the nucleus. When the nuclear translocations of TET2 and p65 were prevented by co-treatment with PAR-1 or ROCK inhibitors, IL-8 levels remained at control levels.

Discussion

The findings of this study are novel because protease activation of neutrophils is specific to pregnancy, and may explain the pathophysiology of preeclampsia. Herein we show that MMP-1 and neutrophil elastase were significantly elevated in the circulation of women with preeclampsia, and that TET2 and p65 were localized to the neutrophil nucleus, as opposed to women with normal pregnancy where TET2 and p65 were localized to the neutrophil cytosol. When neutrophils from normal pregnant women were treated with proteases, TET2 and p65 moved from the cytosol to the nucleus mimicking the nuclear localization in preeclamptic neutrophils, providing evidence that elevated levels of proteases in

preeclamptic women are activating agents. These findings implicate protease activation of PAR-1 and epigenetic mechanisms involving DNA methylation in an inflammatory response specific to preeclampsia.

MMP-1 was significantly elevated 10 weeks before diagnosis of preeclampsia while the women were still considered to have a normal pregnancy. This means the elevation in MMP-1 is occurring well before the appearance of clinical symptoms. Both MMP-1 and neutrophil elastase were significantly elevated after clinical symptoms were diagnosed. This suggests that MMP-1 may be responsible for 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 of preeclamptic women.

The PAR-1 pathway for activation involved ROCK. ROCK is in the protein kinase C family of phosphorylating enzymes and has previously been shown to activate NF- κ B to increase expression of inflammatory genes [12, 13]. TET2 contains several protein kinase C phosphorylation sites [33] (prosite.expasy.org), and we found TET2 translocated from the cytosol to the nucleus in parallel with p65. Furthermore, inhibition of ROCK prevented nuclear translocation stimulated by proteases, indicating that activation of both TET2 and NF- κ B are regulated by ROCK phosphorylation in pregnancy neutrophils. Co-regulation makes sense if activation of TET2 is necessary to first move into the nucleus to erase methylation marks that mask transcription factor binding sites for NF- κ B to increase inflammatory gene expression. This possibility will be tested in a future study using a specific inhibitor recently available for TET2.

We believe the specific role of TET2 in pregnancy is to regulate the expression of inflammatory genes. As long as TET2 is not activated, inflammatory gene expression is suppressed by DNA methylation, but when proteases become elevated in preeclampsia, TET2 is activated and moves into the nucleus to demethylate transcription factor binding sites for NF- κ B resulting in increased expression of inflammatory genes. The fact that PAR-1 is only expressed on neutrophils in pregnant women and that there is extensive infiltration of neutrophils into the mother's systemic vasculature in preeclampsia is important to study because of the elevation of proteases in preeclamptic women. Our findings provide a basis for a new way of thinking about vascular dysfunction in preeclampsia implicating the pregnancy specific activation of neutrophil PAR-1 and epigenetics. This does not discount the potential roles of plasma factors other than proteases or endothelial receptors related to vascular dysfunction but adds a new dimension to the understanding of the underlying mechanisms of vascular phenotype.

We measured IL-8 as an index of NF- κ B activation. IL-8 is of interest in preeclampsia because it is a potent neutrophil chemokine [34], neutrophils infiltrate the vasculature of women with preeclampsia [2–4], and IL-8 expression is increased in blood vessels of preeclamptic women [2]. Both MMP-1 and elastase significantly increased IL-8 as compared to control, whereas inhibition of PAR-1 or ROCK prevented the protease stimulated increases. Therefore, the IL-8 pattern was the same as for the nuclear

translocation of TET2 and p65, suggesting that both TET2 and NF- κ B are involved in the inflammatory response induced by PAR-1 activation.

Immunohistochemistry revealed a remarkable increase in the expression of both PAR-1 and TET2 in omental vessels of preeclamptic women as compared to omental vessels of normal pregnant women. Increased expression was due to, not only increased expression in endothelium and vascular smooth muscle, but also to extensive infiltration of neutrophils into the vessels. Staining for TET2 mirrored the staining for PAR-1. This close relationship may have important implications for vascular inflammation in preeclampsia.

The polymorphonuclear nucleus was clearly discernable in neutrophils of preeclamptic women by the staining of TET2, which suggested TET2 was active in preeclampsia. This was confirmed with confocal studies showing TET2, as well as p65, concentrated in the nucleus of untreated neutrophils isolated from women with preeclampsia. This demonstrates that *in vivo* protease activation persists *in vitro*, which would be consistent with *in vivo* activation by MMP-1 because MMP-1 binds tightly to its substrate [35].

We also tested whether PAR-1 or ROCK inhibition would affect neutrophils isolated from preeclamptic women. We did not expect to see any effect because we thought that once the cells were activated, they would remain activated. We were surprised to find that inhibition of PAR-1 or ROCK resulted in an emptying of TET2 and p65 from the nucleus. This finding has important implications. First, it means the movements of TET2 and p65 between cytosol and nucleus are reversible. Secondly, this has important therapeutic implications for the treatment of preeclampsia based on inhibition of PAR-1 because several lines of evidence indicate protease activation of PAR-1 may play a central role in the pathology of preeclampsia as shown in our study and as reported by others as described below.

As shown in this study, protease activation of PAR-1 promotes an inflammatory response in pregnancy neutrophils, and we previously showed that MMP-1 causes vasoconstriction and enhances vascular reactivity to angiotensin II [36]. These findings implicate protease activation of PAR-1 in vascular inflammation and hypertension of preeclampsia. Activation of PAR-1 may explain other features of preeclampsia as well. For example, as reported by other investigators PAR-1 mediates coagulation abnormalities, platelet aggregation and thromboxane generation. In addition, protease activation of endothelial PAR-1 activates NF- κ B, upregulates cell adhesion molecules (ICAM-1), triggers production of neutrophil chemokines (IL-8), and increases endothelial permeability to trigger edema formation [37–39, 11, 40–42]. PAR-1 may also contribute to the elevation in angiogenic factors because trophoblast and decidual production of sFlt is stimulated by protease activation of PAR-1 [43, 44]. 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, which activates NADPH oxidase to generate reactive oxygen species resulting in the release of sFlt [45]. Activation of NADPH oxidase could explain the placental imbalance of increased thromboxane and decreased prostacyclin because oxidative stress drives this imbalance [46]. Inhibition of PAR-1 may, therefore, have multiple beneficial effects. The treatment of women with

preeclampsia based on inhibition of PAR-1 could be translated to the clinical setting because the FDA has approved a PAR-1 inhibitor for human use [47].

A protease activating mechanism for neutrophil PAR-1 could explain why preeclampsia only occurs in pregnant women. Initial activation of neutrophils likely occurs as they circulate through the intervillous space and are activated by increased lipid peroxides released by the placenta [48–50]. Activated neutrophils produce MMP-1 [24], so a protease feed forward scenario between infiltrated neutrophils and vascular smooth muscle release of MMP-1 could explain why clinical symptoms progressively worsen. Neutrophil activation could explain other features of preeclampsia. For example, since 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 post-partum period. Labor is recognized to be an inflammatory process and we showed that even in normal term labor there is extensive infiltration of neutrophils into maternal systemic vasculature [51]. Women who develop postpartum preeclampsia might have been on the verge of developing preeclampsia and then neutrophil infiltration with labor pushed them over the edge.

Figure 8 summarizes the implications of our study for preeclampsia and the experimental design used to delineate the pathway for protease activation of pregnancy neutrophils. In normal pregnancy circulating proteases are not elevated, TET2 and NF- κ B are localized to the cytosol and inflammatory genes are not expressed (Panel A). In preeclampsia, circulating proteases are elevated and activate neutrophils due to their pregnancy specific expression of PAR-1. Activation of PAR-1 results in the movement of TET2 and NF- κ B from the cytosol to the nucleus and the expression of inflammatory genes (Panel B). The PAR-1 pathway involves ROCK phosphorylation because inhibition of either PAR-1 or ROCK blocks the movement of TET2 and NF- κ B from the cytosol to the nucleus and the inflammatory response (Panel C). We conclude that elevated levels of proteases in the maternal circulation of preeclamptic women activate neutrophils due to their pregnancy-specific expression of PAR-1. PAR-1 activates ROCK which phosphorylates TET2 and NF- κ B causing their translocation from the cytosol to the nucleus. The fact that TET2 moved into the nucleus coincided with NF- κ B implicates epigenetic mechanisms and suggests that TET2 may enzymatically de-methylates DNA, opening up transcription factor binding sites for NF- κ B, resulting in the expression of inflammatory genes.

Acknowledgments

Microscopy was performed at the VCU Department of Anatomy & Neurobiology Microscopy Facility, supported, in part, by funding from the NIH-NINDS Center Core Grant 5 P30 NS047463 and, in part, by funding from the NIH-NCI Cancer Center Support Grant P30 CA016059.

Funding: This work was supported in part by Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD) grants 5R01 HD088386 (SWW), 1U01 HD087198 (SWW), and by the National Heart and Blood Institute (NHLBI) R01 HL069851 (SWW), and by the Dunlap Foundation.

References

1. Cunningham FG, Gant NF, Leveno KJ, Gilstrap LC III, Hauth JC, Wenstrom KD. Williams Obstetrics. 21th ed New York: McGraw-Hill; 2001.

2. Leik CE, Walsh SW. Neutrophils infiltrate resistance-sized vessels of subcutaneous fat in women with preeclampsia. *Hypertension*. 2004;44(1):72–7 [PubMed: 15148293]
3. Mishra N, Nugent WH, Mahavadi S, Walsh SW. Mechanisms of enhanced vascular reactivity in preeclampsia. *Hypertension*. 2011;58(5):867–73.doi:HYPERENSIONAHA.111.176602 [pii] 10.1161/HYPERENSIONAHA.111.176602 [doi]. [PubMed: 21947470]
4. Shah TJ, Walsh SW. Activation of NF-kappaB and expression of COX-2 in association with neutrophil infiltration in systemic vascular tissue of women with preeclampsia. *Am J Obstet Gynecol*. 2007;196(1):48e1–8 [PubMed: 17240230]
5. Wang Y, Gu Y, Lucas MJ. Expression of thrombin receptors in endothelial cells and neutrophils from normal and preeclamptic pregnancies. *J Clin Endocrinol Metab*. 2002;87(8):3728–34 [PubMed: 12161502]
6. Shpacovitch V, Feld M, Hollenberg MD, Luger TA, Steinhoff M. Role of protease-activated receptors in inflammatory responses, innate and adaptive immunity. *J Leukoc Biol*. 2008;83(6):1309–22.doi:10.1189/jlb.0108001. [PubMed: 18347074]
7. Ahn HS, Chackalamannil S, Boykow G, Graziano MP, Foster C. Development of proteinase-activated receptor 1 antagonists as therapeutic agents for thrombosis, restenosis and inflammatory diseases. *Curr Pharm Des*. 2003;9(28):2349–65 [PubMed: 14529396]
8. Trivedi V, Boire A, Tchernychev B, Kaneider NC, Leger AJ, O'Callaghan K et al. Platelet matrix metalloprotease-1 mediates thrombogenesis by activating PAR1 at a cryptic ligand site. *Cell*. 2009;137(2):332–43.doi:S0092-8674(09)00160-3 [pii] 10.1016/j.cell.2009.02.018 [doi]. [PubMed: 19379698]
9. Suzuki T, Moraes TJ, Vachon E, Ginzberg HH, Huang TT, Matthay MA et al. Proteinase-activated receptor-1 mediates elastase-induced apoptosis of human lung epithelial cells. *Am J Respir Cell Mol Biol*. 2005;33(3):231–47.doi:2005-0109OC [pii] 10.1165/rcmb.2005-0109OC [doi]. [PubMed: 15891109]
10. Coughlin SR. Protease-activated receptors in vascular biology. *Thromb Haemost*. 2001;86(1):298–307.doi:01070298 [pii]. [PubMed: 11487018]
11. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost*. 2005;3(8):1800–14.doi:JTH1377 [pii] 10.1111/j.1538-7836.2005.01377.x [doi]. [PubMed: 16102047]
12. Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nature reviews Molecular cell biology*. 2003;4(6):446–56.doi:10.1038/nrm1128. [PubMed: 12778124]
13. Yao L, Romero MJ, Toque HA, Yang G, Caldwell RB, Caldwell RW. The role of RhoA/Rho kinase pathway in endothelial dysfunction. *Journal of cardiovascular disease research*. 2010;1(4):165–70.doi:10.4103/0975-3583.74258. [PubMed: 21264179]
14. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q et al. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science*. 2011 ;333(6047):1303–7.doi:science.1210944 [pii] 10.1126/science.1210944 [doi]. [PubMed: 21817016]
15. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science*. 2011 ;333(6047):1300–3.doi:science.1210597 [pii] 10.1126/science.1210597 [doi]. [PubMed: 21778364]
16. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*. 2009;324(5929):930–5.doi:1170116 [pii] 10.1126/science.1170116 [doi]. [PubMed: 19372391]
17. Yin R, Mao SQ, Zhao B, Chong Z, Yang Y, Zhao C et al. Ascorbic Acid enhances tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. *J Am Chem Soc*. 2013;135(28):10396–403.doi:10.1021/ja4028346 [doi]. [PubMed: 23768208]
18. Nabel CS, Kohli RM. Molecular biology. Demystifying DNA demethylation. *Science*. 2011;333(6047):1229–30.doi:333/6047/1229 [pii] 10.1126/science.1211917 [doi]. [PubMed: 21885763]
19. Klug M, Schmidhofer S, Gebhard C, Andreessen R, Rehli M. 5-Hydroxymethylcytosine is an essential intermediate of active DNA demethylation processes in primary human monocytes. *Genome Biol*. 2013;14(5):R46.doi:gb-2013-14-5-r46 [pii] 10.1186/gb-2013-14-5-r46 [doi]. [PubMed: 23705593]

20. Wu H, Zhang Y. Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. *Genes Dev.* 2011;25(23):2436–52.doi:25/23/2436 [pii] 10.1101/gad.179184.111 [doi]. [PubMed: 22156206]
21. Klug M, Heinz S, Gebhard C, Schwarzfischer L, Krause SW, Andreesen R et al. Active DNA demethylation in human postmitotic cells correlates with activating histone modifications, but not transcription levels. *Genome Biol.* 2010;11 (6):R63.doi:gb-2010-11-6-r63 [pii] 10.1186/gb-2010-11-6-r63 [doi]. [PubMed: 20565882]
22. Wang H, Ogawa M, Wood JR, Bartolomei MS, Sammel MD, Kusanovic JP et al. Genetic and epigenetic mechanisms combine to control MMP1 expression and its association with preterm premature rupture of membranes. *Hum Mol Genet.* 2008;17(8):1087–96 [PubMed: 18178580]
23. Mousa AA, Archer KJ, Cappello R, Estrada-Gutierrez G, Isaacs CR, Strauss JF 3rd et al. DNA Methylation is Altered in Maternal Blood Vessels of Women With Preeclampsia. *Reprod Sci.* 2012;19(12):1332–42.doi:1933719112450336 [pii] 10.1177/1933719112450336 [doi]. [PubMed: 22902744]
24. Mousa AA, Cappello RE, Estrada-Gutierrez G, Shukla J, Romero R, Strauss JF 3rd et al. Preeclampsia is associated with alterations in DNA methylation of genes involved in collagen metabolism. *Am J Pathol.* 2012;181(4):1455–63.doi:S0002-9440(12)00494-4 [pii] 10.1016/j.ajpath.2012.06.019 [doi]. [PubMed: 22863954]
25. Estrada-Gutierrez G, Cappello RE, Mishra N, Romero R, Strauss JF 3rd, Walsh SW. Increased expression of matrix metalloproteinase-1 in systemic vessels of preeclamptic women a critical mediator of vascular dysfunction. *Am J Pathol.* 2011;178(1):451–60.doi:S0002-9440(10)00049-0 [pii] 10.1016/j.ajpath.2010.11.003 [doi]. [PubMed: 21224082]
26. Greer IA, Haddad NG, Dawes J, Johnstone FD, Calder AA. Neutrophil activation in pregnancy-induced hypertension. *Br J Obstet Gynaecol.* 1989;96(8):978–82 [PubMed: 2775697]
27. Gupta AK, Gebhardt S, Hillermann R, Holzgreve W, Hahn S. Analysis of plasma elastase levels in early and late onset preeclampsia. *Arch Gynecol Obstet.* 2006;273(4):239–42.doi:10.1007/s00404-005-0093-z [doi]. [PubMed: 16292578]
28. Shah TJ, Leik CE, Walsh SW. Neutrophil infiltration and systemic vascular inflammation in obese women. *Reprod Sci.* 2010;17 (2):116–24.doi:1933719109348252 [pii] 10.1177/1933719109348252 [doi]. [PubMed: 19820230]
29. Vaughan JE, Walsh SW. Neutrophils from pregnant women produce thromboxane and tumor necrosis factor-alpha in response to linoleic acid and oxidative stress. *Am J Obstet Gynecol.* 2005;193(3 Pt 1):830–5 [PubMed: 16150282]
30. Vaughan JE, Walsh SW, Ford GD. Thromboxane mediates neutrophil superoxide production in pregnancy. *Am J Obstet Gynecol.* 2006;195(5):1415–20 [PubMed: 16643813]
31. Walsh SW. Plasma from preeclamptic women stimulates transendothelial migration of neutrophils. *Reprod Sci.* 2009;16(3):320–5 [PubMed: 19087976]
32. Noursadeghi M, Tsang J, Hausteint T, Miller RF, Chain BM, Katz DR. Quantitative imaging assay for NF-kappaB nuclear translocation in primary human macrophages. *J Immunol Methods.* 2008;329(1-2):194–200.doi:10.1016/j.jim.2007.10.015. [PubMed: 18036607]
33. Bauer C, Gobel K, Nagaraj N, Colantuoni C, Wang M, Muller U et al. Phosphorylation of TET proteins is regulated via O-GlcNAcylation by the glycosyltransferase OGT. *J Biol Chem.* 2015.doi:M114.605881 [pii] 10.1074/jbc.M114.605881 [doi].
34. Luster AD. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med.* 1998;338(7):436–45 [PubMed: 9459648]
35. Welgus HG, Jeffrey JJ, Stricklin GP, Roswit WT, Eisen AZ. Characteristics of the action of human skin fibroblast collagenase on fibrillar collagen. *J Biol Chem.* 1980;255(14):6806–13 [PubMed: 6248534]
36. Nugent WH, Mishra N, Strauss JF 3rd, Walsh SW. Matrix metalloproteinase 1 causes vasoconstriction and enhances vessel reactivity to angiotensin II via protease-activated receptor 1. *Reprod Sci.* 2016;23(4):542–8.doi:10.1177/1933719115607998. [PubMed: 26438597]
37. Henriksen RA, Samokhin GP, Tracy PB. Thrombin-induced thromboxane synthesis by human platelets. Properties of anion binding exosite I-independent receptor. *Arterioscler Thromb Vasc Biol.* 1997;17(12):3519–26 [PubMed: 9437201]

38. Austin KM, Covic L, Kuliopulos A. Matrix metalloproteases and PAR1 activation. *Blood*. 2013; 121 (3):431–9.doi:blood-2012-09-355958 [pii] 10.1182/blood-2012-09-355958 [doi]. [PubMed: 23086754]
39. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature*. 2000;407(6801):258–64.doi: 10.1038/35025229 [doi]. [PubMed: 11001069]
40. Rahman A, True AL, Anwar KN, Ye RD, Voyno-Yasenetskaya TA, Malik AB. Galpha(q) and Gbetagamma regulate PAR-1 signaling of thrombin-induced NF-kappaB activation and ICAM-1 transcription in endothelial cells. *Circ Res*. 2002;91 (5):398–405 [PubMed: 12215488]
41. Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. *Pharmacol Rev*. 2001 ;53(2):245–82 [PubMed: 11356985]
42. Goerge T, Barg A, Schnaeker EM, Poppelmann B, Shpacovitch V, Rattenholl A et al. Tumor-derived matrix metalloproteinase-1 targets endothelial proteinase-activated receptor 1 promoting endothelial cell activation. *Cancer Res*. 2006;66(15):7766–74.doi:66/15/7766 [pii] 10.1158/0008-5472.CAN-05-3897 [doi]. [PubMed: 16885380]
43. Lockwood CJ, Toti P, Arcuri F, Norwitz E, Funai EF, Huang ST et al. Thrombin regulates soluble fms-like tyrosine kinase-1 (sFlt-1) expression in first trimester decidua: implications for preeclampsia. *Am J Pathol*. 2007;170(4):1398–405.doi:S0002-9440(10)61353-3 [pii] 10.2353/ajpath.2007.060465 [doi]. [PubMed: 17392178]
44. Zhao Y, Koga K, Osuga Y, Nagai M, Izumi G, Takamura M et al. Thrombin enhances soluble Fms-like tyrosine kinase 1 expression in trophoblasts; possible involvement in the pathogenesis of preeclampsia. *Fertil Steril*. 2012;98(4):917–21 .doi:S0015-0282(12)00691-7 [pii] 10.1016/j.fertnstert.2012.06.038 [doi]. [PubMed: 22819145]
45. Huang QT, Chen JH, Hang LL, Liu SS, Zhong M. Activation of PAR-1/NADPH oxidase/ROS signaling pathways is crucial for the thrombin-induced sFlt-1 production in extravillous trophoblasts: possible involvement in the pathogenesis of preeclampsia. *Cell Physiol Biochem*. 2015;35(4):1654–62.doi:000373979 [pii] 10.1159/000373979 [doi]. [PubMed: 25824463]
46. Walsh SW. Eicosanoids in preeclampsia. *Prostaglandins Leukot Essent Fatty Acids*. 2004;70(2):223–32 [PubMed: 14683695]
47. Poole RM, Elkinson S. Vorapaxar: first global approval. *Drugs*. 2014;74(10):1153–63.doi:10.1007/s40265-014-0252-2 [doi]. [PubMed: 24962425]
48. Walsh SW. Maternal-placental interactions of oxidative stress and antioxidants in preeclampsia. *Semin Reprod Endocrinol*. 1998;16(1):93–104 [PubMed: 9654611]
49. Walsh SW, Vaughan JE, Wang Y, Roberts LJ, II. Placental isoprostane is significantly increased in preeclampsia. *FASEB J*. 2000;14(10):1289–96 [PubMed: 10877821]
50. Mellembakken JR, Aukrust P, Olafsen MK, Ueland T, Hestdal K, Videm V. Activation of leukocytes during the uteroplacental passage in preeclampsia. *Hypertension*. 2002;39(1):155–60 [PubMed: 11799095]
51. Leik CE, Walsh SW. Systemic activation and vascular infiltration of neutrophils with term labor. *J Soc Gynecol Investig*. 2006;13(6):425–9

1. Representative images of immunohistochemical staining for PAR-1 and TET2 in omental fat vessels of women with normal pregnancy and women with preeclampsia and percentage of vessels stained. In normal pregnancy, weak brown staining for PAR-1 was present in the endothelial cells (EC) and in neutrophils (arrows) present in the vessel lumen (VL) (Panel A). Little staining was present for TET2 in normal pregnancy (Panel C). In preeclampsia, there was significantly more staining for PAR-1 and TET2 than in normal pregnancy. PAR-1 and TET2 were both expressed in endothelial cells, vascular smooth muscle cells (VSM) and

in neutrophils flattened and adhered to the endothelium, infiltrated into the vessel and present in the lumen of the vessel (Panels B and D). Dark brown staining of the polymorphonuclear nucleus (polys) of neutrophils was evident in preeclamptic vessels (Panel F), as opposed to diffuse staining of neutrophils in normal pregnancy vessels (Panel E). Approximately 80 - 90% of vessels stained positive for TET2 and PAR-1 in women with preeclampsia (PE, n = 8) as compared to only about 15% of vessels in women with normal pregnancy (NP, n = 8). In all of the preeclamptic patients, the polymorphonuclear nucleus of neutrophils was evident by darker staining than the cytosol. Pictures for Panels A through D were taken with a 40X lens and pictures for Panels E and F with a 100X lens. A, adipose cell, **** P < 0.0001.

2.

Maternal plasma levels of MMP-1 and neutrophil elastase in women with normal pregnancy (NP) and women who developed preeclampsia (PE). Samples were collected longitudinally throughout pregnancy. MMP-1 was significantly elevated in the second trimester (weeks 13 – 28, $n = 20$ for NP and $n = 16$ for PE) and third trimester (weeks 29 – 40, $n = 24$ for NP and $n = 16$ for PE) in women who developed preeclampsia and neutrophil elastase was significantly elevated in the third trimester. MMP-1 was significantly elevated an average of 10 weeks before the diagnosis of preeclampsia ($n = 17$), as well as after the diagnosis of preeclampsia ($n = 14$), whereas neutrophil elastase was significantly elevated only after diagnosis of preeclampsia, ($n = 15$ for NP subjects and $n = 20$ for PE subjects, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Normal Pregnancy Neutrophils

Preeclamptic Pregnancy Neutrophils

3.

Representative images of TET2 immunofluorescence staining for neutrophils isolated from women with normal pregnancy and women with preeclampsia. Green fluorescence identifies TET2 and DAPI blue identifies the location of the nucleus. TET2 was strongly localized to the nucleus of untreated neutrophils of preeclamptic women, but not in neutrophils of normal pregnant women where TET2 was localized to the cytosol. When neutrophils of normal pregnant women were treated with MMP-1, TET2 localized to the nucleus mimicking the nuclear localization of untreated preeclamptic pregnancy neutrophils. The

intense localization of TET2 to the nucleus caused by MMP-1 in normal pregnancy neutrophils was prevented by co-treatment with either PAR-1 inhibitor or ROCK inhibitor. When neutrophils of preeclamptic women were treated with inhibitors, there was a remarkable decrease in the amount of TET2 in the nucleus with most of the fluorescence for TET2 now in the cytosol. N, nucleus

Normal Pregnancy Neutrophils

Preeclamptic Pregnancy Neutrophils

4.

Representative images of immunofluorescence staining for the p65 subunit of NF- κ B in neutrophils isolated from women with normal pregnancy and women with preeclampsia. Red fluorescence identifies p65 and DAPI blue the location of the nucleus. As for TET2, p65 was strongly localized to the nucleus of untreated preeclamptic pregnancy neutrophils, but in untreated normal pregnancy neutrophils it was localized to the cytosol. MMP-1 caused intense localization of p65 to the nucleus of normal pregnancy neutrophils, whereas co-treatment with PAR-1 inhibitor or ROCK inhibitor prevented the translocation of p65 from

the cytosol to the nucleus. Also similar to TET2, treatment of neutrophils of preeclamptic women with inhibitors markedly decreased nuclear p65 with the brightest staining now in the cytosol. N, nucleus

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

5.

Percent of nuclear localization of TET2 or p65 in response to treatment of neutrophils from normal pregnant women with MMP-1 or elastase in the absence and presence of inhibitors of PAR-1 or ROCK. Nuclear localization was quantitated by the density of immunofluorescence in the area of the nucleus as a percentage of the density of the entire cell using Image J software. MMP-1 caused a dose response increase in the translocation of TET2 from the cytosol to the nucleus, which was prevented by inhibition of PAR-1 or ROCK (Panel A). MMP-1 also caused a significant increase in the nuclear localization of

p65, which was prevented by PAR-1 or ROCK inhibition (Panel B). TET2 results for elastase and inhibitors were similar to those for MMP-1 (Panel C). (n = 7 for MMP-1 TET2, n = 11 for elastase TET2, n = 5 for MMP-1 p65, mean $\pm$ SE, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001)

Preeclamptic Pregnancy Neutrophils

6. Percent of nuclear localization of TET2 or p65 in neutrophils isolated from women with preeclampsia as compared to normal pregnancy and in the presence of inhibitors of PAR-1 or ROCK. Immunofluorescence intensities for TET2 and p65 were strongly localized to the nucleus in untreated neutrophils of preeclamptic women (PE) as compared to untreated neutrophils of normal pregnant women (NP) for which little immunofluorescence was present in the nucleus. Treatment with inhibitors of PAR-1 or ROCK significantly reduced the amount of TET2 or p65 localized to the nucleus of preeclamptic neutrophils. In the

presence of PAR-1 or ROCK inhibition, nuclear localization was comparable to normal pregnancy, (n = 6, mean $\pm$ SE, **** P < 0.0001. Data for NP neutrophils is from Figure 5 and shown here for the sake of comparison with PE and inhibitors)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Normal Pregnancy Neutrophils

7.

Media concentrations of IL-8, an NF- κ B regulated gene, in response to MMP-1 or elastase alone and in the presence of inhibitors of PAR-1 or ROCK. Both MMP-1 and elastase significantly stimulated IL-8, but the IL-8 response was prevented in the presence of inhibitors of PAR-1 or ROCK, (n = 14 for MMP-1 and n = 9 for elastase, mean $\pm$ SE, * P < 0.05, *** P < 0.001)

8.

Proposed mechanism whereby elevated levels of proteases in the maternal circulation of preeclamptic women activate neutrophils via their pregnancy-specific expression of PAR-1. In normal pregnancy, proteases are not elevated and TET2 and NF- κ B are localized to the cytosol so inflammatory gene expression does not occur (Panel A). In preeclampsia, circulating proteases are elevated and activate neutrophils due to their pregnancy specific expression of PAR-1. Activation of PAR-1 results in the movement of TET2 and NF- κ B from the cytosol to the nucleus. In the nucleus, TET2 enzymatically de-methylates DNA

opening up transcription factor binding sites for NF- κ B resulting in increased expression of preeclamptic inflammatory genes (Panel B). The PAR-1 pathway involves ROCK phosphorylation of TET2 and NF- κ B because inhibition of either PAR-1 or ROCK blocks the movement of TET2 and NF- κ B from the cytosol to the nucleus and the expression of inflammatory genes (Panel C).

Table 1:

Clinical Characteristics of Patient Groups

Variable	Normal Pregnant n = 40	Preeclamptic n = 36
Maternal age (years)	27.6 ± 5.3	27.8 ± 5.7
Pre-pregnancy BMI (kg/m ²)	27.5 ± 6.1	31.5 ± 8.6
BMI at sample collection (kg/m ²)	32.3 ± 6.1	36.6 ± 8.7
Systolic blood pressure (mmHg)	119 ± 10	161 ± 19 ^{***}
Diastolic blood pressure (mmHg)	73 ± 10	99 ± 14 ^{***}
Proteinuria (mg/24 h)	ND	259 ± 72
Dipstick	ND	2.1 ± 0.9
Protein/creatinine Ratio	ND	0.6 ± 0.4
Primiparous	14	16
Multiparous	26	20
Race		
White	15	8
Black	20	21
Hispanic	2	7
Other	3	
Gestational age at delivery (weeks)	39.5 ± 1.1	32.1 ± 4.5 ^{***}
Infant birth weight (grams)	3334 ± 447	1768 ± 996 ^{***}

Values are mean ± SD

P < 0.001

ND = not determined