Reduced Methylation of the Thromboxane Synthase Gene Is Correlated With Its Increased Vascular Expression in Preeclampsia

Ahmad A. Mousa, Jerome F. Strauss III, Scott W. Walsh

Abstract—Preeclampsia is characterized by increased thromboxane and decreased prostacyclin levels, which predate symptoms, and can explain some of the clinical manifestations of preeclampsia, including hypertension and thrombosis. In this study, we examined DNA methylation of the promoter region of the thromboxane synthase gene (TBXAS1) and the expression of thromboxane synthase in systemic blood vessels of normal pregnant and preeclamptic women. Thromboxane synthase is responsible for the synthesis of thromboxane A₂, a potent vasoconstrictor and activator of platelets. We also examined the effect of experimentally induced DNA hypomethylation on the expression of thromboxane synthase in a neutrophil-like cell line (HL-60 cells) and in cultured vascular smooth muscle and endothelial cells. We found that DNA methylation of the TBXAS1 promoter was decreased and thromboxane synthase expression was increased in omentum of preeclamptic women as compared with normal pregnant women. Increased thromboxane synthase expression was observed in vascular smooth muscles, endothelial cells, and infiltrating neutrophils. Experimentally induced DNA hypomethylation only increased expression of thromboxane synthase in the neutrophil-like cell line, whereas tumor necrosis factor-α, a neutrophil product, increased its expression in cultured vascular smooth muscle cells. Our study suggests that epigenetic mechanisms and release of tumor necrosis factor-α by infiltrating neutrophils could contribute to the increased expression of thromboxane synthase in maternal systemic blood vessels, contributing to the hypertension and coagulation abnormalities associated with preeclampsia. (Hypertension. 2012;59:00-00.) • Online Data Supplement

Key Words: preeclampsia • DNA methylation • thromboxane synthase • epigenetics • omental blood vessels • thromboxane A₂

Preeclampsia occurs in 5% to 7% of pregnancies and is a leading cause of maternal and infant mortality and morbidity. It is diagnosed clinically by the onset of hypertension and proteinuria, usually occurring after 20 weeks’ gestation. Preeclampsia is also associated with increased activation of the coagulation system evidenced by an increase in formation of fibrin, activation of the fibrinolytic system, expression of platelets, and a decrease in platelet count. In 1985, increased thromboxane and decreased prostacyclin levels were reported in placentas of women with preeclampsia and later confirmed for maternal blood and maternal urine. The imbalance in thromboxane, a potent vasoconstrictor and activator of platelets, and prostacyclin, a vasodilator and inhibitor of platelet activation, could explain hypertension, reduced uteroplacental blood flow, and hypercoagulopathy observed in women with preeclampsia.

Thromboxane and prostacyclin have a common precursor, prostaglandin H₂, but are synthesized by different enzymes. Thromboxane synthase is the enzyme that catalyzes the isomerization of prostaglandin H₂ into thromboxane. An increase in thromboxane synthase has been demonstrated in trophoblast and decidual cells of placentas of preeclamptic women, but increased expression in maternal tissue has heretofore not been shown. If thromboxane synthase was increased in maternal blood vessels, vasoconstriction and platelet activation could result because of increased thromboxane.

Increased thromboxane production in preeclampsia could be related to altered expression of the thromboxane synthase gene (TBXAS1) resulting from genomic variation or transcriptional activation. The latter could encompass epigenetic regulation, including DNA methylation. DNA methylation is a major epigenetic mechanism controlling gene expression. In general, hypomethylation is associated with increased gene expression, whereas hypermethylation is associated with decreased gene expression. It has been reported that DNA...
methylation is involved in the regulation of \(TBXAS1\).\(^{11}\) Thus, reduced methylation of the \(TBXAS1\) gene could result in increased thromboxane synthase and increased thromboxane \(A_2\) production. DNA methylation status in preeclampsia may be related to oxidative stress. Oxidation of DNA causes loss of methylation,\(^{12-14}\) and preeclampsia is associated with oxidative stress.\(^{15,16}\) Consistent with this notion is a preliminary report that found increased urinary levels of 8-hydroxy-2-deoxyguanosine, an indicator of DNA oxidation, in preeclamptic women.\(^{17}\)

In the present study, we tested the hypothesis that reduced DNA methylation of the \(TBXAS1\) gene leads to increased vascular expression of thromboxane synthase in preeclampsia. To test this, we examined the DNA methylation status of the \(TBXAS1\) gene and correlated it with gene and protein expression of thromboxane synthase in omental arteries obtained from preeclamptic and normal pregnant women. We then experimentally induced hypomethylation in vascular smooth muscle and endothelial cells and in a neutrophil-like cell line and determined gene and protein expression of thromboxane synthase. We examined the expression of thromboxane synthase in omental arteries, because these arteries are a component of the maternal systemic vasculature, and they play a role in blood pressure regulation by contributing to the total peripheral vascular resistance. We examined the effect of hypomethylation in the neutrophil cell line because of the extensive vascular infiltration of neutrophils that occurs in preeclampsia,\(^{18-20}\) and neutrophils are a source of thromboxane.\(^{21}\)

**Materials and Methods**

**Study Subjects**

Omental fat biopsies of \(\sim 2.0 \text{~cm} \times 2.0 \text{~cm} \times 0.5 \text{~cm}\) in size were collected from normal pregnant (\(n=16\)) and preeclamptic (\(n=22\)) women (28–38 weeks of gestation) during cesarean section at Medical College of Virginia Hospital at Virginia Commonwealth University Medical Center. All of the subjects gave informed consent, and the procedures followed were in accordance with institutional guidelines. This study was approved by the Office of Research Subjects Protection, Virginia Commonwealth University. Please see the online-only Data Supplement expanded Materials and Methods for clinical characteristics of the patient groups (Table S1), methylation assay, immunohistochemistry, HL-60 cell culture and treatments, vascular smooth muscle and endothelial cell culture and treatments, quantitative RT-PCR, Western blotting, enzyme immunoassay, and statistical analysis.

**Results**

The Illuma Infinium HumanMethylation27 BeadChip assay revealed 4184 CpG sites, with significant differential methylation when comparing normal pregnant and preeclamptic omental arteries at a \(P\) value of <0.05.\(^{22}\) Many of these genes were genes involved in inflammation. Of these genes, thromboxane synthase (\(TBXAS1\)) was the most significantly less methylated with an average difference in methylation (\(\Delta \beta\)) of 0.24 at a \(P\) value of 0.00037 corresponding with a false discovery rate of 0.042. There was no overlap in the methylation values (\(\beta\)-values) between the 2 groups demonstrating that all of the preeclamptic samples were less methylated as compared with normal pregnant samples (Figure 1).

[Figure 1. Box plot of proportion methylated (\(\beta\)-values) in omental arteries by subject group for the \(TBXAS1\) gene as determined by the HumanMethylation27 BeadChip. Methylation was significantly lower in preeclamptic patients (PE; \(n=7\)) than in normal pregnant patients (NP; \(n=5\)) with no overlap between the groups. **\(P=0.00037; \text{false discovery rate (FDR)}=0.042.**]

Representative staining images for thromboxane synthase are shown in Figure 2. Negative controls showed no staining for thromboxane synthase (Figure 2A). There was little or no staining in vessels of normal pregnant women (Figure 2B). However, preeclamptic vessels showed significant staining for thromboxane synthase (Figure 2C through 2F). Staining for thromboxane synthase in preeclamptic vessels was present in endothelium, vascular smooth muscle, and in leukocytes, which were in the lumen, adhered to the endothelium and infiltrated into the walls of the vessels (Figure 2F).

The staining intensity score for thromboxane synthase was significantly greater for preeclamptic women as compared with normal pregnant women (3.0±0.1 versus 0.5±0.1, respectively; \(P<0.001\); Figure 3A), as was OD of staining (88.0±6.0 versus 19.0±2.0 OD, respectively; \(P<0.001\); Figure 3D). Staining intensity scores and ODs were highly correlated (\(r=0.93\)). The percentage of vessels with staining for thromboxane synthase was significantly greater for preeclamptic women than for normal pregnant women (95.0±2.0% versus 25.0±4.0%, respectively; \(P<0.001\); Figure 3C), as was the percentage of vessels with leukocytes stained for thromboxane synthase (80.0±2.0% versus 12.0±3.0%, respectively; \(P<0.001\); Figure 3D).

To verify the immunohistochemistry results, we examined \(TBXAS1\) gene expression in omental arteries of normal pregnant and preeclamptic women. \(TBXAS1\) gene expression was 2.5-fold higher in omental arteries of preeclamptic women as compared with normal pregnant women (2.6±0.2 versus 1.0±0.1, respectively; \(P<0.01\); Figure 4A). Western blotting confirmed that increased gene expression for \(TBXAS1\) was associated with increased thromboxane synthase protein (Figure 4B and 4C). Thromboxane synthase protein expression was 3-fold greater in preeclamptic arteries as compared with normal pregnant arteries, as determined by Western blot density measurements (\(P<0.01\)).

To examine the role of DNA methylation in regulating the expression of thromboxane synthase in neutrophils, it was necessary to use a neutrophil-like cell line (HL-60), because neutrophils isolated from patients do not divide and, therefore, the 5-Aza-2-deoxycytidine (5-Aza) could not be incorporated into the genomic DNA to induce hypomethylation. Treatment of HL-60 cells with 5-Aza resulted in a significant
increase in TBXAS1 gene expression (2.6±0.2-fold; *P*<0.001). Treatment with phorbol 12-myristate 13-acetate (PMA) to activate the cells resulted in a 1.4±0.1-fold increase in TBXAS1. Activation of the cells by PMA was evidenced by cell clumping and adhesion to the floor of the flask. Combining 5-Aza treatment with PMA resulted in a significant increase in TBXAS1 gene expression as compared with control (3.8±0.4-fold; *P*<0.001), PMA alone (*P*<0.001), or 20 µm

**Figure 2.** Representative sections for blood vessels in omental fat from normal pregnant and preeclamptic women immunostained for thromboxane synthase. A, There was no brown staining for thromboxane synthase in negative control sections. B, Blood vessels of normal pregnant women showed little or no staining for thromboxane synthase. C through F, Vessels of preeclamptic women showed significant brown staining for thromboxane synthase. Staining for thromboxane synthase in preeclamptic blood vessels was observed in endothelium (black arrows), vascular smooth muscle cells (red arrows), and leukocytes (blue arrows), which were either adhered to the endothelium or infiltrated into the wall of the vessel (F). A indicates adipocyte; VL, vessel lumen. Magnification and scale bar are shown on each image.

<table>
<thead>
<tr>
<th><strong>NP</strong></th>
<th><strong>PE</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>50</td>
<td>75</td>
</tr>
<tr>
<td>75</td>
<td>100</td>
</tr>
</tbody>
</table>

***

**Figure 3.** Results for immunohistochemical staining for thromboxane synthase in omental blood vessels from normal pregnant (NP) and preeclamptic (PE) women. A, Visual staining score for thromboxane synthase was significantly higher in blood vessels of PE women vs NP women. B, Optical density of staining for thromboxane synthase was also significantly higher in PE blood vessels and significantly correlated with the visual score (*r*=0.93). C, PE women had a significantly higher percentage of blood vessels stained for thromboxane synthase vs NP women. D, PE women had a significantly higher percentage of blood vessels with leukocyte stained for thromboxane synthase vs NP women. Data are presented as mean±SEM. *** *P*<0.001.
5-Aza alone (P<0.001; Figure 5A). Western blotting confirmed that protein expression was altered in concert with gene expression (Figure 5B and 5C). Treatment with 5-Aza significantly increased thromboxane synthase protein expression (347±11% average density measurement of Western blot as compared with control; P<0.001). Combining 5-Aza treatment with PMA resulted in significantly increased thromboxane synthase protein expression (515±15% of control; P<0.001), PMA alone (P<0.001), or 5-Aza alone (P<0.001).

To evaluate the effect of the same treatments on the production of thromboxane by the neutrophil-like HL-60 cells, cells were cultured with 70 μmol/L of linoleic acid, the precursor of arachidonic acid. Treatment with 5-Aza significantly increased the production of thromboxane B2, the stable metabolite of thromboxane A2, as compared with controls (619±32 versus 115±20 ng/μg DNA, respectively; P<0.001; Figure 6). PMA treatment caused a significant increase in the production of thromboxane B2 as compared with control (745±36 versus 115±20 ng/μg DNA, respectively; P<0.001). Combining 5-Aza and PMA treatments caused an even greater increase in the production of thromboxane B2 (1228±140 ng/μg of DNA; P<0.001).

In contrast to HL-60 cells, 5-Aza treatment increased TBXAS1 gene expression by only 40% in cultured vascular smooth muscle cells (VSMCs) and only 13% in cultured human umbilical vein endothelial cells, which were not statistically significant (data not shown). However, treatment of VSMCs with tumor necrosis factor (TNF) α, a neutrophil product, significantly increased TBXAS1 gene expression as compared with controls (3.0±0.2-fold; P<0.001; Figure 7A). Western blotting confirmed increased protein expression induced by TNFα (Figure 7B and 7C). TNFα resulted in a 2.6-fold increase in thromboxane synthase protein in VSMCs (263±37% average density of control; P<0.01).
cells. However, treatment of VSMCs with TNF-α neutrophils but not in vascular smooth muscle or endothelial cells. Hypomethylation resulted in significantly increased expression of thromboxane synthase in the neutrophil-like cell line. Increased expression of thromboxane synthase would lead to increased production of thromboxane A2 locally in the vessel, which could explain hypertension and coagulation abnormalities in preeclamptic patients, because thromboxane is a potent vasoconstrictor and platelet activator.

Leukocyte infiltration requires leukocyte activation, which most likely occurs as they circulate through the intervillous space and are exposed to increased lipid peroxides secreted by the placenta. The infiltrating leukocytes are most likely neutrophils, because neutrophils normally compose 60% to 70% of all leukocytes, their numbers increase 2.5-fold by 30 weeks of gestation, and their numbers are further increased in preeclampsia. In addition, we reported previously that neutrophils but not lymphocytes or monocytes primarily infiltrate systemic blood vessels of preeclamptic women.

To study the role of DNA methylation in the regulation of thromboxane synthase, we experimentally induced DNA hypomethylation in a neutrophil-like cell line and in cultured human VSMCs and endothelial cells. Hypomethylation resulted in significantly increased expression of thromboxane synthase only in the neutrophil-like cell line. Increased expression of thromboxane synthase in the neutrophil-like cell line was associated with a parallel increase in the production of the stable metabolite of thromboxane A2, thromboxane B2. These data suggest that DNA methylation is important in regulating thromboxane synthase expression in neutrophils but not in vascular smooth muscle or endothelial cells. However, treatment of VSMCs with TNFα, a neutrophil product, did significantly increase thromboxane synthase expression. We previously observed increased expression of thromboxane synthase in vascular tissue of preeclamptic women. In this study we report a significant reduction in DNA methylation in the promoter region of the TBXAS1 gene associated with a significant increase in thromboxane synthase expression in omental fat arteries of preeclamptic women as compared with normal pregnant women. Increased expression of thromboxane synthase was observed in the endothelium, in the VSMCs, and in leukocytes, which were flattened and adhered to the endothelium and infiltrated into the wall of the vessel. Increased expression of thromboxane synthase would lead to increased production of thromboxane A2 locally in the vessel, which could explain hypertension and coagulation abnormalities in preeclamptic patients, because thromboxane is a potent vasoconstrictor and platelet activator.

Leukocyte infiltration requires leukocyte activation, which most likely occurs as they circulate through the intervillous space and are exposed to increased lipid peroxides secreted by the placenta. The infiltrating leukocytes are most likely neutrophils, because neutrophils normally compose 60% to 70% of all leukocytes, their numbers increase 2.5-fold by 30 weeks of gestation, and their numbers are further increased in preeclampsia. In addition, we reported previously that neutrophils but not lymphocytes or monocytes primarily infiltrate systemic blood vessels of preeclamptic women.

To study the role of DNA methylation in the regulation of thromboxane synthase, we experimentally induced DNA hypomethylation in a neutrophil-like cell line and in cultured human VSMCs and endothelial cells. Hypomethylation resulted in significantly increased expression of thromboxane synthase only in the neutrophil-like cell line. Increased expression of thromboxane synthase in the neutrophil-like cell line was associated with a parallel increase in the production of the stable metabolite of thromboxane A2, thromboxane B2. These data suggest that DNA methylation is important in regulating thromboxane synthase expression in neutrophils but not in vascular smooth muscle or endothelial cells. However, treatment of VSMCs with TNFα, a neutrophil product, did significantly increase thromboxane synthase expression, so increased expression of thromboxane synthase in vascular tissue of preeclamptic women may be because of inflammation caused by neutrophil infiltration. Reduced DNA methylation in leukocytes has been reported in other diseases involving the cardiovascular system, such as atherosclerosis, ischemic heart disease, and stroke.

Pertinent to our findings of increased expression of thromboxane synthase are previous findings of increased levels of serum arachidonic acid in preeclamptic women and significant activation of nuclear factor κB and increased expression of cyclooxygenase 2 in preeclamptic blood vessels. Similar to thromboxane synthase expression, activation of nuclear factor κB and increased expression of cyclooxygenase 2 were observed in the endothelium, vascular smooth muscle, and infiltrating neutrophils. A possible scenario in preeclamptic blood vessels is that increased cyclooxygenase 2 converts increased arachidonic acid into prostaglandin H2 and increased thromboxane synthase then converts prostaglandin H2 into thromboxane.

Preeclampsia is associated with oxidative stress and increased plasma levels of linoleic acid, the fatty acid

Discussion

In this study we report a significant reduction in DNA methylation in the promoter region of the TBXAS1 gene associated with a significant increase in thromboxane synthase expression in omental fat arteries of preeclamptic women as compared with normal pregnant women. Increased expression of thromboxane synthase was observed in the endothelium, in the VSMCs, and in leukocytes, which were flattened and adhered to the endothelium and infiltrated into the wall of the vessel. Increased expression of thromboxane synthase would lead to increased production of thromboxane A2 locally in the vessel, which could explain hypertension and coagulation abnormalities in preeclamptic patients, because thromboxane is a potent vasoconstrictor and platelet activator.

Leukocyte infiltration requires leukocyte activation, which most likely occurs as they circulate through the intervillous space and are exposed to increased lipid peroxides secreted by the placenta. The infiltrating leukocytes are most likely neutrophils, because neutrophils normally compose 60% to 70% of all leukocytes, their numbers increase 2.5-fold by 30 weeks of gestation, and their numbers are further increased in preeclampsia. In addition, we reported previously that neutrophils but not lymphocytes or monocytes primarily infiltrate systemic blood vessels of preeclamptic women.

To study the role of DNA methylation in the regulation of thromboxane synthase, we experimentally induced DNA hypomethylation in a neutrophil-like cell line and in cultured human VSMCs and endothelial cells. Hypomethylation resulted in significantly increased expression of thromboxane synthase only in the neutrophil-like cell line. Increased expression of thromboxane synthase in the neutrophil-like cell line was associated with a parallel increase in the production of the stable metabolite of thromboxane A2, thromboxane B2. These data suggest that DNA methylation is important in regulating thromboxane synthase expression in neutrophils but not in vascular smooth muscle or endothelial cells. However, treatment of VSMCs with TNFα, a neutrophil product, did significantly increase thromboxane synthase expression, so increased expression of thromboxane synthase in vascular tissue of preeclamptic women may be because of inflammation caused by neutrophil infiltration. Reduced DNA methylation in leukocytes has been reported in other diseases involving the cardiovascular system, such as atherosclerosis, ischemic heart disease, and stroke.

Pertinent to our findings of increased expression of thromboxane synthase are previous findings of increased levels of serum arachidonic acid in preeclamptic women and significant activation of nuclear factor κB and increased expression of cyclooxygenase 2 in preeclamptic blood vessels. Similar to thromboxane synthase expression, activation of nuclear factor κB and increased expression of cyclooxygenase 2 were observed in the endothelium, vascular smooth muscle, and infiltrating neutrophils. A possible scenario in preeclamptic blood vessels is that increased cyclooxygenase 2 converts increased arachidonic acid into prostaglandin H2 and increased thromboxane synthase then converts prostaglandin H2 into thromboxane.

Preeclampsia is associated with oxidative stress and increased plasma levels of linoleic acid, the fatty acid

Discussion

In this study we report a significant reduction in DNA methylation in the promoter region of the TBXAS1 gene associated with a significant increase in thromboxane synthase expression in omental fat arteries of preeclamptic women as compared with normal pregnant women. Increased expression of thromboxane synthase was observed in the endothelium, in the VSMCs, and in leukocytes, which were flattened and adhered to the endothelium and infiltrated into the wall of the vessel. Increased expression of thromboxane synthase would lead to increased production of thromboxane A2 locally in the vessel, which could explain hypertension and coagulation abnormalities in preeclamptic patients, because thromboxane is a potent vasoconstrictor and platelet activator.

Leukocyte infiltration requires leukocyte activation, which most likely occurs as they circulate through the intervillous space and are exposed to increased lipid peroxides secreted by the placenta. The infiltrating leukocytes are most likely neutrophils, because neutrophils normally compose 60% to 70% of all leukocytes, their numbers increase 2.5-fold by 30 weeks of gestation, and their numbers are further increased in preeclampsia. In addition, we reported previously that neutrophils but not lymphocytes or monocytes primarily infiltrate systemic blood vessels of preeclamptic women.

To study the role of DNA methylation in the regulation of thromboxane synthase, we experimentally induced DNA hypomethylation in a neutrophil-like cell line and in cultured human VSMCs and endothelial cells. Hypomethylation resulted in significantly increased expression of thromboxane synthase only in the neutrophil-like cell line. Increased expression of thromboxane synthase in the neutrophil-like cell line was associated with a parallel increase in the production of the stable metabolite of thromboxane A2, thromboxane B2. These data suggest that DNA methylation is important in regulating thromboxane synthase expression in neutrophils but not in vascular smooth muscle or endothelial cells. However, treatment of VSMCs with TNFα, a neutrophil product, did significantly increase thromboxane synthase expression, so increased expression of thromboxane synthase in vascular tissue of preeclamptic women may be because of inflammation caused by neutrophil infiltration. Reduced DNA methylation in leukocytes has been reported in other diseases involving the cardiovascular system, such as atherosclerosis, ischemic heart disease, and stroke.

Pertinent to our findings of increased expression of thromboxane synthase are previous findings of increased levels of serum arachidonic acid in preeclamptic women and significant activation of nuclear factor κB and increased expression of cyclooxygenase 2 in preeclamptic blood vessels. Similar to thromboxane synthase expression, activation of nuclear factor κB and increased expression of cyclooxygenase 2 were observed in the endothelium, vascular smooth muscle, and infiltrating neutrophils. A possible scenario in preeclamptic blood vessels is that increased cyclooxygenase 2 converts increased arachidonic acid into prostaglandin H2 and increased thromboxane synthase then converts prostaglandin H2 into thromboxane.

Preeclampsia is associated with oxidative stress and increased plasma levels of linoleic acid, the fatty acid
precursor of arachidonic acid. Neutrophils from normal pregnant women exposed to an oxidizing solution enriched with linoleic acid showed increased production of TNFα and thromboxane. Also, exposure of cultured VSMCs to an oxidizing solution enriched with linoleic acid increased production of thromboxane.

Our study has several limitations. First, our findings are correlative in that they show that reduced methylation is associated with increased expression of thromboxane synthase in omental arteries of preeclamptic women, but they do not prove cause and effect. In addition, we were not able to determine the cell types where methylation changes were occurring in the omental arteries because of cellular heterogeneity, which included endothelial cells, VSMCs, and infiltrated neutrophils. Another limitation is that we cannot prove that reduced methylation in the TBXAS1 gene promoter, per se, is responsible for increased expression, as opposed to changes in the expression of other factors that regulate TBXAS1 (eg, transcription factors or other regulatory factors) for which levels might be altered by changes in DNA methylation. However, by experimentally inducing hypomethylation in a neutrophil-like cell line we were able to demonstrate a strong association between DNA methylation status and thromboxane synthase expression, which is significant, because neutrophils have the highest thromboxane synthase content per cell in the vessels.

In summary, we found that reduced methylation in the promoter region of TBXAS1 is correlated with increased gene and protein expression of thromboxane synthase in systemic blood vessels of preeclamptic women. Increased expression was present in endothelium, VSMCs, and infiltrating neutrophils. We also showed that experimentally induced DNA hypomethylation increases the expression of thromboxane synthase in a neutrophil-like cell line and that TNFα, a neutrophil product, increases thromboxane synthase expression in cultured VSMCs. These data suggest that reduced DNA methylation is responsible for increased expression of thromboxane synthase in neutrophils that infiltrate maternal systemic blood vessels in preeclampsia and that vascular inflammation caused by infiltrating neutrophils is responsible for increased expression of thromboxane synthase in the endothelium and vascular smooth muscle. Increased expression of thromboxane synthase in systemic vasculature of preeclamptic women may help explain hypertension and coagulation abnormalities.

Perspectives
These findings suggest possible treatments for preeclampsia involving inhibition of thromboxane synthase, blockade of thromboxane receptors, or dietary supplementation with folate to increase methylation donors to protect against adverse changes in DNA methylation that affect thromboxane synthase expression. In this regard, a large study of ~3000 pregnant women found supplementation with multivitamins containing folic acid was associated with reduced risk of preeclampsia.

Sources of Funding
This research was supported in part by the National Heart, Lung, and Blood Institute grant RO1 HL069851 (to S.W.W.) and the National Center on Minority Health and Health Disparities grant P60 MD002256 (to J.F.S.). Methylation analysis was performed at the VCU Nucleic Acids Research Facility core laboratory, which is supported in part by funding from NIH-NCI Cancer Center Support Grant P30 CA166720.

Disclosures
None.

References
Reduced Methylation of the Thromboxane Synthase Gene Is Correlated With Its Increased Vascular Expression in Preeclampsia
Ahmad A. Mousa, Jerome F. Strauss III and Scott W. Walsh

Hypertension. published online April 9, 2012;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2012/04/09/HYPERTENSIONAHA.111.188730

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2012/04/09/HYPERTENSIONAHA.111.188730.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click RequestPermissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

REDUCED METHYLATION OF THROMBOXANE SYNTHASE GENE IS CORRELATED WITH ITS INCREASED VASCULAR EXPRESSION IN PREECLAMPSIA

Ahmad A. Mousa\textsuperscript{1,2}, B.D.S., Jerome F. Strauss, III\textsuperscript{1}, M.D. ,Ph.D., Scott W. Walsh\textsuperscript{1,2}, Ph.D.

\textsuperscript{1}Departments of Obstetrics and Gynecology, and \textsuperscript{2}Physiology and Biophysics
Virginia Commonwealth University, Richmond, VA

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) 828-8468-Phone; (804) 828-0573-Fax
swwalsh@vcu.edu
EXPANDED MATERIALS and METHODS

Study Subjects
Omental fat biopsies of approximately 2 cm x 2 cm x 0.5 cm in size were collected from normal pregnant (n=16) and preeclamptic (n=22) women (28-38 weeks of gestation) during cesarean section at MCV Hospital, Virginia Commonwealth University Medical Center, Richmond, VA. Due to the small size, samples were processed for DNA, RNA, protein or immunohistochemistry. Preeclampsia was diagnosed by new onset of hypertension (systolic blood pressure of ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg) and proteinuria (300 mg or more of protein in the urine per 24 h collection) that occur in women who are otherwise normal1. Women with chorioamnionitis, infections, active sexually transmitted diseases, lupus or diabetes, and women who were smokers or in labor were excluded because these conditions are associated with inflammatory changes. Patient’s clinical data are shown in Table S1. All subjects gave informed consent and the procedures followed were in accordance with institutional guidelines. This study was approved by the Office of Research Subjects Protection, Virginia Commonwealth University, Richmond, VA.

Methylation Assay
Omental arteries from 5 normal pregnant and 7 preeclamptic women were processed for DNA extraction. DNA was extracted from the arteries (~10 mg by weight) using QuickGene DNA tissue kit and QuickGene-Mini80 system (AutoGen, Holliston, MA). DNA was treated with RNase A (Qiagen, Valencia, CA). DNA (1µg) was bisulfite treated and used in Illumina Infinium HumanMethylation27 BeadChip assay (Illumina, San Diego, CA) for DNA methylation analysis. The BeadChip was run by the Nucleic Acids Research Facilities at Virginia Commonwealth University using the protocol provided by Illumina.

Immunohistochemistry
Omental fat samples from 4 normal pregnant and 5 preeclamptic women were cut into small pieces approximately 0.5 cm x 0.5 cm x 0.5 cm in size, fixed immediately in 10% neutral buffered formalin and embedded in paraffin. Tissues were cut into 8 µm sections. Tissue slides were stained for rabbit antihuman polyclonal antibody specific for thromboxane synthase (1:50 titer, Proteintech, Chicago, IL). Rabbit primary antibody isotype control (Invitrogen, Camarillo, CA) was used as a negative control. A kit was used for immunohistochemical staining (SuperPicTure Polymer Detection Kit Broad Spectrum (DAB), Invitrogen, Camarillo, CA). 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. Tissue slides were counterstained with 1:5 dilution of hematoxylin QS (Vector Laboratories, Burlingame, CA).

For data analysis, vessels between 10 µm and 200 µm were examined. An average of 35 vessels was analyzed per slide. Vessel staining for thromboxane synthase was
evaluated using a visual intensity score of 0, 1, 2, 3 or 4 where 0 was assigned for no staining and 4 for dark and extensive staining. Visual scoring was verified by a second investigator and by measuring the optical density of staining (OD) using image analysis software (cellSens Imaging Software, Olympus America, Center Valley, PA) as previously described²,³. Optical density of staining for thromboxane synthase in vessels was normalized to the optical density of the background. Slides were also analyzed for percentage of vessels stained and percentage of vessels with leukocyte staining for thromboxane synthase.

**HL-60 Cell Culture and Treatments**

HL-60 cells (ATCC, Manassas, VA), a neutrophil-like cell line, were used to evaluate the effect of DNA hypomethylation on the expression of thromboxane synthase. Cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM, ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA) and 1% antibiotics and antmycotics (100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin B, Gibco, Invitrogen, Carlsbad, CA) as recommended by ATCC. Approximately 500,000 cells per ml were seeded in 5 ml of media in a T-25 flask for treatments. Cell treatments were: 1) 10 µM 5-Aza-2-deoxycytidine (5-Aza, Sigma-Aldrich, Saint-Louis, MO), an agent that inhibits DNA methylation when incorporated into DNA during cell division⁴, for 48 h followed by 10⁻⁸ M of phorbol 12-myristate 13-acetate (PMA, an activator of protein kinase C, Sigma-Aldrich, Saint-Louis, MO) for 24 h; 2) 10 µM 5-Aza for 48 h followed by no treatment for 24 h; 3) no treatment for 48 h followed by 10⁻⁸ M PMA for 24 h; or 4) 72 h without treatment to serve as control. To study the effect of these treatments on the production of thromboxane B₂, cells were also treated with 70 µM linoleic acid (Cayman Chemical, Ann Arbor, MI). Cells were collected for DNA, RNA or protein extraction and media was saved for enzyme immunoassay.

**Vascular Smooth Muscle and Endothelial Cell Culture and Treatments**

Human vascular smooth muscle cells (VSMC) were cultured from chorionic plate arteries of placentas collected at cesarean section from healthy pregnant women at term deliveries as previously described⁵. Human umbilical vein endothelial cells were purchased from Lifeline Cell Technology (Grand Island, NY) and cultured in T-25 flasks according to their protocol with their medium. VSMCs were cultured in T-25 flasks using Medium-199 (M-199, Gibco, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA) and 1% antibiotics and antmycotics (100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin B, Gibco, Invitrogen, Carlsbad, CA). Cells were treated with 1 ng/ml of human recombinant TNFα (R&D Systems, Minneapolis, MN) or with 5-Aza for 48 hours. Control flasks contained M-199 media with 10% FBS and 1% antibiotics and antmycotics. Treatments were refreshed every day. Cells were 50% confluent at the time of treatment and 100% confluent at the time of harvesting. Cells were washed with PBS and harvested for RNA and protein extraction.
Quantitative RT-PCR
Omental arteries (25-30 mg) from 5 normal pregnant and 8 preeclamptic patients were homogenized with a rotor stator homogenizer (PRO200, PRO Scientific, Oxford, CT) and total RNA was extracted using Tri-reagent with spin columns (RiboPure kit, Ambion, Austin, TX). For cell cultures, RNA extraction was performed using QuickGene RNA cultured cell kit with QuickGene Mini-80 system (AutoGen, Holliston, MA). DNase treatment was performed using Turbo DNase kit (Ambion, Austin, TX). RNA (0.25-1 µg) was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (SABiosciences, Frederick, MD) on Eppendorf Realplex Thermal Cycler. For each reaction, 8 ng of cDNA was used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. For TBXAS1 gene, commercial primers were used (SABiosciences, Frederick, MD) and for GAPDH gene the following primers were used: forward primer: GATTCCACCCATGGCAAATT; reverse primer: AGATGGTGATGGGGATTCCATT. GAPDH primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Data were normalized to GAPDH by the \(\Delta\Delta\)Ct method. Melting curve analysis confirmed specificity of the primers.

Western Blotting
Omental arteries (45-50 mg) from 4 normal pregnant and 6 preeclamptic patients were homogenized in RIPA buffer containing 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris (pH 8.0), and 1X Halt protease inhibitor (Thermo Scientific, Pittsburgh, PA) using a rotor stator homogenizer (PRO200, PRO Scientific, Oxford, CT) at 300 rpm for 1 min. VSMC or HL-60 cells were homogenized in the same buffer using a probe sonicator (EpiShear Probe Sonicator, Active Motif, Carlsbad, CA) at 25% amplitude for 10 one sec bursts. Protein concentrations were measured using BCA assay (Thermo Fisher Scientific, Rockford, IL). Denatured protein lysates (50-100 µg) were resolved by electrophoresis on sodium dodecyl sulphate polyacrylamide gel and electrotransferred to polyvinylidene fluoride membrane (Immobilon-FL, Millipore, Billerica, MA). Membranes were probed for thromboxane synthase and β-actin. Primary antibodies used were rabbit anti-human thromboxane synthase (1:1000, Proteintech, Chicago, IL); mouse anti-human β-actin (1:1,000, Sigma, Saint Louis, MO). Secondary antibodies used included: Alexa Fluor 680 donkey anti-rabbit (1:10,000, Invitrogen, Carlsbad, CA) for the detection of thromboxane synthase and IRDye800 goat anti-mouse (1:20,000, Rockland Immunochemicals, Gilbertsville, PA) for the detection of β-actin. LI-COR Odyssey Infrared Imaging System (Thermo Scientific, Pittsburgh, PA) was used to detect and analyze the immunoreactive proteins. Density values of protein bands were measured by multiplying the intensity of the bands by their area. Density values for thromboxane synthase bands were normalized by dividing them by the density values of their corresponding β-actin bands. The normalized thromboxane synthase density value of
the reference group (control or normal pregnant) was considered 100% and changes in
other groups were represented as relative to the reference group.

Enzyme Immunoassay
The Thromboxane B₂ (TXB₂) Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI)
was used to measure TXB₂ secretion into the media. TXB₂ is the stable metabolite of
thromboxane A₂. The procedure for the assay was done as recommended by the
manufacturer. Media was diluted by a factor of 1:1 before it was used in the assay.
TXB₂ concentrations were measured in pg/ml using FLUOstar OPTIMA plate reader
(BMG Labtech, Ortenberg, Germany). For normalization, the total amount of TXB₂ (pg)
in 5ml (volume of medium used in a T-25 flask) was divided by the total amount of DNA
(µg) extracted from the cells of the same flask. DNA was extracted as described above.

Statistical Analysis
Data analysis of the HumanMethylation27 BeadChip assay was performed using the
beadarray package in R programming environment. To control for multiple hypothesis
testing, the p-values were subsequently used in estimating the false discovery rates
(FDR) using the q-value method. Methylation values (β values) are expressed as
range from 0 to 1 where 0 means not methylated and 1 means fully methylated. Δβ-
values indicate the difference in methylation between normal pregnant and preeclamptic
women.

Student’s t test was used to make comparisons of parameters between two groups and
one-way ANOVA with Newman-Keuls test was used to make comparisons of
parameters between more than two groups for normally distributed data. Mann-Whitney
U test was used for visual intensity score data. Quantitative results were presented as
mean ± SEM. We considered a p-value of < 0.05 statistically significant. A statistical
software program was used for data analysis (GraphPad Prism version 4.0, San Diego,
CA).
REFERENCES

Table S1: Clinical Characteristics of Patient Groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal Pregnant (n=16)</th>
<th>Preeclamptic (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>26.8 ± 6.2</td>
<td>25.6 ± 4.9</td>
</tr>
<tr>
<td>Pre-pregnancy BMI (kg/m²)</td>
<td>24.4 ± 3.0</td>
<td>30.2 ± 7.4</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>114.2 ± 8.6</td>
<td>169.5 ± 14.8*</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>70.0 ± 8.1</td>
<td>96.1 ± 11.4*</td>
</tr>
<tr>
<td>Proteinuria (mg/24 h)</td>
<td>ND</td>
<td>260.1 ± 103.6 (n=11)</td>
</tr>
<tr>
<td>Dipstick</td>
<td>ND</td>
<td>2.6 ± 1.4 (n=11)</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primiparous</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Multiparous</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>38.8 ± 1</td>
<td>33.0 ± 4*</td>
</tr>
<tr>
<td>Infant birth weight (g)</td>
<td>3226 ± 322</td>
<td>2060 ± 908*</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD.

*p < 0.001 by t-test.

ND, not determined.