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

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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 A_2, 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 omental arteries of preeclamptic women as compared to normal pregnant women. Increased thromboxane synthase expression was observed in vascular smooth muscles cells, 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.

Keywords

preeclampsia; DNA methylation; thromboxane synthase; epigenetics; omental blood vessels; thromboxane A_2

INTRODUCTION

Preeclampsia occurs in 5–7% of pregnancies, and is a leading cause of maternal and infant mortality and morbidity[1]. It is diagnosed clinically by the onset of hypertension and proteinuria, usually occurring after twenty weeks gestation. Preeclampsia is also associated

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CONFLICT OF INTEREST/DISCLOSURE
None

with increased activation of the coagulation system evidenced by an increase in formation of fibrin, activation of the fibrinolytic system, activation of platelets and a decrease in platelet count[2].

In 1985, increased thromboxane and decreased prostacyclin levels were reported in placentas of women with preeclampsia[3] and later confirmed for maternal blood[4] and maternal urine[5]. 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 [6].

Thromboxane and prostacyclin have a common precursor, prostaglandin H_2, but are synthesized by different enzymes[7]. Thromboxane synthase is the enzyme that catalyzes the isomerization of prostaglandin H_2 into thromboxane[8]. An increase in thromboxane synthase has been demonstrated in trophoblast and decidua cells of placentas of preeclamptic women[9] 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 due to 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[10]. 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-like 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 approximately 2 cm × 2 cm × 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. 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. Please see the Online Supplement at http:/hyper.ajajournals.org for 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 Illumina Infinium HumanMethylation27 BeadChip assay revealed 4,184 CpG sites, corresponding to 3,736 genes, with significant differential methylation when comparing between normal pregnant and preeclamptic omental arteries at p-value of less than 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 p-value of 0.00037 corresponding to a false discovery rate (FDR) of 0.042. There was no overlap in the methylation values (β-values) between the two groups demonstrating that all preeclamptic samples were less methylated as compared to normal pregnant samples (Figure 1).

Representative staining images for thromboxane synthase are shown in Figure 2. Negative controls showed no staining for thromboxane synthase (Panel A). There was little or no staining in vessels of normal pregnant women (Panel B). However, preeclamptic vessels showed significant staining for thromboxane synthase (Panels C, D, E and F). 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 (Panel F).

The staining intensity score for thromboxane synthase was significantly greater for preeclamptic women as compared to normal pregnant women (3.0 ± 0.1 vs 0.5 ± 0.1, respectively, $p < 0.001$, Figure 3A), as was optical density (OD) of staining (88.0 ± 6.0 vs 19.0 ± 2.0 OD, respectively, $p < 0.001$, Figure 3B). 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 ($95.0 \pm 2.0\%$ vs $25.0 \pm 4.0\%$ respectively, $p < 0.001$, Figure 3C), as was the percentage of vessels with leukocytes stained for thromboxane synthase ($80.0 \pm 2.0\%$ vs $12.0 \pm 3.0\%$ 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 to normal pregnant women (2.6 ± 0.2 vs 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 4, B and C). Thromboxane synthase protein expression was 3-fold greater in preeclamptic arteries as compared to 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 to control (3.8 ± 0.4-fold, $p < 0.001$), PMA alone ($p < 0.001$) or 5-Aza alone ($p < 0.001$) (Figure 5A). Western blotting confirmed that protein expression was altered in concert with gene expression (Figure 5, B and C). Treatment with 5-Aza significantly increased thromboxane synthase protein expression (347 ± 11% average density measurement of Western blot as compared to 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 μM linoleic acid, the precursor of arachidonic acid. Treatment with 5-Aza significantly increased the production of TXB_2, the stable metabolite of TXA_2, as compared to controls (619 ± 32 vs 115 ± 20 ng/μg DNA, respectively, $p < 0.001$, Figure 6). PMA treatment caused a significant increase in the production of TXB_2 as compared to control (745 ± 36 vs 115 ± 20 ng/μg DNA, respectively, $p < 0.001$). Combining 5-Aza and PMA treatments caused an even greater increase in the production of TXB_2 (1228 ± 140 ng/μg 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 TNFα, a neutrophil product, significantly increased *TBXAS1* gene expression as compared to controls (3.0 ± 0.2-fold, $p < 0.001$, Figure 7A). Western blotting confirmed increased protein expression induced by TNFα (Figure 7, B and C). TNFα resulted in a 2.6-fold increase in thromboxane synthase protein in VSMCs (263 ± 37% average density of control, $P < 0.01$).

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 to normal pregnant women. Increased expression of thromboxane synthase was observed in the endothelium, in the vascular smooth muscle cells 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 A_2 locally in the vessel, which could explain hypertension and coagulation abnormalities in preeclamptic patients because thromboxane is a potent vasoconstrictor and platelet activator[23].

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[15]. The infiltrating leukocytes are most likely neutrophils because neutrophils normally comprise approximately 60%–70% of all leukocytes[24], their numbers increase 2.5-fold by 30 weeks of gestation[25] and their numbers are further increased in preeclampsia[26]. In addition, we previously reported that neutrophils, but not lymphocytes or monocytes, infiltrate systemic blood vessels of preeclamptic women[18–20,27].

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 TXA_2, TXB_2. 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 vascular smooth muscle cells with TNFα, a neutrophil product, did significantly increase thromboxane synthase, so increased expression of thromboxane synthase in vascular tissue of preeclamptic women may be due to inflammation caused by neutrophil infiltration. Reduced DNA methylation in leukocytes has been reported in other diseases involving the cardiovascular system such as atherosclerosis[28], ischemic heart disease and stroke[29].

Pertinent to our findings of increased expression of thromboxane synthase are previous findings of increased levels of serum arachidonic acid in preeclamptic women[30] and significant activation of NF-κB and increased expression of cyclooxygenase-2 (COX-2) in preeclamptic blood vessels[19]. Similar to thromboxane synthase expression, activation of NF-κB and increased expression of COX-2 were observed in the endothelium, vascular smooth muscle and infiltrating neutrophils[19]. A possible scenario in preeclamptic blood vessels is that increased COX-2 converts increased arachidonic acid into prostaglandin H_2 and increased thromboxane synthase then converts prostaglandin H_2 into thromboxane.

Preeclampsia is associated with oxidative stress[15,16] and increased plasma levels of linoleic acid, the fatty acid precursor of arachidonic acid[31]. Neutrophils from normal pregnant women exposed to an oxidizing solution enriched with linoleic acid showed increased production of TNFα and thromboxane[21]. Also, exposure of cultured VSMCs to an oxidizing solution enriched with linoleic acid increased production of thromboxane[32].

Our study has several limitations. First, our findings are correlative in that they show 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, vascular smooth muscle cells 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* (e.g., transcription factors or other regulatory factors) whose 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, vascular smooth muscle cells 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 vascular smooth muscle cells. 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 of 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.

PERSPECTIVE

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 almost 3,000 pregnant women found supplementation with multivitamins containing folic acid was associated with reduced risk of preeclampsia[33].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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NOVELTY and SIGNIFICANCE

1) What Is New?

- Thromboxane synthase is increased in maternal blood vessels in preeclampsia.
- Increased expression is correlated with reduced DNA methylation in the promoter of the *TBXAS1* gene.
- Methylation appears to be an important regulator of thromboxane synthase in neutrophils, which extensively infiltrate the vessels.

2) What Is Relevant?

- The product of thromboxane synthase, thromboxane A_2, is a potent vasoconstrictor and stimulator of platelet aggregation. Increased synthesis of thromboxane A_2 in maternal blood vessels could contribute to hypertension and coagulation abnormalities of preeclamptic women.
- Our findings open up a new concept in the etiology of preeclampsia relating to epigenetics.

3) Summary

- Reduced methylation of the *TBXAS1* promoter is correlated with increased thromboxane synthase expression in systemic blood vessels of women with preeclampsia.

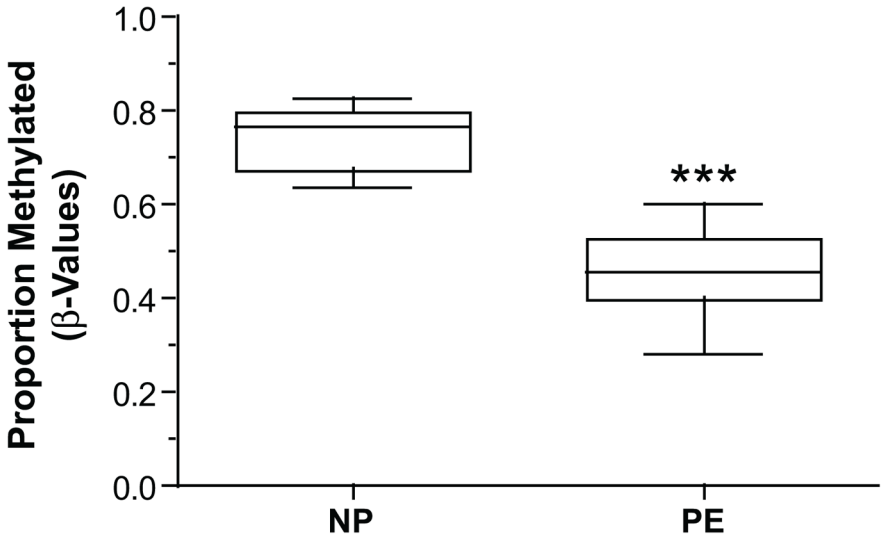

Figure 1.

Boxplot of proportion methylated (β-values) in omental arteries by subject group for *TBXAS1* gene as determined by the HumanMethylation27 BeadChip. Methylation was significantly lower in preeclamptic patients (n=7) than in normal pregnant patients (n=5) with no overlap between the groups. ***p = 0.00037, FDR = 0.042.

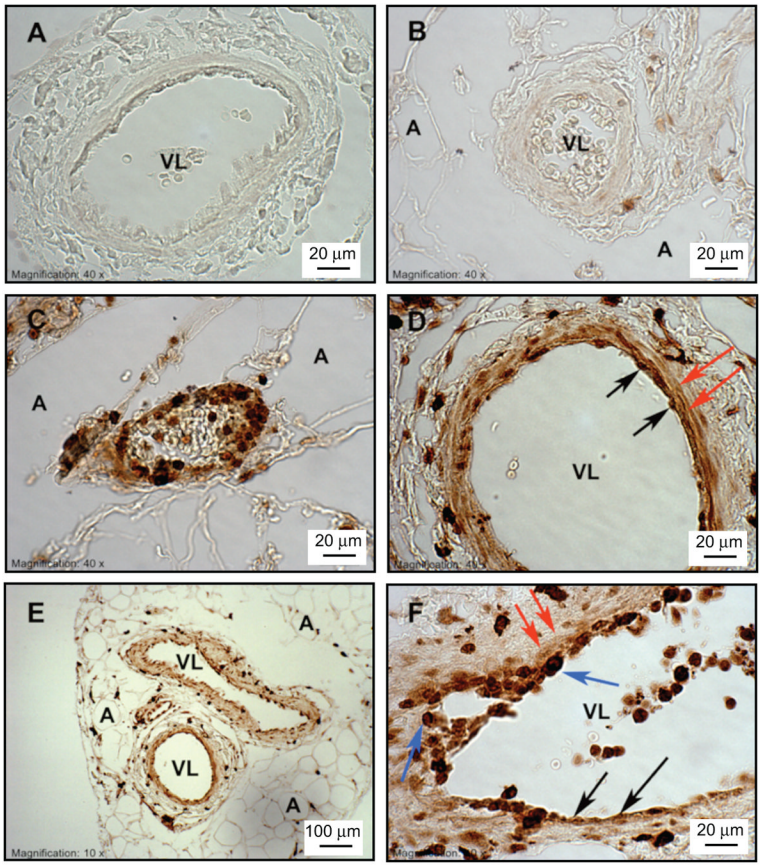

Figure 2.

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, D, E and 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: adipocyte; VL: vessel lumen. Magnification and scale bar are shown on each image.

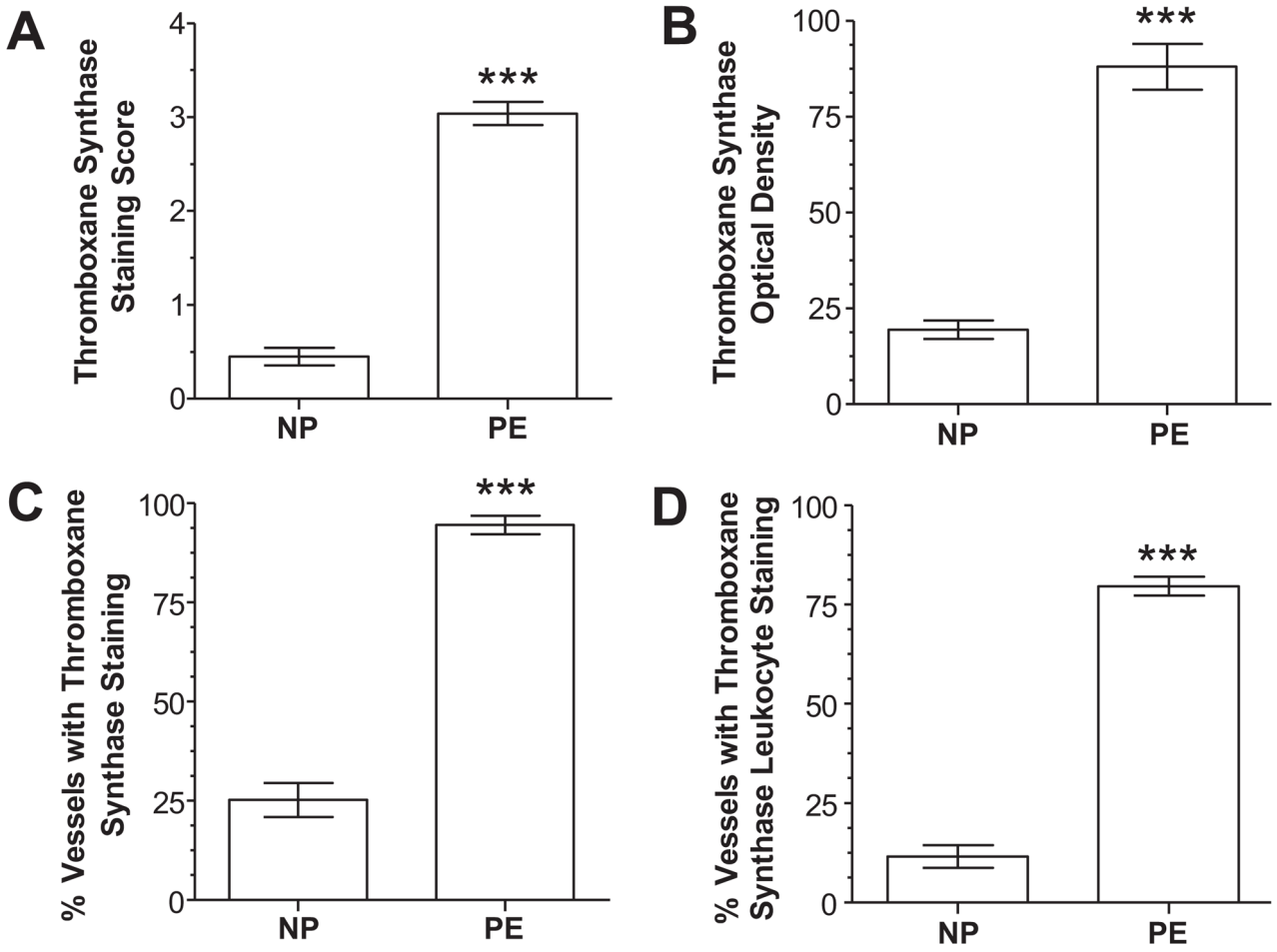

Figure 3.

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 preeclamptic women as compared to normal pregnant women. B) Optical density of staining for thromboxane synthase was also significantly higher in preeclamptic blood vessels and significantly correlated with the visual score (r=0.93). C) Preeclamptic women had significantly higher percentage of blood vessels stained for thromboxane synthase as compared to normal pregnant women. D) Preeclamptic women had significantly higher percentage of blood vessels with leukocyte stained for thromboxane synthase as compared to normal pregnant women. Data are presented as means ± SEM. ***p< 0.001.

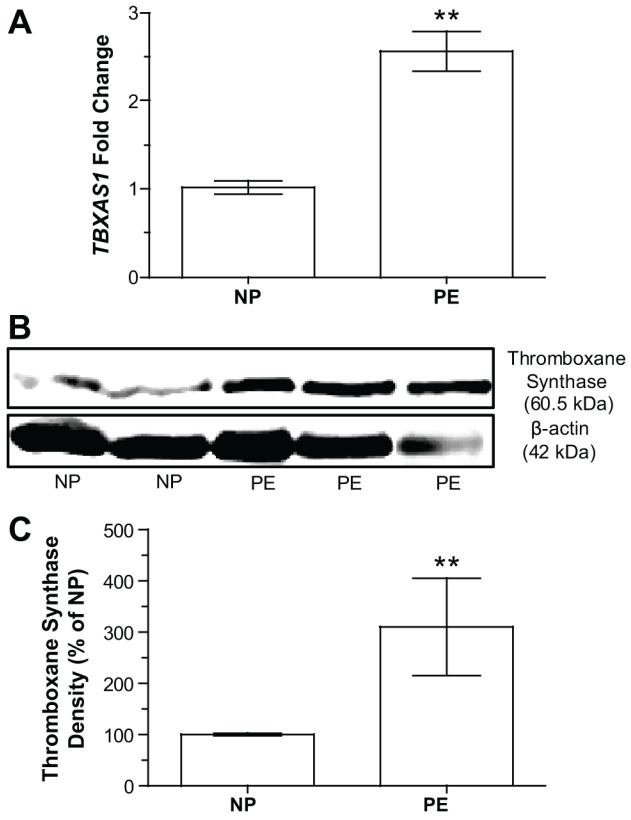

Figure 4.

Thromboxane synthase expression in omental arteries from normal pregnant and preeclamptic women. A) Fold change in gene expression of the *TBXAS1* in omental fat arteries of normal pregnant (NP) and preeclampsia (PE) women. Gene expression of *TBXAS1* was significantly increased in omental arteries from preeclamptic women (n=8) as compared to normal pregnant women (n=5). B) Representative Western blot for thromboxane synthase protein expression in omental arteries of normal pregnant and preeclamptic women. C) Density of thromboxane synthase plotted as percentage of average normal pregnant. Thromboxane synthase expression was significantly increased in arteries of preeclamptic women (n=6) as compared to normal pregnant women (n=4). Data are shown as means ± SEM. **$p < 0.01$.

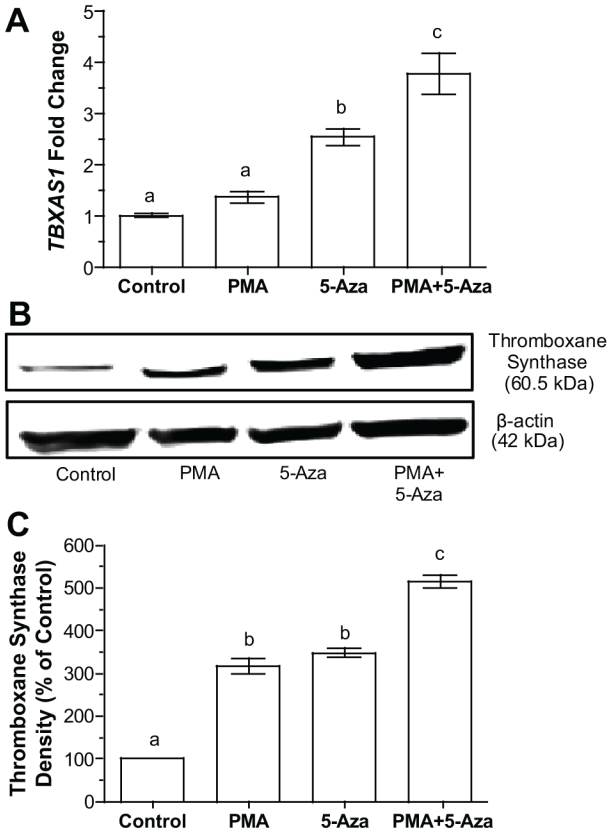

Figure 5.

Thromboxane synthase expression in HL-60 cells. A) Fold change in the expression for the *TBXAS1* gene in HL-60 cells treated with PMA for 24 hours, 5-Aza for 48 hours or with 5-Aza for 48 hours and then with PMA for 24 hours (n=8). 5-Aza is an agent that inhibits DNA methylation when incorporated into DNA during cell division. The expression of *TBXAS1* was significantly increased in 5-Aza treated cells as compared to controls. Activation with PMA after treatment with 5-Aza caused an even greater increase in *TBXAS1* expression as compared to control, PMA alone or 5-Aza alone. B) Representative Western blot for thromboxane synthase expression in HL-60 cells. C) Density of thromboxane synthase plotted as percentage of control (n=5). Treatment with 5-Aza significantly increased thromboxane synthase expression as compared to control. Activation with PMA after treatment with 5-Aza resulted in significant increase in thromboxane synthase expression as compared to control, PMA alone and 5-Aza alone. Data are shown as means ± SEM. Groups with different letters are significantly different, $p<0.001$.

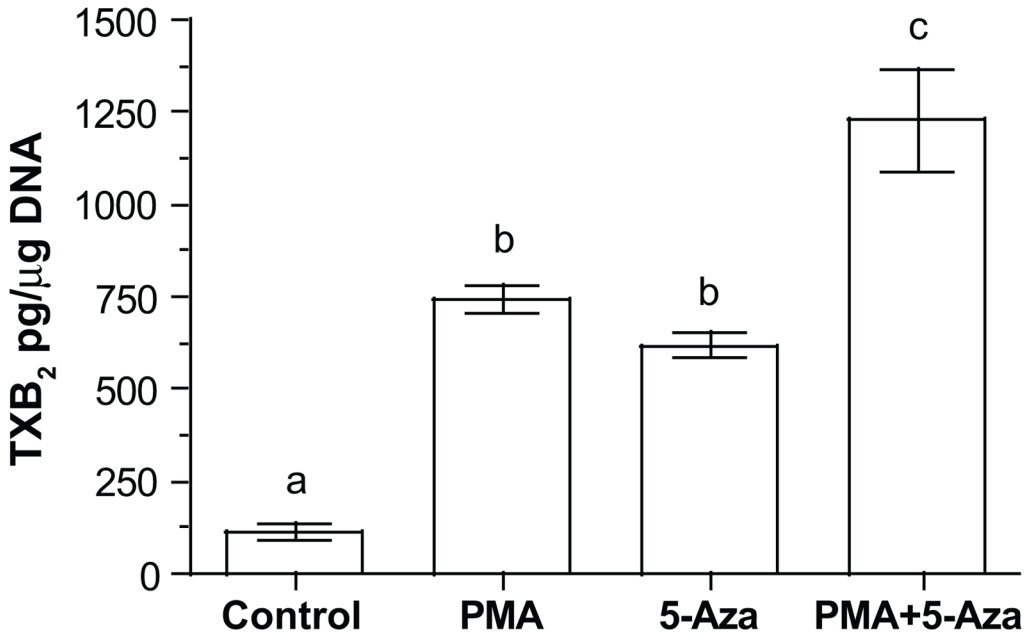

Figure 6.

Thromboxane B_2 (TXB_2) secretion into the media by HL-60 cells treated with PMA for 24 hours, 5-Aza for 48 hours or with 5-Aza for 48 hours and then with PMA for 24 hours. TXB_2 secretion was significantly increased in 5-Aza or PMA treated cells as compared to controls. Combined treatment of PMA and 5-Aza caused an even greater increase in TXB_2 secretion as compared to control, PMA alone or 5-Aza alone. Data are shown as means ± SEM, n=6. Groups with different letters are significantly different, $p<0.001$.

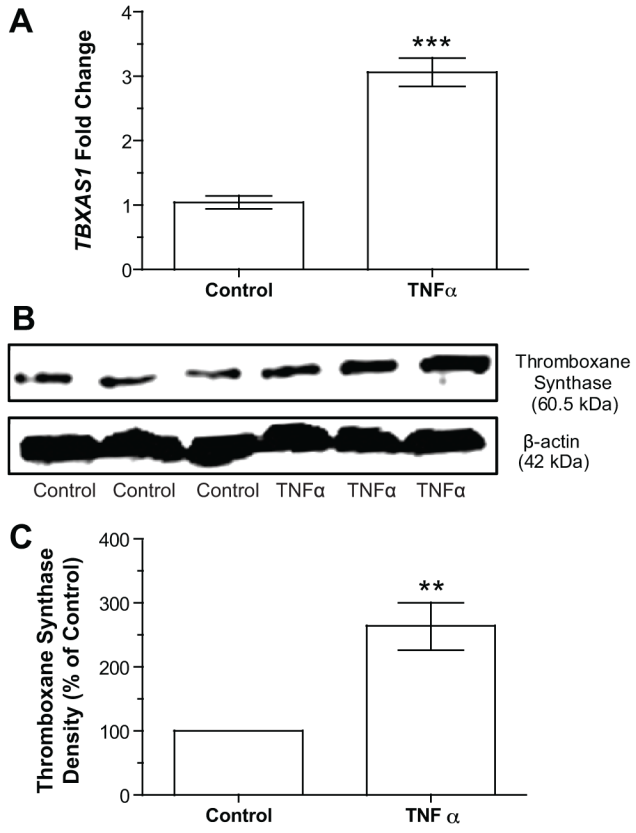

Figure 7.

Thromboxane synthase expression in human vascular smooth muscle cells treated with TNFα. A) Fold change in the expression for *TBXAS1* gene in cultured VSMCs treated with TNFα for 48 hours. The expression of *TBXAS1* was significantly increased in TNFα treated cells as compared to controls. B) Representative Western blot for thromboxane synthase protein expression in cultured VSMCs. C) Density of thromboxane synthase plotted as percentage of control. Treatment with TNFα significantly increased thromboxane synthase protein expression as compared to control. Data are shown as means ± SEM, n=6. ***$p < 0.001$, **$p<0.01$.