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Nitric Oxide Formation Is Inversely Related to Serum Levels of Antiangiogenic Factors Soluble Fms-Like Tyrosine Kinase-1 and Soluble Endogline in Preeclampsia

Valeria C. Sandrim, Ana C.T. Palei, Ingrid F. Metzger, Valeria A. Gomes, Ricardo C. Cavalli, Jose E. Tanus-Santos

Abstract—Deficient NO formation has been implicated in hypertensive disorders of pregnancy. However, no previous study has compared the circulating nitrite concentrations in healthy pregnant women with those found in hypertensive disorders of pregnancy. Moreover, 2 antiangiogenic factors produced in the placenta (soluble fms-like tyrosine kinase-1 and soluble endogline) may affect NO formation during pregnancy. Here, we hypothesized that lower concentrations of markers of NO formation exist in hypertensive disorders of pregnancy and that inverse relationships exist between these markers and soluble fms-like tyrosine kinase-1 or soluble endogline. In this cross-sectional study, we compared 58 healthy pregnant women with 56 gestational hypertensive subjects and 45 preeclamptic patients. We measured plasma and whole blood nitrite concentrations using an ozone-based chemiluminescence assay and serum soluble fms-like tyrosine kinase-1 and soluble endogline concentrations using enzyme immunoassays. Whole blood nitrite levels were significantly lower in gestational hypertensive subjects and preeclamptic patients (−36% and −58%, respectively; both \( P<0.05 \)) compared with healthy pregnant women. The plasma nitrite levels were 37% lower in both groups with hypertensive disorders of pregnancy compared with the group with normotensive pregnancies (both \( P<0.05 \)).

As expected, we found higher circulating soluble fms-like tyrosine kinase-1 and soluble endogline concentrations in preeclampsia compared with gestational hypertensive subjects or with healthy pregnancies (both \( P<0.05 \)). We found negative correlations between antiangiogenic factors and plasma or whole blood nitrite concentrations (Spearman’s \( r \) from −0.175 to −0.226; all \( P<0.05 \)). Our results show clinical evidence for impaired NO formation in preeclampsia or gestational hypertension. The negative correlations between markers of NO formation and antiangiogenic factors in preeclamptic patients suggest an inhibitory effect for these factors on NO formation. (Hypertension. 2008;52:402-407.)

Key Words: NO ■ nitrite ■ whole blood nitrite ■ preeclampsia ■ sEng ■ sFLT-1

Normal human pregnancy is accompanied by increased blood volume that is accommodated within the cardiovascular system by systemic vasodilatation.¹ This vasodilatation involves increased NO formation, thus decreasing peripheral vascular resistance in healthy pregnant women.²,³ On the other hand, deficient NO formation has been implicated in hypertensive disorders of pregnancy, such as preeclampsia and gestational hypertension.⁴–⁷ Indeed, previous studies compared the circulating concentrations of NO metabolites (nitrite+nitrate) in the plasma from preeclamptic women with those found in healthy pregnant women.⁸–¹³ Conflicting results were reported, and some studies showed higher,¹⁴ similar,¹⁰,¹² or lower⁶ nitrite+nitrate levels in preeclamptic women compared with healthy pregnant women. A possible explanation for these discrepancies is that nitrite+nitrate may not accurately reflect endogenous NO formation in vivo, and there is mounting evidence that measuring the circulating concentrations of nitrite is an improved alternative to assess endogenously produced NO.¹⁵–¹⁹ However, no previous study has compared the circulating nitrite concentrations in healthy pregnant women with those found in women with preeclampsia.

The pathophysiology of preeclampsia is not completely known. However, there is evidence that a failure of cytotrophoblast invasion and absence of dilatation of uterine arteries lead to a high-resistance uteroplacental circulation, thus reducing placental perfusion and causing hypoxia, which, in turn, induces the release of factors to the maternal circulation that causes systemic endothelial dysfunction.²⁰–²² Two important antiangiogenic factors produced in the placenta gain access to the maternal circulation and are involved in the pathogenesis of preeclampsia.²³–²⁵ First, soluble fms-like tyrosine kinase-1 (sFLT-1) is a splice variant of the vascular endothelial growth factor receptor that captures vascular...
endothelial growth factor, thus preventing its interaction with ligands and downregulating the biological effects of vascular endothelial growth factor, such as angiogenesis and stimulation of NO synthesis by endothelial cells. Second, although soluble endogline (sEng) is found in healthy pregnancies, its concentrations increase in preeclampsia. This is relevant because sEng is a transforming growth factor (TGF)-β1 and -β3 coreceptor that is highly expressed in vascular endothelial cells and may inhibit TGF-β1 signaling in the vasculature. 

In the present study, we hypothesized that lower concentrations of relevant markers of NO formation (plasma and whole blood nitrite) would be found in hypertensive disorders of pregnancy compared with those found in healthy pregnancies. In addition, given the fact that sFLT-1 and sEng interfere with eNOS activity, we hypothesized that inverse relationships exist between the circulating concentrations of markers of NO formation and sFLT-1 or sEng during pregnancy.

### Materials and Methods

#### Subjects

Approval for the use of human subjects was obtained from the institutional review board at the Faculty of Medicine of Ribeirão Preto, University of São Paulo, and subjects gave informed consent. The procedures followed were in accordance with institutional guidelines. A total of 159 pregnant were enrolled in the University Hospital of the Faculty of Medicine of Ribeirão Preto Department of Obstetrics and Gynecology. Of these, 58 were healthy pregnant women with uncomplicated pregnancies, 56 were gestational hypertensive subjects, and 45 had preeclampsia. Hypertensive disorders were defined in accordance with the guidelines of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. Gestational hypertension was defined as pregnancy-induced hypertension (≥140 mm Hg systolic or ≥90 mm Hg diastolic on ≥2 measurements ≥6 hours apart) without significant proteinuria in a woman after 20 weeks of gestation and returning to normal by 12 weeks postpartum. Preeclampsia was defined as increased blood pressure with significant proteinuria in a woman after 20 weeks of gestation. No women with preexisting hypertension, with or without superimposed preeclampsia, were included in the present study. Exclusion criteria included twin or multiple pregnancies or any evidence of previous medical illness.

At the time of clinic attendance, maternal venous blood samples were collected into standard Vacutainer tubes (Becton-Dickinson) containing heparin or clot activator (serum). The tubes were immediately centrifuged at 1000g for 3 minutes at room temperature, and plasma/serum samples were stored at −70°C until used to measure plasma nitrite and serum sFLT-1 and sEng concentrations. An aliquot of whole blood was mixed with a nitrite preservation solution in a 4:1 dilution, as described previously, and stored at −70°C until analyzed to measure whole blood nitrite concentrations. Briefly, this solution contains 0.8 mol/L of ferricyanide and 1% Nonidet P-40.

#### Enzyme Immunoassays of Angiogenic Factors

Serum concentrations of sFLT-1 and sEng were measured with commercially available ELISA kits (R&D Systems) according to the manufacturer’s instructions.

#### Measurement of Plasma and Whole Blood Nitrite Concentrations

Plasma aliquots were analyzed in triplicate for their nitrite content using an ozone-based chemiluminescence assay, as described previously.

### Table. Characteristics of Study Participants

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy Pregnant</th>
<th>Gestational Hypertensive</th>
<th>Preeclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>58</td>
<td>56</td>
<td>45</td>
</tr>
<tr>
<td>Age, y</td>
<td>25.9 ± 8.2</td>
<td>27.9 ± 6.4</td>
<td>27.5 ± 7.8</td>
</tr>
<tr>
<td>Primigravida, %</td>
<td>45</td>
<td>36</td>
<td>40</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.5 ± 4.9</td>
<td>29.4 ± 9.7*</td>
<td>26.5 ± 7.8</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>110.6 ± 10.7</td>
<td>133.3 ± 17.0*</td>
<td>142.3 ± 16.7*</td>
</tr>
<tr>
<td>DPB, mm Hg</td>
<td>70.6 ± 8.6</td>
<td>85.0 ± 12.2*</td>
<td>89.6 ± 12.4*</td>
</tr>
<tr>
<td>24-h-Pr, mg/24 h</td>
<td>ND</td>
<td>151.2 ± 68.6</td>
<td>1010.6 ± 1249.0</td>
</tr>
<tr>
<td>GA at sampling</td>
<td>34.9 ± 3.4</td>
<td>33.8 ± 5.6</td>
<td>33.1 ± 4.9</td>
</tr>
<tr>
<td>GAD, wk</td>
<td>40.0 ± 2.5</td>
<td>39.1 ± 2.1</td>
<td>34.8 ± 4.1*</td>
</tr>
<tr>
<td>Newborn weight, g</td>
<td>3350 ± 585</td>
<td>3166 ± 517</td>
<td>2445 ± 928*</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; GA, gestational age; SBP, systolic blood pressure; DBP, diastolic blood pressure; GAD, gestational age at delivery; 24-h-Pr, 24-hour proteinuria; ND, not determined (however, with negative dipstick test). Values are the means ± SDs.

*P < 0.05 vs healthy pregnant group.

### Statistical Analysis

Data were reported as the means ± SEMs. The clinical and biochemical characteristics of study groups were compared, respectively, by 1-way ANOVA followed by the Dunnett multiple comparisons test, and by the Kruskal-Wallis test followed by the Dunn’s multiple comparison test. The correlations among plasma or whole blood nitrite concentrations and sFLT-1 or sEng concentrations were analyzed using Spearman’s correlation (r and P values). Statistical analysis was performed with StatView for Windows. A P value of < 0.05 was considered the minimum level of statistical significance.

### Results

The Table summarizes the clinical characteristics of the 159 subjects enrolled in the present study. There were no significant differences in age, gestational age at sampling, and percentage of primigravida between the hypertensive groups and the control (healthy) group (all P > 0.05). However, women with gestational hypertension had higher body mass index than those in the other groups (P < 0.05; Table). As expected, higher systolic and diastolic blood pressures were found in women with gestational hypertension or with preeclampsia compared with the control group (both P < 0.05; Table). Lower birth weights and gestational ages at delivery were found in the group of preeclamptic women compared with the other groups (P < 0.05; Table).

Whole blood nitrite levels were significantly lower in the gestational hypertensive subjects and preeclamptic patients (−36% and −58%, respectively; both P < 0.05; Figure 1).
plasma nitrite levels were compared with those found in healthy pregnancies. The plasma nitrite levels were \( \approx 37\%\) lower in both groups of hypertensive pregnancies compared with the group of normotensive pregnancies (both \( P<0.05\); Figure 1). As expected,\(^{34,46–50}\) we found higher circulating sFLT-1 and sEng concentrations in preeclampsia compared with gestational hypertensive subjects or with healthy pregnancies (both \( P<0.05\); Figure 2).

To address the possibility that inverse relationships exist between the circulating concentrations of markers of NO formation and sFLT-1 or sEng, we carried out correlation analysis between sFLT-1 or sEng levels and whole blood or plasma nitrite concentrations. Taking into consideration all of the pregnant women, we found significant negative correlations between both antiangiogenic factors and plasma nitrite or whole blood nitrite concentrations (Figure 3; all \( P<0.05\)). Interestingly, when we excluded gestational hypertensive women from the analysis, the correlations strengthened even more.

When we examined whether significant correlations exist between these markers and the severity of preeclampsia, we found that proteinuria correlated significantly with both sFLT-1 and sEng (\( r=0.490, P=0.002\), and \( r=0.621, P<0.0001\), respectively), but not with whole blood or plasma nitrite (both \( P>0.05\)). In addition, systolic and diastolic blood pressures correlated significantly with sEng (\( r=0.5310, P<0.0001\), and \( r=0.353, P=0.008\), respectively) but not with other markers (all \( P>0.05\)).

**Discussion**

This is the first study to measure the circulating nitrite levels in hypertensive disorders of pregnancy. A main novel finding reported here is that patients with preeclampsia or with gestational hypertension have lower whole blood and lower plasma nitrite levels compared with normotensive healthy pregnant women, thus indicating impaired NO formation during hypertensive pregnancies. Moreover, our findings suggest that the circulating concentrations of these markers of NO formation correlate negatively with the increased circulating levels of the antiangiogenic factors sFLT-1 and sEng, a usual finding during preeclampsia.\(^{34,46–50}\)

Important changes in maternal cardiovascular hemodynamics take place during normal pregnancy. These modifications include increased blood volume and cardiac output without increases in arterial blood pressure, and 1 of the main reasons for the lack of increase in arterial blood pressure during normal pregnancy is NO-induced systemic vascular dilation.\(^2\) Although impaired NO formation has been implicated in the pathogenesis of hypertensive disorders of pregnancy,\(^4–7\) no previous study has examined whether these conditions downregulate the circulating levels of relevant markers of NO formation. Because NO is rapidly oxidized to nitrite, recent studies have indicated that measuring plasma nitrite levels may reflect endogenous NO formation. This is because \( \approx 70\%\) of plasma nitrates derive from NO synthase activity in the endothelium,\(^{15}\) and inhibition of NO synthase activity was associated with corresponding decreases in circulation nitrite concentrations.\(^{15,17}\) The reduced concentrations of both plasma and whole blood nitrite give support to the idea that preeclampsia and gestational hypertension are disorders of pregnancy characterized by impaired NO formation.\(^4–7\)

The antiangiogenic factors sFLT-1 and sEng have been widely studied in preeclampsia.\(^{46,49,51}\) Our results corroborate previous studies showing strikingly elevated sFLT-1 and sEng levels in preeclampsia compared with healthy pregnan-
cies or with gestational hypertension. These factors probably play a role in the pathophysiology of preeclampsia and have been associated with endothelial dysfunction, which is usually found in this disorder. The impaired endothelial function could result from sFLT-1–induced antagonism of vascular endothelial growth factor actions, which include eNOS activation via phosphorylation of Ser-1177 by Akt and NO synthesis by endothelial cell. In fact, our findings showing an inverse correlation between plasma or whole blood nitrite concentrations and sFLT-1 levels are consistent with this suggestion and provide clinical evidence for a pathophysiological mechanism probably playing a role in preeclampsia.

Endoglin is a TGF-β1 and -β3 coreceptor highly expressed in vascular endothelial cells, and it may be cleaved in preeclampsia, thus releasing its extracellular domain into the circulation forming sEng. This soluble form of endoglin, in turn, decreases the arterial vasodilatation induced by TGF-β1 and -β3, which is mediated by NO. Indeed, TGF-β receptor activation increases NO formation through increased eNOS expression and activation, and sEng attenuates eNOS activation by TGF-β by interfering with the Thr495 dephosphorylation of eNOS, thus contributing to decreased NO synthesis. The negative correlations that we found between plasma or whole blood nitrite concentrations and sEng support the suggestion that sEng plays a role in the pathogenesis of preeclampsia by decreasing NO formation. Finally, it is possible that increased concentrations of asymmetrical dimethyl arginine (an endogenous NO synthesis inhibitor) contribute to decreased NO signaling in preeclampsia.

In conclusion, we found clinical evidence for impaired NO formation in patients with preeclampsia or with gestational hypertension. Moreover, the negative correlations that we found between NO markers and sFLT-1 or sEng in preeclamptic patients suggest relevant mechanisms possibly involved in the pathogenesis of this life-threatening condition. These findings may have important therapeutic implications.

Perspectives
In this study, we show clinical evidence for impaired NO formation in patients with preeclampsia or with gestational hypertension. Importantly, the decreased concentrations of markers of NO formation were negatively associated with the circulating levels of relevant antiangiogenic factors produced in the placenta. Although these antiangiogenic factors have been implicated in the pathogenesis of preeclampsia, this is the first study reporting clinical evidence for a possible inhibitory effect caused by these factors on the endogenous formation of NO in patients with preeclampsia. These findings may have important therapeutic implications. Although the cause of upregulation of antiangiogenic factors in preeclampsia remains unknown, it is possible that therapeutic approaches focusing on upregulating NO bioavailability may be useful targets in patients with gestational disorders of pregnancy.

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Disclosures
None.
References


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