Clinical Research

Elevated Levels of S-Nitrosoalbumin in Preeclampsia Plasma


Abstract—The availability of nitric oxide (NO), which is required for the normal regulation of vascular tone, may be decreased in preeclampsia, thus contributing to the vascular pathogenesis of this pregnancy disorder. Because ascorbate is essential for the decomposition of S-nitrothiols and the release of NO, we speculated that the ascorbate deficiency typical of preeclampsia plasma might result in decreased rates of decomposition of S-nitrosothiols. We tested the hypothesis that total S-nitrosothiol and S-nitrosoalbumin concentrations are increased in preeclampsia plasma, reflecting a decreased release of NO from these major reservoirs of NO. Gestationally matched plasma samples were obtained (before labor or intravenous MgSO4) from 21 women with preeclampsia and 21 women with normal pregnancy, and plasma samples were also obtained from 12 nonpregnant women of similar age and body mass index during the follicular phase of the menstrual cycle. All were nonsmokers. The assay included ultraviolet-induced decomposition of S-nitrosothiols to liberate NO captured by a fluorogenic reagent, 4,5-diaminofluoresceine, to produce diaminofluorescin-triazole. Preeclampsia plasma contained significantly higher concentrations of total S-nitrosothiols (11.1±2.9 nmol/mL) than normal pregnancy samples (9.4±1.5 nmol/mL). Even greater differences were found between preeclampsia plasma and plasma samples from normal pregnancies and nonpregnant women (294±110, 186±25, and 151±25 pmol/mg protein, respectively) when S-nitrosothiol content was expressed per milligram protein. The albumin fraction contained 49.4% of total plasma S-nitrosothiols in the control samples and 53.7% and 56.8% of plasma S-nitrosothiols in normal pregnancy and preeclampsia, respectively. The level of S-nitrosoalbumin was significantly higher in preeclampsia than in normal pregnancy or nonpregnancy plasma (6.3±1.4, 5.1±0.7, and 4.2±1.0 nmol/mL, respectively). The increased concentration of S-nitrosoalbumin in preeclampsia almost completely accounted for the increased levels of S-nitrosothiols in total plasma. Due to combined increases in nitrosothiols and decreases in protein, the preeclampsia plasma concentration of S-nitrosoalbumin was greatly increased on a per milligram of protein basis (271% and 186% compared with normal nonpregnancy and normal pregnancy plasma, respectively). We conclude that S-nitrosoalbumin and total S-nitrosothiol concentrations are significantly increased in preeclampsia plasma and may reflect insufficient release of NO groups in this condition. (Circ Res. 2001;88:1210-1215.)

Key Words: preeclampsia ■ plasma ■ nitrosothiols ■ nitrosoalbumin ■ ascorbate

Preeclampsia, a pregnancy-specific disorder characterized by placental abnormalities and maternal vascular endothelial dysfunction, is the major cause of both maternal and fetal/neonatal morbidity and mortality.1–3 Oxidative stress accompanied by a pronounced depletion of ascorbate4 is thought to contribute to the endothelial dysfunction of preeclampsia.4–10 Although specific pathways through which a shortage of ascorbate translates into functional endothelial deficiency are not completely understood,11 ascorbate has been shown to have nitric oxide (NO)–dependent endothelial dysfunction in atherosclerosis,12–14 hypertension, hypercholesterolemia, and diabetes.15–18

A decreased availability of the NO required for the normal regulation of vascular tone, which may contribute to the pathogenesis of preeclampsia, could be due to suppressed NO production by endothelium and/or improper storage and delivery to its targets. In plasma, nitrosylated thiols, mainly S-nitrosoalbumin, are the major reservoirs of NO groups.19–21 Notably, S-nitrosothiols are potent vasodilators whose action is commonly associated with their ability to release NO in physiologically specified locations.22 Transition metals (eg, copper) in the presence of appropriate reductants, such as ascorbate, are often required for this physiological function of S-nitrosothiols.23,24

On the basis of these facts, we speculated that oxidative stress–induced ascorbate deficiency might result in decreased rates of decomposition of S-nitrosothiols in preeclampsia plasma. This hypothesis thus predicts that elevated levels of S-nitrosothiols are characteristic of preeclampsia plasma. In the current work, we determined the levels of S-nitrosothiols...
in total plasma and the albumin-enriched fraction of plasma obtained from normal pregnant women and women with preeclampsia and compared them with nonpregnant controls. We found that increased levels of \( \text{S-nitrosothiols} \) and \( \text{S-nitrosoalbumin} \) are typical of preeclampsia plasma compared with normal pregnancy and nonpregnancy control plasma samples.

### Materials and Methods

#### Study Subjects and Sample Collection

A total of 42 nulliparous white women were studied; 21 had preeclampsia, and 21 had an uncomplicated pregnancy. These subjects were recruited at Magee–Women’s Hospital as part of our ongoing investigation of preeclampsia, either early in pregnancy or at the time of admittance to the labor and delivery ward. The study was approved by the hospital Institutional Review Board, and all subjects gave informed, written consent. Clinical data were collected at routine obstetric visits and are summarized in Table 1. Preeclampsia was defined using the criteria of gestational hypertension, proteinuria, and hyperuricemia and reversal of hypertension and proteinuria after delivery. Gestational hypertension was defined as an increase of 30 mm Hg in systolic blood pressure or 15 mm Hg in diastolic blood pressure compared with values obtained before 20 weeks gestation or as having an absolute blood pressure \( \geq 140/90 \text{ mm Hg} \) that developed after 20 weeks of gestation at the time of admittance to the labor and delivery ward. The study population was approved by the hospital Institutional Review Board, and subjects gave informed, written consent.

Blood samples were obtained before labor and the therapeutic intravenous administration of magnesium sulfate. Pregnancy cases and controls were individually matched for gestational age at time of blood sampling (Table 1). The average gestational age difference between pairs was 1.0 week; the maximum was 3.0 weeks. Plasma was prepared from whole venous blood that was drawn into sterile tubes, immediately separated by centrifugation, and frozen at \(-70^\circ \text{C} \) until assayed.

#### Fluorometric Assay for \( \text{S-nitrosothiols} \)

The content of \( \text{S-nitrosothiols} \) in plasma samples was determined fluorometrically using 4,5-diaminofluoresceine (DAF-2), which specifically reacts with NO (but not with \( \text{NO}_2^-, \text{ONOO}^-, \text{NO}_3^- \) or \( \text{N}_2\text{O}_3^- \)) to yield fluorescent DAF-2–Triazole (DAF-2T).\(^25\) The assay\(^26\) included decomposition of nitrosothiols by ultraviolet (UV) irradiation (>330 nm). Aliquots (50 \( \mu \text{L} \)) of plasma were mixed with DAF-2 (5 \( \mu \text{mol/L} \)) in 2.5 mL of PBS and exposed to UV radiation (15 minutes, 80 \( \mu \text{W/cm}^2 \)) using an Oriel UV lightsource (model 66002) and cutoff filter (Balzers; >330 nm). After UV irradiation, aliquots of the mixture were heated (80°C for 4 minutes) and centrifuged at 14 000 \( \text{g} \) for 5 minutes. The fluorescence emission intensity of DAF-2T was determined at 515 nm, with excitation at 495 nm (slits: excitation, 1.5 nm; emission, 5.0 nm) in 2.5 mL cuvettes using a Shimadzu RF-5301 PC spectrofluorophotometer. The data obtained were exported and processed using Shimadzu RF-5301 PC personal software. A standard curve was established by using nitrosylated glutathione as the standard. The detection limit for \( \text{S-nitrosothiols} \) was 50 mmol/L.

#### Purification of Albumin

The albumin-enriched fraction of plasma was obtained by affinity-column chromatography.\(^27\) Plasma (100 \( \mu \text{L} \)) was loaded on a HiTrap column (HiTrap Blue 1 mL, Pharmacia Biotech) and collected in 2 fractions. Fraction 1 of plasma was collected in 5 mL of buffer A (50 mmol/L phosphate buffer and 0.1 mol/L KCl; \( \text{pH} 7.0 \)). Fraction 2 (albumin-enriched fraction) was collected in 5 mL of buffer B (50 mmol/L phosphate buffer and 1.5 mol/L KCl; \( \text{pH} 7.0 \)). The elution rate was 0.5 mL/min. After column chromatography, the amount of \( \text{S-nitrosothiols} \) was estimated in each fraction as described above.

#### Polyacrylamide Gel Electrophoresis

Native polyacrylamide gel electrophoresis was performed for electrophoretic separation of proteins in plasma and plasma fractions using the method of Sambrook et al\(^28\) with an 8% acrylamide gel.

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**TABLE 1. Characteristics of the Pregnant Population**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Preeclampsia (n=21)</th>
<th>Normal Pregnancy (n=21)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, y</td>
<td>29.1±5.6</td>
<td>26.3±7.0</td>
<td>NS</td>
</tr>
<tr>
<td>Prepregnancy body mass index, kg/m²</td>
<td>24.1±4.2</td>
<td>25.1±5.0</td>
<td>NS</td>
</tr>
<tr>
<td>Weeks of gestation at delivery</td>
<td>33.0±4.8</td>
<td>38.7±1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weeks of gestation at sampling</td>
<td>32.6±4.8</td>
<td>32.9±5.1</td>
<td>NS</td>
</tr>
<tr>
<td>Blood pressure at &lt;20 weeks gestation, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>113±8</td>
<td>117±9</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic</td>
<td>70±7</td>
<td>74±5</td>
<td>NS</td>
</tr>
<tr>
<td>Predelivery blood pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>160±9</td>
<td>121±9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic</td>
<td>96±8</td>
<td>74±6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Uric acid, mg/dL</td>
<td>6.4±1</td>
<td>Not measured</td>
<td></td>
</tr>
<tr>
<td>Infant birth weight, g</td>
<td>1846±953</td>
<td>3281±366</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are given as means±SD. NS indicates not significant.

*First trimester blood pressures were not available for 2 women with preeclampsia.
Gels were stained with Coomassie blue (R-250) and scanned using Fluor-S Multilamger (Bio-Rad).

Quantification of Protein
The protein concentration in plasma samples and chromatographic fractions of plasma were determined with the BioRad protein assay kit. A standard curve was established by adding bovine serum albumin to the Bio-Rad kit, and protein content was calculated.

Statistical Evaluation
Clinical characteristics of pregnancy subjects (Table 1) were compared using an unpaired Student’s t test (2-tailed). The data for each experiment done to determine S-nitrosothiols in the fractions and whole plasma samples obtained from preeclampsia patients, women with normal pregnancies, and nonpregnant controls were analyzed by a one-way ANOVA, with Tukey’s family error rate of \( P \leq 0.05 \) applied to post hoc differences between group means. Because the preeclampsia group exhibited several variables that were relatively higher than most of the data in each of the experiments measuring S-nitrosothiol content, we performed all ANOVA analyses both with and without the 2 extreme data points.

Results

Content of S-Nitrosothiols in Total Plasma
Decomposition of nitrosothiols via homolytic (using UV irradiation) or heterolytic (using excess mercury or copper ions) cleavage is commonly used in assays of nitrosothiols in biological systems. Separate control experiments demonstrated that using UV radiation for the decomposition of S-nitrosothiols in plasma is preferable to using Cu or Hg, because these ions may yield erroneous results. This is due to the presence of a high concentration of proteins (including albumin) in plasma (50 to 70 mg of protein/mL), which are capable of high-affinity binding of metals. Because the protein content of plasma may vary (eg, proteinuria and albuminuria in preeclampsia), concentrations of free Cu (Hg) available for the reaction with nitrosothiols should be experimentally established for each set of samples. We found that the intensity of DAF-2T fluorescence was strongly dependent on the concentration of added Cu in the range of 0.1 to 1.0 mmol/L and that a saturation of the DAF-2T fluorescence response from a plasma sample (50 \( \mu \)L of undiluted plasma) was only achieved at Cu concentrations >1.0 mmol/L (data not shown). Therefore, to determine the content of S-nitrosothiols in plasma samples, we used a sensitive procedure based on UV-induced decomposition of S-nitrosothiols\(^{19,20}\) and subsequent detection of released NO with a specific reagent, DAF-2,\(^{25}\) that produces DAF-2T with a characteristic fluorescence emission spectra (Figure 1). The method permits reliable quantitative measurements of S-nitrosothiols at concentrations in plasma as low as 50 nmol/L.\(^{26}\) We found that plasma samples from nonpregnant women (n=12) contained 9.2±1.6 nmol/mL (151±25 pmol/mg protein) S-nitrosothiols. This is within the range of concentrations previously reported for human plasma.\(^{19-21}\)

Scatter plots of our fluorescence response determinations are shown in Figure 2. Preeclampsia plasma samples (n=21) contained significantly higher concentrations of total S-nitrosothiols (11.1±2.9 \( \mu \)mol/L) than normal pregnancy samples (9.4±1.5 \( \mu \)mol/L) (n=21). Because proteinuria is a defining feature of preeclampsia pregnancy, we performed measurements of protein concentrations in plasma samples (Table 2) and normalized concentrations of S-nitrosothiols to the content of protein in each sample (Figure 3). The results of this experiment showed statistically significant differences between preeclampsia patients compared with both women with normal pregnancies and nonpregnant controls in plasma S-nitrosothiol content. The concentration of total S-nitrosothiols expressed this way remained significantly higher in women with preeclampsia than in women with normal pregnancies or in the nonpregnant controls, even when the extreme values were removed from the ANOVA analysis.

Content of S-Nitrosoalbumin in Plasma
Because albumin is said to be the major source of S-nitrosylated thiols in plasma,\(^{19-21}\) we determined the levels

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Typical fluorescence emission spectra of DAF-2T formed after UV-induced decomposition of nitrosothiols in the presence of DAF-2. Line 1 indicates DAF-2 alone; line 2, plasma plus DAF-2; line 3, S-nitrosoalbumin plus DAF-2; and line 4, nitrosylated glutathione plus DAF-2. Control (nonpregnancy) plasma (50 \( \mu \)L), S-nitrosoalbumin (0.16 \( \mu \)mol/L), or nitrosylated glutathione (0.24 \( \mu \)mol/L) was mixed with DAF-2 (5 \( \mu \)mol/L) in 2.5 mL of PBS and exposed to UV irradiation, and released NO was detected as described in Materials and Methods. AU indicates arbitrary units.

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** S-nitrosothiols (RSNO) in total plasma samples and fractions obtained from plasma by affinity-column chromatography. A, A total of 50 \( \mu \)L of plasma was mixed with DAF-2 in PBS and exposed to UV irradiation, and S-nitrosothiols were determined as described in Materials and Methods. A, Whole plasma samples; B, fraction 1 obtained from plasma by affinity-column chromatography; C, fraction 2 (albumin-enriched fraction) obtained from plasma by affinity-column chromatography. Open circles indicate control (nonpregnancy) samples (n=11); open diamonds, normal pregnancy samples (n=21); and open triangles, preeclampsia samples (n=21). \( *P<0.001 \) vs normal pregnancy. **P<0.001 vs control (nonpregnancy) or normal pregnancy.
of nitrosylated thiols in albumin-enriched fractions. Affinity chromatography of plasma samples using a HiTrap column resulted in 2 fractions (Figure 4A). The first one was essentially albumin-free, and the second one was enriched with albumin, as evidenced by native polyacrylamide gel electrophoresis (Figures 4B and 4C). In a separate series of control experiments, we showed that the recovery of S-nitrosothiols from plasma fractions after chromatography was 99.8% compared with the direct measurements of S-nitrosothiols in nonfractionated total plasma samples.26 Analysis of S-nitrosothiols revealed that the albumin fraction contained 49.4% of total plasma S-nitrosothiols in the control samples and 53.7% and 56.8% of S-nitrosothiols in normal pregnancy and preeclampsia samples, respectively (Figures 2B and 2C). Importantly, the level of S-nitrosoalbunmin on a per milliliter of plasma basis was significantly higher in preeclampsia samples than in normal pregnancy plasma or nonpregnancy plasma. In fact, the increased concentration of S-nitrosoalbumin in preeclampsia plasma was almost 3- and 2-fold higher than that in nonpregnant hypoproteinemia (Table 2 and Figure 3), group differences were even more pronounced after normalization to protein content in each plasma sample. The data in Figure 3 indicate that the concentration of S-nitrosoalbumin in preeclampsia plasma was almost 3- and 2-fold higher than that in nonpregnant controls when the extreme values were removed from the ANOVA analysis. There was not a significant relationship between concentrations of S-nitrosylated proteins and blood pressure in preeclamptic patients [S-nitrosoalbumin (nmol/mL) and systolic blood pressure: r=0.43, P=0.06; S-nitrosoalbumin and diastolic blood pressure: r=0.10, P=0.78].

Given that preeclampsia is accompanied by proteinuria and hypoprothrombinemia (Table 2 and Figure 3), group differences were even more pronounced after normalization to protein content in each plasma sample. The data in Figure 3 indicate that the concentration of S-nitrosoalbumin in preeclampsia plasma was almost 3- and 2-fold higher than that in nonpregnant controls and normal pregnancy plasma samples, respectively. These differences were statistically significant and remained so, despite outlier exclusion. On a per milligram of protein basis, the content of S-nitrosothiols in the albumin-free fraction of preeclampsia plasma was also significantly higher than in normal pregnancy samples and nonpregnancy controls. These differences remained significant, even after the removal of the extreme preeclampsia values from the comparison of the group means.

### Table 2. Concentration of Protein in Total Plasma Samples and Fractions 1 and 2

<table>
<thead>
<tr>
<th>Plasma Samples</th>
<th>Total Plasma</th>
<th>Fraction 1</th>
<th>Fraction 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (nonpregnancy)</td>
<td>67.0±7.8</td>
<td>20.0±5.0</td>
<td>47.0±5.4</td>
</tr>
<tr>
<td>Normal pregnancy</td>
<td>53.5±8.2</td>
<td>15.1±3.0</td>
<td>38.4±5.1</td>
</tr>
<tr>
<td>Preeclampsia</td>
<td>37.2±8.2*</td>
<td>10.4±3.5</td>
<td>26.8±6.2†</td>
</tr>
</tbody>
</table>

Values are mean±SD. Control (nonpregnancy), n=11; normal pregnancy, n=21; and preeclampsia, n=21.

*P<0.001, †P<0.05 vs control (nonpregnancy) or normal pregnancy.
One would suspect that the 2 extreme values in the preeclampsia group would, in all cases, cause a greater difference between this group and the normal pregnancies. However, in each experiment, except for total plasma not corrected for protein, the exclusion of these outliers sharpened the statistical differences between the preeclampsia group and the 2 other groups. This was due to the extra variability in the observations being removed from the ANOVA.

Discussion

In this study, we used a sensitive fluorescence assay to measure S-nitrosothiols in plasma. The assay is based on a combination of a commonly used decomposition of S-nitrosothiols by UV irradiation and subsequent capture of released NO by a fluorogenic reagent, DAF-2. We found that the use of UV irradiation is particularly appropriate for measurements in plasma, where the traditional use of Cu or Hg to decompose S-nitrosothiols is complicated by the high metal-binding capacity of plasma proteins, including albumin. DAF-2 was developed and introduced as a specific reagent for NO that does not react with NO, nitrates, or ONOO-. We found that our protocol was sensitive enough to detect S-nitrosothiols in plasma at concentrations as low as 50 nmol/L. Thus, the assay we used was adequate for quantitative determinations of S-nitrosothiols in plasma samples. We detected low micromolar concentrations of S-nitrosothiols in normal plasma samples; these concentrations were well within the range previously reported by several investigators in human plasma.

Our results demonstrate, for the first time, that preeclampsia plasma contains significantly higher levels of S-nitrosothiols than normal pregnancy plasma or nonpregnancy control plasma. This was true for S-nitrosothiol concentrations expressed per volume of plasma (nmol/mL) and even more so when S-nitrosothiol levels were normalized to the amount of protein in the samples (pmol/mg protein). The latter was likely due to the proteinuria and hypoproteinemia that accompany preeclampsia. This suggests that the plasma thiols (proteins) lost during preeclampsia were those that were relatively less S-nitrosylated.

We further obtained albumin-free and albumin-enriched fractions of plasma and conducted measurements of S-nitrosothiols in them. We found that albumin was responsible for 50% to 57% of the S-nitrosothiols in total plasma samples from control, normal pregnancy, and preeclampsia samples. This is in line with previous reports, in which ≈80% of S-nitrosylated proteins were found in albumin (and ≈20% of total S-nitrosothiols were nitrosylated glutathione). Remarkably, we found that the greatest increase in S-nitrosothiols in preeclampsia was due to S-nitrosoalbumin. Our estimates indicate that ≈0.1 mol% albumin was S-nitrosylated in control plasma samples and that this level increased ≈3-fold (≈0.3 mol% albumin) in preeclampsia. We did not observe a significant relationship between concentrations of S-nitrosylated proteins and blood pressure in preeclamptic patients. This result is not surprising; although systemic hypertension is a component of the preeclampsia syndrome, it is not the overriding pathological mechanism, nor is it a major predictive marker for the morbidity or mortality associated with preeclampsia. Focal vasospasm and ischemia (eg, cerebral, intrarenal) often exceed the degree of systemic blood pressure elevation.

Given that plasma ascorbate levels are substantially reduced in preeclampsia and that ascorbate is essential for the decomposition of S-nitrosothiols, it is tempting to speculate that decomposition of S-nitrosothiols is impaired in preeclampsia plasma due to subnormal plasma ascorbate reserves. Normally, ascorbate levels in plasma are within the range of 30 to 50 μmol/L, but this amount is decreased by half in preeclampsia. Because stoichiometric amounts of ascorbate are required for the decomposition of S-nitrosothiols, the steady-state concentrations of ascorbate in preeclampsia plasma may be insufficient to act as a source of reducing equivalents in the reaction with S-nitrosothiols. It is noteworthy that the depletion of ascorbate may also be associated with the redox-cycling activity of albumin-bound Cu and its mishandling during preeclampsia.

We postulate that the elevated levels of S-nitrosothiols and S-nitrosoalbumin in preeclampsia plasma reflect insufficient nitrosothiol decomposition and release of NO groups at sites critical for normal regulation of vascular tone. Although low ascorbate is one possible mechanism for NO group release, the role of ascorbate in nitrosothiol metabolism remains unclear. Future studies will be necessary to test whether antioxidant vitamins, which seem to reduce the incidence of preeclampsia, might also enhance the release of NO groups from protein-bound reservoirs.

Acknowledgments

Supported by grants from the NIH (1RO1HL64145-01A1, 3PO1HD30367-06, and 1RO1HL56829-01) and by the Magee-Womens Research Institute Fellowship (to V.A.T.).

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Elevated Levels of S-Nitrosoalbumin in Preeclampsia Plasma
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Circ Res. 2001;88:1210-1215; originally published online May 24, 2001;
doi: 10.1161/hh1101.092179

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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