L-Arginine Depletion in Preeclampsia Orients Nitric Oxide Synthase Toward Oxidant Species

Marina Noris, Marta Todeschini, Paola Cassis, Fabio Pasta, Anna Cappellini, Samantha Bonazzola, Daniela Macconi, Raffaella Maucci, Francesca Porrati, Ariela Benigni, Claudio Piccoli, Giuseppe Remuzzi

Abstract—Less nitric oxide (NO)-dependent vasodilation and excess formation of reactive oxygen species could explain poor placenta perfusion in preeclampsia, but the pathways involved are unknown. We tested the hypothesis that reduced NO activity and increased oxidative stress in preeclamptic placenta is related to a low bioavailability of L-arginine. Placental endothelial NO synthase (ecNOS) expression (by immunoperoxidase) and activity (by diaphorase and [3H]L-citrulline formation) were comparable in normotensive pregnancy and in preeclampsia, whereas nitrotyrosine staining, a marker of peroxynitrite, was stronger in preeclamptic villi, confirming previously reported data. Oxidative tissue damage was documented in preeclamptic villi by strong 4-hydroxynonenal-lysine staining (by immunoperoxidase), which closely colocalized with nitrotyrosine. Concentration of the NO precursor L-arginine (by HPLC) in umbilical blood and in villous tissue was lower in preeclampsia than in normotensive pregnancy. This was not caused by a defective L-arginine transport, because gene expression of the CAT-1, 4F2hc, and LAT-1 cationic amino acid transporters (by real-time reverse-transcription polymerase chain reaction [RT-PCR]) was normal. Instead, gene expression (by real-time RT-PCR) and protein tissue content (by immunoperoxidase and Western blot) of arginase II—the enzyme that degrades arginine to ornithine—were higher in preeclamptic villi than in normotensive pregnancy. These results provide a biochemical explanation for defective NO activity and increased oxidative stress in preeclamptic placenta. In normal placenta, adequate concentration of L-arginine orients ecNOS toward NO. In preeclampsia, a lower than normal L-arginine concentration caused by arginase II overexpression redirects ecNOS toward peroxynitrite. (Hypertension. 2004;43:614-622.)

Key Words: arginine ■ nitric oxide synthase ■ nitrites ■ oxidative stress ■ preeclampsia

In the past few years, evidence has accumulated suggesting that nitric oxide (NO), a potent endothelial-derived vasodilator, might be implicated in gestational vasodilation.1,2 NO is synthesized from the amino acid L-arginine by a family of enzymes, the NO synthases (NOS).3 While human placenta does not form inductive NOS (iNOS) or neuronal NOS (nNOS) mRNAs and proteins,1 the endothelial isoform of NOS (ecNOS) is actually detectable in healthy placenta and is localized to the endothelium of the umbilical cord, chorionic plate, and stem villous vessels.1 Conceivably, locally formed NO serves to maintain low vascular resistance besides attenuating the action of vasoconstrictors.1 Besides, ecNOS is localized in villous cytotrophoblasts,1,4 the specialized epithelial cells that aggregate into cell columns and invade the uterine interstitium and vasculature, anchoring the fetus to the mother and establishing blood flow to the placenta.5 By virtue of its unique angiogenic/vasculogenic properties,6,7 locally generated NO may be instrumental to promote this cytotrophoblast endovascular invasion that is an essential feature of normal placentation,5 although whether extravillous invading trophoblasts express ecNOS is still a controversial issue.8,9

Unlike in normal pregnancy, in preeclampsia the cytotrophoblast fails to adopt a vascular adhesion phenotype,10 which compromises the supply of blood flow to the maternal–fetal interface. It has been hypothesized that a reduced formation of NO could account for abnormal placental perfusion in preeclampsia.1,11 Most data indicate that preeclamptic placenta has a normal capacity to synthesize ecNOS as compared with normal placenta and the formation of NO is also comparable in preeclamptic and in normal placenta.1,2 However, one study12 found that inhibiting NO synthesis raised the perfusion pressure of isolated human cotyledon preparations from normal pregnant women, but not from preeclamptic women. Moreover, in preeclampsia the concentration of the NO second messenger, cGMP, in the placental circulation was lower than normal.1,13 Thus, pre-
TABLE 1. Major Clinical Characteristics of Women With Normotensive Pregnancy and Preeclampsia

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normotensive Pregnancy</th>
<th>Preeclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>Mean 33.2, SEM 1.8 (26–42)</td>
<td>Mean 32.6, SEM 1.8 (27–43)</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>Mean 125.4, SEM 2.7 (115–140)</td>
<td>Mean 157.3*, SEM 3.3 (130–170)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>Mean 70.3, SEM 1.7 (60–83)</td>
<td>Mean 99.1*, SEM 1.9 (90–110)</td>
</tr>
<tr>
<td>Proteinuria (g/24 h)</td>
<td>Absent</td>
<td>2.54, 1.78 (0.34–5.20)</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>Mean 37.6, SEM 0.8 (32.2–41.0)</td>
<td>Mean 32.4†, SEM 1.3 (25.5–40.0)</td>
</tr>
<tr>
<td>Placenta pathology score</td>
<td>Mean 0.4, SEM 0 (0–3)</td>
<td>Mean 2†, SEM 1 (1–3)</td>
</tr>
<tr>
<td>Nulliparous/multiparous</td>
<td>6/7</td>
<td>7/4</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>Mean 2880, SEM 149 (1920–3650)</td>
<td>Mean 1683†, SEM 343 (510–3330)</td>
</tr>
<tr>
<td>Apgar score</td>
<td>Mean 8.5§, SEM 0.4 (7–10)</td>
<td>Mean 7.6, SEM 0.4 (7–10)</td>
</tr>
</tbody>
</table>

*P<0.0001; †P<0.005; ‡P<0.001; §P<0.01 vs normotensive pregnancy.

Preeclampsia is a condition of normal placental expression of eNOS and normal generation of NO, whose activity, however, is abnormally reduced. This apparent inconsistency can be reconciled considering that the relative activity of NO in a given organ/tissue depends on the rates of synthesis and degradation. Beside NO, NOS enzymes also generate superoxide anion (O2−) and the rate of NO versus O2− formation and disposal is closely regulated by intracellular levels of L-arginine, tetrahydrobiopterin, and superoxide dismutase (SOD).14–16 NO, by interacting with O2−, forms peroxynitrite, a cytotoxic anion that inhibits mitochondrial electron transport, oxidizes proteins, initiates lipid peroxidation, and nitrates aromatic amino acids.17 Markers of oxidative stress are increased in placentas of women with preeclampsia and nitrotyrosine staining, a marker of peroxynitrite, has been found in the preeclamptic placenta.18,19

The present study was designed: (1) to test whether lower placental NO bioavailability of preeclampsia is caused more by increased NO degradation to peroxynitrite than to low eNOS expression or activity and (2) to investigate whether excessive peroxynitrite formation is related to a low bioavailability of the NO precursor L-arginine and to find out the biochemical determinants of that.

Methods

Patients

Pregnant women (normotensive pregnancy, NP; n=13; preeclampsia: n=11) were recruited among those referred to the Obstetrics and Gynecology Division of the Ospedale San Gerardo in Monza. Healthy age-matched nonpregnant women (n=6) served as controls. The Institutional Review Committee approved the study and all participants gave informed consent to the study. Criteria for defining NP were: no history of hypertension, diastolic blood pressure (BP) ≤90 mm Hg, systolic BP ≤140 mm Hg, and no significant proteinuria. Four women underwent premature delivery for ruptured membranes (n=2), oligohydramnios (n=1), and fetal distress (n=1). According to previously published criteria,20 preeclampsia was diagnosed as increase in diastolic BP of 15 mm Hg and systolic BP of 30 mm Hg at two measurements at least 4 hours apart compared with BP obtained before 20 weeks of gestation, proteinuria >0.3 g/24 hours in the absence of urinary tract infection, return to normal BP, resolution of proteinuria by 12 weeks postpartum, and edema. One preeclamptic pregnancy was complicated by the HELLP syndrome and two were complicated by intrauterine growth restriction (IUGR). The patients’ clinical characteristics are summarized in Table 1.

Tissue Collection and Histology

For placenta tissue collection, see the online data supplement available at http://www.hypertensionaha.org.

Placenta pathology score was graded according to the presence (in at least 30% of the tissue) of each of the following abnormalities: intervillous thrombosis, subchorionic thrombosis, infarcts, chronic hypoxia, and intima hyperplasia, according to the guidelines of the College of American Pathologists (Table 1).21

NADPH-Diaphorase, Immunoperoxidase, and Western Blotting

NADPH-diaphorase reaction was performed on 7-µm thick frozen sections, as described.22 Immunoperoxidase for eNOS, nitrotyrosine, HNE-lysine, and arginine was performed on 3-µm paraffin sections.22 Western blot analysis for arginase II was performed in placenta homogenates. An expanded Methods section can be found in online data supplement available at http://www.hypertensionaha.org.

NOS Activity and L-arginine Levels in Placenta Tissue

Homogenates of total placentas or villous and decidua tissues from NP (n=9) and preeclamptic women (n=6) were used to evaluate NOS activity and L-arginine levels, respectively (see online data supplement available at http://www.hypertensionaha.org).

Conjugated Dienes

Total lipids extracted from frozen tissues were resuspended in cyclohexane and the absorbance read at 233 nm. The extinction coefficient of 2.52×104 mol/L·cm−1 was used to calculate the diene concentration.23

Plasma L-arginine and Nitrites/Nitrates (NO3−/NO2−)

Plasma was obtained from maternal (antebrachial vein) and fetal blood (umbilical vein) collected on heparin and was stored at −80°C until assayed. Women fasted at least 8 hours before blood collection. In preliminary experiments, we found that in this condition, the interference of dietary intake to NO2−/NO3− plasma levels is negligible (not shown). Plasma L-arginine was measured by HPLC.24 NO3−/NO2− plasma levels were measured as reported in online data supplement http://hypertensionaha.org.

Real-Time Quantitative RT-PCR

To analyze gene expression of the cationic amino acid transporters CAT-1,25 4F2hc,25 LAT-1,25 and arginase II,26 and of the housekeep-
ing gene β-actin, RNA was treated with DNase and reverse transcribed to cDNA. Quantitative real-time polymerase chain reaction (PCR) was performed on a TaqMan ABI PRISM 5700 Sequence Detection System (PE Applied Biosystems, Monza, Italy) with SYBR Green PCR Core Reagents (Applied Biosystems), in combination with optimal primer concentrations. Detailed methods are available in the online data supplement http://www.hypertensionaha.org.

Statistical Analysis
Results are means±SEM. Groups were compared by 1-way ANOVA using the StatView 4.01 software. Linear regression analysis was used to correlate immunoperoxidase and mRNA expression data with clinical and experimental parameters. The ρ Spearman Rank test was used to correlate immunoperoxidase data with the placenta pathology score and Apgar score. The statistical level of significance of the two-tailed test was defined as P<0.05.

Results
Placental NADPH-Diaphorase Staining, NOS Activity, and ecNOS Expression and Localization Are Comparable in NP and in Preeclampsia
Comparable diaphorase staining, NOS activity, and ecNOS expression were seen in placentas from NP and preeclampsia (online Figure I and Table 2). Detailed results and online Figure I are in online data supplement available at http://www.hypertensionaha.org.

Exuberant Peroxynitrite Formation in Preeclampsia
Nitrotyrosine staining was absent or very faint in villi and decidua vessels of placentas from NP (Figure 1A, B), whereas it was intense in preeclamptic villous tissue, mainly localized in the syncytiotrophoblast (Figure 1C and Table 2). The endothelium of villous vessels also showed moderate nitrotyrosine staining. A faint nitrotyrosine staining was also seen in the vessels of the decidua in preeclamptic placenta (Figure 1D and Table 2), whereas no staining was found in the cells of the stroma. Nitrotyrosine and ecNOS staining colocalized in preeclamptic villi (Figure 1E, F), indicating that peroxynitrite was formed within the very cells that synthesized NO. Moderate specific ecNOS staining was also found in villous vascular endothelium (Figure 1G). No signal was found in negative control (Figure 1H).

The mean gestational age of NP was significantly greater than in the preeclamptic group. However, we noted no differences in nitrotyrosine staining in placental tissue with differing gestational age in either group. The intensity of nitrotyrosine staining in pre-term NP (n=4) was not different from NP at term (n=9) (syncytiotrophoblast: 0.55±0.29 versus 0.28±0.10; endothelium of villi: 0.38±0.38 versus 0.16±0.10), but was significantly weaker (P<0.05) than in preeclamptic villi. An inverse correlation was found between nitrotyrosine staining in the syncytiotrophoblast and birth weight (r=−0.63, P=0.01) and Apgar score (r=−0.47, P<0.05), whereas a positive correlation was found between nitrotyrosine and placenta pathology score (r=0.48, P<0.05).

Excess Lipid Peroxidation in the Preeclamptic Placenta
As compared with NP, a larger amount of conjugated dienes was extracted from preeclamptic villi (NP: 24±1; preeclampsia: 47±3 pmol/μg phospholipid; P<0.0001) and umbilical cord tissue (NP: 263±2; preeclampsia: 497±54 pmol/μg phospholipid; P<0.001). Dienes concentration in preeclamptic deciduas was also increased, although to a lesser degree (NP: 22±2; preeclampsia: 36±4 pmol/μg phospholipid; P>0.05). Within the NP group, the amount of conjugated dienes was comparable for preterm and for term pregnancies, indicating that placental lipid peroxidation does not depend on the week of gestation.

4-HNE-lysine staining, a marker of cell oxidative stress, was mostly faint in the syncytiotrophoblast and villous vessels (Figure 2A) as well as in the endothelium of NP decidua vessels (Figure 2B). A higher 4-HNE-lysine staining intensity was found in the syncytiotrophoblast and in endothelial cells of preeclamptic villous vessels than in NP villi (Figure 2C, Table 2). Moderate staining was also focally found on preeclamptic decidua vessels (Figure 2D and Table 2). 4-HNE-lysine and nitrotyrosine staining showed the same

### Table 2. Mean Scores of ecNOS, NADPH-Diaphorase, Nitrotyrosine, 4-HNE-lysine, CuZn-SOD Immunoperoxidase, and Arginase II Staining in Villi and Decidua From Normotensive Pregnancy (n=13) and Preeclampsia (n=11)

<table>
<thead>
<tr>
<th>Markers</th>
<th>Normotensive Pregnancy</th>
<th>Preeclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chorionic Villi</td>
<td>Decidua</td>
</tr>
<tr>
<td></td>
<td>Endothelium</td>
<td>Syncytiotrophoblast</td>
</tr>
<tr>
<td>ecNOS</td>
<td>1.6±0.2</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>NADPH-diacarbose</td>
<td>2.2±0.1</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>0.3±0.1</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>4-HNE-lysine</td>
<td>0.8±0.2</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>CuZn-SOD</td>
<td>1.5±0.3</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>Arginase II</td>
<td>0.4±0.1</td>
<td>0.5±0.2</td>
</tr>
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</table>

Each section was scored for intensity of immunostaining (absent, faint, moderate, intense: 0 through 3). Data are mean±SEM.

ecNOS indicates endothelial nitric oxide synthase; 4-HNE-lysine, 4-hydroxynonenal-(4-HNE)-lysine; superoxide dismutase, CuZn-SOD.

*P<0.05; †P<0.001; §P<0.0001; ‡P<0.005 vs normotensive pregnancy.
cell localization (Figure 2E and F), and the intensity of 4-HNE-lysine staining in the syncytiotrophoblast ($r = 0.79, P < 0.0001$) and in villous endothelium ($r = 0.64, P < 0.005$) correlated with the intensity of nitrotyrosine staining, confirming the association between lipid peroxidation and peroxynitrite formation. No signal was found in negative control (Figure 2G). The intensity of 4-HNE-lysine staining in villous tissue was inversely correlated with birth weight (syncytiotrophoblast: $r = -0.69, P < 0.001$, endothelium: $r = -0.58, P < 0.01$) and Apgar score (syncytiotrophoblast: $r = -0.61, P < 0.01$, endothelium: $r = -0.62, P < 0.01$), whereas a positive correlation was found between 4-HNE-lysine staining and placenta pathology score (syncytiotrophoblast: $r = 0.54, P < 0.05$).

Immunostaining for CuZn–SOD showed no differences in intensity and localization in either villous tissue or decidua vessels between NP and preeclamptic placentas (Table 2).

**Fetal–Maternal Gradient of l-arginine and NO$_2^-$/NO$_3^-$ Is Lost in Preeclampsia**

In line with previous data, the mean concentration of l-arginine in maternal blood from NP was significantly lower than in nonpregnant women (Figure 3A). The same was true in preeclampsia (Figure 3A). No significant differences were observed in maternal plasma l-arginine in NP and preeclampsia (Figure 3A). In NP, l-arginine was significantly higher in fetal than in maternal blood (Figure 3A), which is in line with the existence of an active placental l-arginine transport from the mother to the fetus. By contrast, in preeclampsia, fetal l-arginine concentrations were almost identical to those in maternal blood (Figure 3A) and significantly lower than NP (Figure 3A). As previously reported, we found no correlation between maternal ($r = 0.03, P = 0.93$) or fetal ($r = 0.16, P = 0.6$) l-arginine concentrations and gestational age, thus excluding that the lower fetal l-arginine in preeclampsia depends on shorter gestation.

Consistent with l-arginine data, in NP the concentration of the NO metabolites, NO$_2^-$/NO$_3^-$, in the fetal blood was significantly higher than in the maternal blood (Figure 3B). In preeclampsia, NO$_2^-$/NO$_3^-$ concentration in maternal blood was normal whereas levels in fetal blood were significantly ($P < 0.05$) lower than in NP (Figure 3B). An inverse correlation was found between fetal blood l-arginine concentration and the intensity of nitrotyrosine staining in the syncytiotrophoblast (framed panel at upper right of Figure 3A) and in the endothelium of villous vessels ($r = -0.6, P < 0.05$).

**Expression of Cationic Amino Acid Transporters Is Not Reduced in Preeclamptic Placenta**

To search for the possible causes of loss of fetal–maternal l-arginine and NO$_2^-$/NO$_3^-$ gradients in preeclampsia, we...
evaluated gene expression of two cationic amino acid transporters highly expressed in human placenta, CAT-1 (y+ system transporter) \(^{25}\) and 4F2hc and LAT-1 (the 2 subunits of the y+L system transporter) \(^ {25}\). As shown in Figure 4A, CAT-1 expression in villous tissue was higher in preeclampsia than in NP. Expression of CAT-1 in decidua and of 4F2hc and LAT-1 (Figure 4A, 4B) in villous tissue and decidua was comparable in NP and in preeclampsia.

**Excess Expression of Arginase II in Preeclamptic Placenta**

We then looked for placental expression of arginase II—the extrahepatic isoform of arginase that degrades arginine into ornithine and urea. \(^ {26}\) Real-time RT-PCR analysis of villous tissue specimens showed a higher expression of arginase II in preeclampsia than in NP, whereas arginase II expression in decidua tissue was comparable in the two groups (Figure 5A). The expression of arginase II in preterm NP villous tissue (3.08 ± 1.32) was not different from arginase II in villous tissue from NP at term (4.08 ± 1.05), indicating that placental arginase II expression does not depend on the week of gestation. The levels of arginase II mRNA in villous tissue were inversely correlated with fetal L-arginine concentrations \((r = -0.8, P < 0.005)\), indicating that arginase II expressed in placenta may modulate L-arginine availability to the fetus. Immunostaining and Western blot for arginase II was performed to evaluate tissue localization and protein expression. Arginase II staining was mostly faint in chorionic villi and in decidua from NP (Figure 5B, Table 2). An intense arginase II staining was instead found in syncytiotrophoblast and in the villous endothelium (C and E). F, Immunostaining with anti-nitrotyrosine Ab of villous tissue from the same preeclamptic woman, showing colocalization of nitrotyrosine and 4-HNE-lysine staining. Moderate staining is also focally evident on vessels of preeclamptic decidua (D). No signal is found in the negative control (G). V, indicates villous tissue; D, decidua; closed arrow, syncytiotrophoblast; open arrow, endothelium. Original magnifications: A, B, C, D, and G: x200; E and F: x400.

**Discussion**

An excess formation of peroxynitrite was found in preeclamptic placentas, despite the fact that ecNOS was expressed at comparable extent in NP and in preeclampsia, confirming previously reported data. \(^ {19}\) That this can be taken to reflect an excess lipid peroxidation and oxidation of...
structural proteins and enzymes in preeclampsia is consistent with other findings that conjugated dienes and 4-HNE-lysine adducts were detected in large amounts in preeclamptic villous tissue but not in normal placenta. To clarify the nature of the excess peroxynitrite in preeclamptic placenta, we first studied the tissue content of SOD, a major oxidant scavenger in human placenta; however, experimental findings excluded a role for a SOD deficiency in the observed phenomenon (see present data). However, the finding that nitrotyrosine and 4-HNE-lysine staining closely co-localized with ecNOS suggests the possibility that peroxynitrite is generated by NOS. This can occur in cells and tissues in conditions of low L-arginine availability. Whereas cells cultured with adequate concentrations of L-arginine only formed NO from NO synthase, cells depleted of L-arginine generated both NO and $O_2^-$, which then reacted to form peroxynitrite, as shown by the strong nitrotyrosine immunostaining in L-arginine-depleted cells. The formation of both $O_2^-$ and nitrotyrosine was virtually abolished by the specific NO synthase blocker, N-nitro-L-arginine-methylester (L-NMMA), confirming that NOS was actually the source of these toxic oxidants.

Pertinent to the matter are data that in endothelial cells, ecNOS and the arginine transporter CAT-1 form a complex that localizes in plasma membrane caveolae, confirming the importance of adequate L-arginine delivery to ecNOS for NO synthesis.

The relevance of this biochemical pathway to the pathogenesis of preeclampsia rests on present and previous findings that in NP the L-arginine concentration is higher in fetal than in maternal blood and that the fetal-maternal L-arginine gradient was completely lost in preeclampsia. This abnormality was not because of a defect in L-arginine transporters within the syncytiotrophoblast because expres-
sion of \( y^+ \) and \( y^+L \) system cationic transporters was normal or even increased in preeclamptic placenta. Based on the observation that \( L \)-arginine levels in preeclamptic villi were lower than in NP villi, we then addressed the possibility that \( L \)-arginine deficiency was caused by consumption of the amino acid in the preeclamptic villous tissue by arginase II, the extrahepatic isoform of arginase expressed in human placenta.\(^{26}\) Both arginase and NOS use arginine as common substrate and arginase inhibits NO synthesis by reducing arginine bioavailability. Transfection of endothelial cells with arginase II increased \( L \)-arginine consumption and reduced NO synthesis\(^{26}\) and in vivo administration of arginase to experimental animals significantly depleted plasma \( L \)-arginine.\(^{32}\) Arginase II mRNA expression was more than 4-fold higher in preeclamptic than in NP villous tissue; also, protein expression was increased, as documented by immunostaining and Western blot data. In addition, levels of arginase II mRNA in villous tissue inversely correlated with fetal \( L \)-arginine concentration. We hypothesize that in preeclamptic placenta, higher than normal expression of arginase II causes less \( L \)-arginine available for ecNOS in trophoblast cells and in the villous endothelium, which can lead to the aberrant catalytic activity described, giving a rapid NO degradation by reactive oxygen species. This possibility is also supported by finding in our patients that the intensity of nitrotyrosine and 4-HNE-lysine staining in villous tissue and fetal \( L \)-arginine concentration were inversely correlated. Deficiency in tetrahydrobiopterin (BH\(_4\)) can also poise ecNOS toward producing superoxide anion;\(^{33}\) however, levels of BH\(_4\) were found to be normal in preeclamptic placentas.\(^{33}\)

Further investigation is required to clarify the mechanism responsible for arginase II upregulation in preeclampsia. Testosterone could be a possible candidate mediator of the aforementioned phenomenon. Indeed in studies in female rats and mice, testosterone stimulates arginase activity and patients with preeclampsia have been shown to have higher levels of testosterone than NP women.\(^{11}\)
Previous reports\(^1\) of higher resistance in the fetal–placental circulation in the preeclamptic placenta are consistent with the reduced placental NO availability we observed, because NO is a potent vasodilator in the vascular district.\(^1\) In addition, there is evidence of a major role for NO as an angiogenic and vascular remodelling factor.\(^6\)–\(^9\) Exposure of human endothelial cells to NO donors leads to a dose-dependent increase in endothelial cell migration and differentiation,\(^34\) and NO is a mediator of growth factor-induced angiogenesis.\(^6,34,35\) That NO may be instrumental to endovascular invasion and vessel remodeling of developing placenta is suggested by a number of experimental findings. First, NO release is coupled to VEGF and hepatocyte growth factor (HGF)-induced trophoblast invasion and motility.\(^36,37\) Second, in trophoblast cells, NO upregulates the expression and the activity of the matrix-degrading proteases MMP-2 and MMP-9, which are required for invasion during embryo implantation.\(^38\) Finally, NO causes dilation of the uteroplacental arteries, which is another prerequisite for trophoblast invasion and remodeling of the endothelium.\(^1\) It is tempting to speculate that low NO availability could contribute to impaired cytotrophoblast invasion in preeclampsia, a hypothesis that should be formally tested in developing placenta obtained before 20 weeks of gestation. However, the present study, as most previously published reports on preeclamptic placenta\(^10,18,19\) have been necessarily performed at the time of disease onset, usually in the third trimester of pregnancy, represents a limitation that cannot be easily overcome.

In conclusion, our findings indicate that normal placenta with enough tissue L-arginine sustains adequate generation of NO by eNOS. By contrast, in preeclampsia, when the placental L-arginine concentration is low because of excessive arginase II expression, activation of eNOS leads to excessively high generation of superoxide anion, which reduces NO half-life by forming peroxynitrite. This may promote microvascular oxidative damage and favor abnormal placenta perfusion.

**Perspectives**

The present findings provide a rationale for clinical trials with L-arginine or antioxidant supplementation\(^39\) that, either by providing more substrate to eNOS or by lowering the rate of NO degradation to peroxynitrite, could help prevent this disease, which remains one of the leading causes of maternal and fetal morbidity and mortality.

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Dr Paola Cassis is a recipient of a fellowship from Associazione Ricerca Malattie Rare (ARMR, Bergamo) through the generosity of Banco di Brescia. Dr Francesca Porrati received a fellowship in memory of Libera Dossi Grana. This work has been partially supported by a grant from Farmaceutici Damor S.p.A, Napoli, Italy.

**References**


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Figure I

Figure I
ONLINE DATA SUPPLEMENT

In Preeclampsia L-arginine Depletion Orients Nitric Oxide Synthase Toward Oxidant Species

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Short title: arginine and peroxynitrite in preeclampsia

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Tissue collection and histology

Placentas were obtained following vaginal delivery (5 with normotensive pregnancy, 1 with preeclampsia) or during cesarian delivery (8 with NP, 10 with preeclampsia). Tissue blocks were taken from the fetal and maternal surfaces of the placenta to include umbilical cord, chorionic villi (stem and terminal villi) and decidua. Some blocks were snap-frozen in liquid nitrogen and stored at –80°C for NADPH diaphorase, conjugated diene, Western blot and real-time RT-PCR assays, NOS activity, L-arginine levels, others were fixed with 10% formalin and paraffin-embedded for immunoperoxidase and conventional histological examination.

For NADPH diaphorase staining, sections were counterstained with methyl green. Negative controls were run without NADPH or in the presence of the NO synthesis inhibitor DPI,\(^1\) reactivity was totally NADPH-dependent and was abolished by DPI.

For immunoperoxidase, mouse monoclonal antibodies against the following antigens: human-ecNOS (Transduction Laboratories, Exeter, UK),\(^1\) nitrotyrosine (Upstate Biotechnology Inc, Lake Placid, NY),\(^2\) human-CuZn-SOD (Sigma Chemicals Co, St Louis, MO) and 4-hydroxynonenal-(4-HNE)-lysine adduct (NA59, kindly provided by Dr Witzum, The Scripps Research Institute, La Jolla, CA),\(^3\) and a rabbit polyclonal antibody against human-arginase II (Santa Cruz Biotechnology, Inc., USA)\(^4\) were used.

After blocking aspecificities with non-immune horse serum (for ecNOS, nitrotyrosine, CuZn-SOD, 4-HNE-lysine) or with normal goat serum (for arginase II), slides were incubated overnight at 4°C with the primary antibody (anti-ecNOS 1:150, anti-nitrotyrosine 1:300, anti-CuZn-SOD 1:500, anti-4-HNE-lysine 1:500, anti-arginase II 1:25) in PBS/1%-BSA, followed by the biotinylated horse anti-mouse IgG or goat anti-rabbit IgG, ABC solution (Vector Laboratories, Burlingame, CA), and developed with
diaminobenzidine (for ecNOS) or diaminobenzidine-Nickel (for nitrotyrosine, CuZn-SOD, 4-HNE-lysine and arginase II). For ecNOS, sections were incubated before adding the ABC solution with a monoclonal antibody anti-biotin. The sections were counterstained with Harris hematoxylin. Negative controls included the omission of the primary antibody and replacement with a non-immune antibody on a second section on the same slide. Each experiment randomly included samples from normotensive pregnancy and from preeclampsia. In preliminary experiments anti-nitrotyrosine antibody at 1:300 dilution brightly stained a monolayer of human microvascular endothelial cells (HMEC) pre-incubated with 200 µmol/L peroxynitrite, while no signal was found on HMEC incubated with vehicle (not shown).

Multiple sections from each patient were examined. Each section was scored for intensity of immunostaining (absent, faint, moderate, intense: 0 through 3). At least 8-10 fields per section were examined. The final score (S) per section was calculated as a weighted mean:

\[ S = \frac{N_0 \times 0 + N_1 \times 1 + N_2 \times 2 + N_3 \times 3}{N_0 + N_1 + N_2 + N_3} \]

where \((N_0+N_1+N_2+N_3)\) is the number of villi or of decidua vessels in each category. Two pathologists blinded to the nature of the experimental groups assigned the scores, and the mean of the two assessments was calculated.
Western blot

For Western blotting analysis the frozen placental tissues were pulvarizated and resuspended in 1 mL lysis buffer (50 mmol/L *-glicerolphosphate, 2 mmol/L MgCl₂, 1 mmol/L EGTA, 0.5% Triton X-100, 0.5% NP-40, 1 mmol/L DTT, 100 mL protease inhibitor cocktail) and sonicated. The whole lysate was stored at -80°C. Protein concentration was determined by using the Bradford method (Biorad). The proteins (40 mg for each lane from either normal or preeclamptic placentas) were separated on denaturating sodium dodecyl sulfate 10% polyacrylamide gel by electrophoresis and then blotted on nitrocellulose membrane by wet electroblotting for 90 minutes. Blots were blocked overnight at 4°C with 3% BSA, 1% goat serum in PBS at pH 7.3 (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 1.8 KH₂PO₄, 0.05% Tween 20) and then incubated for 2.5 hours with a rabbit polyclonal antibody directed against human arginase II (1:200, Santa Cruz Biotechnology) followed by the secondary antibody (HRP-conjugated goat anti-rabbit IgG 1:30000, Vector Laboratories). Antibody binding was visualized by the enhanced chemiluminescence detection system (ECL).

NOS activity in placenta tissue

Placental tissue (3-4 g) for NOS activity was finely minced and homogenized in buffer as previously described.⁵ The homogenate (about 20 mg protein each) was incubated at 37°C for 24 hours with 1.2 mL Hank’s buffer containing 2 mmol/L NADPH (Sigma Chemical Co, St Louis, MO), 0.45 mmol/L Ca²⁺ (1 *mol/L free calcium), 200 *mol/L L-arginine (12 nmol L-arginine/mg protein) and 0.5 *Ci [³H]L-arginine.⁵ After
centrifugation the supernatant was extracted with 15%TCA (1:1, vol:vol) to evaluate the conversion of [3H]L-arginine to [3H]L-citrulline.¹

**Measurement of L-arginine levels in placenta tissue**

A portion (0.2-0.3 g) of villi or decidua was washed in 0.9% saline buffer. Tissue was weighted and homogenized with buffer containing sucrose 0.25 mol/L, Tris 50 mmol/L, EDTA 1 mmol/L, aprotinine 2 *g/mL, PMSF 1 *g/mL, STI 5 *g/mL, DTT 80 *g/mL, pH 7. After centrifugation at 11000 rpm for 5 minutes at 4°C the supernatants were used to measure levels of L-arginine as previously described.⁶

All results were corrected for mg protein concentration in each sample.

**Measurement of NO₂⁻/NO₃⁻ plasma levels**

NO₂⁻/NO₃⁻ plasma levels were measured using HPLC¹ (MN System Gold, Beckman Instruments Inc, Berkeley, CA). Briefly, plasma samples were treated with zincum sulphate (60 *mol/L, final concentration) and centrifuged to eliminate proteins. Supernatants were eluted onto a Dowex AG50WX-8 column followed by a cadmium column, which catalysed the reduction of nitrate to nitrite (eluent: borate buffer, pH=8.5). The post-column eluate reacted with Griess reagent (1% sulphanilic acid in 5% H₃PO₄, 0.1% n-[1-naphthyl]-ethylenediamine, vol:vol) (Sigma Chemical Co, St Louis, MO) to form a purple azo dye and the color was detected at * 540nm.

**Real time PCR**

The amplification profile consisted of 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles of 95°C for 15 minutes, then 60°C for 1 minute. All samples were run in
triplicate. To assess the overall cDNA content, *-actin served as housekeeping gene. The following oligonucleotide primers were used: human CAT-1 (200 nmol/L): 5’-ACACATCAGGCGTGCTTGTGTT-3’ and (5’-AACCCGAAGGCGATGAATC-3’; human 4F2hc (100 nmol/L): 5’-TGAGATTGGCCTGGATCCA-3’ and 5’-CATGACTGGAGCCTCCATAGG-3’; human LAT-1 (600nmol/L): 5’-TCGTTGCAGGCATTGTAGACT-3’ and 5’-GATGAACCCTTCAAAAGGATTTCTCA-3’; human arginase II (100 nmol/L): 5’-GAAGAAATCCGTCCACTCCG-3’ and 5’-GGACCATGCTCCACTCCTTTT-3’; human *-actin (50 nmol/L): 5’-TCACCCACACTGTGCCCATCTACGA-3’ and 5’-CAGCGAACCCTCATGCAATGG-3’.
Similar amplification efficiencies for targets and housekeeping genes were demonstrated by analysing serial cDNA dilutions, showing an absolute value of the slope of log input cDNA amount versus *Ct (Ct target - Ct housekeeping gene) of <0.1. Thus, we used the **Ct technique (user bulletin # 2, PE Applied Biosystems and reference7) to calculate cDNA content in each sample using the cDNA expression in human umbilical vein endothelial cells (HUVECs, for CAT-1 and 4F2hc and LAT-1) or in peripheral blood mononuclear cells (PBMC, for arginase II) as reference (calibrator). Melting temperature analysis evidenced no primer dimers or aspecific amplification products. Controls consisting of ddH2O or RT- RNAs were negative for targets and housekeeping.

Results

Placental NADPH-diaphorase staining, NOS activity and ecNOS expression and localization are comparable in NP and in preeclampsia
Intense diaphorase staining, which detects catalytic NOS activity irrespective of the enzyme isoform, was evident in the chorionic villi of placentas from NP, mainly in the syncytiotrophoblast (Figure IA and B). Staining was very faint in the decidua (Figure IA). The intensity and distribution of staining in villous tissue (Figure IC) and in the decidua (Table 2) were similar in NP and in preeclampsia. Since NADPH-diaphorase activity is not specific for NOS and enzyme involved in sex steroid metabolism are also diaphorase enzymes, NOS activity was also evaluated by measuring the conversion of $^3$HL-arginine to $^3$HL-citrulline in placental tissue homogenate in the presence of 4 to 5 fold excess of the substrate L-arginine. Results shown in Figure ID are in line with the diaphorase staining and document comparable NOS activity in placental tissue from NP and preeclampsia.

In villous tissue ecNOS staining was intense throughout the syncytiotrophoblast in NP and in preeclamptic women (Figure 1G and F). Moderate specific ecNOS staining was also found in villous vascular endothelium (Figure 1G). No significant differences in intensity and localization were seen in either villous tissue or decidua vessels from the placentas from NP and preeclampsia (Figure 1F and Table 2). EcNOS was only slightly reduced in the endothelium of preeclamptic villous vessels, probably depending on the reduced number of viable cells.

**Legend to Figure I:**

panels A-C: Representative photomicrographs of NADPH-diaphorase staining (blue colour) of placenta tissue from normotensive (A and B) and preeclamptic women (C). No difference in intensity and distribution of NADPH-diaphorase staining can be observed between normotensive pregnancy and preeclampsia. (v): villi, (d): decidua. Original magnification: A, 100x; B and C, 400x.
(panel D): Formation of [³H]L-citrulline from [³H]L-arginine in homogenate placental tissue from normotensive pregnancy (n=9) and preeclamptic women (n=6). Data are mean±SEM.
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Figure I


D

- normotensive pregnancy
- preeclampsia

Figure I