Increased L-Citrulline/L-Arginine Plasma Ratio in Severe Preeclampsia

CHIARA BENEDETTO, MD, PhD, LUCA MAROZIO, MD, ISABELLA NERI, MD, MAURIZIO GIAROLA, MD, ANNIBALE VOLPE, MD, AND FABIO FACCHINETTI, MD

Objective: **To evaluate nitric oxide (NO) production in patients with pregnancy-induced hypertension or preeclampsia and in controls.**

Methods: **Four groups of pregnant women were included: 17 patients with pregnancy-induced hypertension, ten with mild or moderate preeclampsia, 17 with severe preeclampsia, and 44 normotensive women matched for weeks of gestation at blood sampling with the cases. Plasma levels of L-citrulline and L-arginine were measured by using high-performance liquid chromatography.**

Results: **The mean plasma levels of L-citrulline and the ratio of L-citrulline to L-arginine, which reflects NO production, were higher in women with severe preeclampsia than in controls, patients with pregnancy-induced hypertension, and patients with mild or moderate preeclampsia.**

Conclusion: **Nitric oxide production is enhanced in severe preeclampsia, possibly as a compensatory phenomenon for the increased synthesis and release of vasoconstrictors and platelet-aggregating agents. (Obstet Gynecol 2000;96:395–9.**

Nitric oxide (NO) is released by the vascular endothelium and controls blood pressure (BP) by direct vasodilatory action and by blunting the responsiveness to vasoconstrictors. Moreover, it is produced in the platelets, where it inhibits aggregation through the second messenger cyclic guanosine monophosphate (cGMP).[1] Because of such properties, it has been hypothesized that derangement of the NO pathway might be involved in the pathogenesis of preeclampsia.[2]

Animal experiments have shown that administration of NO-synthetase inhibitors to rats produces signs similar to those of preeclampsia, such as sustained hypertension, proteinuria, thrombocytopenia, and fetal growth restriction (FGR).[3–6] Furthermore, these effects are reversed by administration of L-arginine substrate of NO; or sodium nitroprusside, an NO donor that increases the endogenous production of NO.[5,7–9]

Nitric oxide is extremely labile and cannot be measured directly because it rapidly undergoes oxidation to the inorganic end-products nitrite and nitrate.[1] In humans, indirect evaluation of NO production through measurement of nitrite, nitrate, and cGMP levels in plasma and urine samples during pregnancy has produced conflicting results.[10–19] NO production was found to be higher, lower, or unchanged in preeclamptic women compared with normotensive pregnant women. Such discrepancies could be due to different assays; patient heterogeneity; or confounding factors, such as dietary intake, infections, cigarette smoking, alcohol consumption, atmospheric pollution, and exercise.[1] Another possible explanation is that measurement of NO metabolites (nitrite and nitrate) or second messenger (cGMP) is not a reliable method to assess NO production and metabolism.

L-Citrulline is the stoichiometric metabolite resulting from the conversion of L-arginine to NO. Measurement of L-citrulline may be the most specific and reliable method to assess NO synthetase activity in vivo. Such conversion has proven to be consistent with the evaluation of mRNA of NO synthetase, both in platelet preparation[2] and placental tissue.[20] It has been observed that L-citrulline production after L-arginine loading in preeclamptic patients is smaller than in normotensive pregnant women.[21]

To verify the hypothesized derangement of the NO pathway in preeclampsia, we measured plasma levels of L-citrulline and L-arginine in normotensive pregnant women and in patients with pregnancy-induced hypertension or preeclampsia.

From the Department of Gynaecology and Obstetrics, University of Turin, Turin, and the Department of Gynaecology, Obstetrics and Pediatrics Sciences, University of Modena, Modena and Reggio Emilia, Italy.

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Materials and Methods

We studied four groups of subjects with singleton pregnancy at 22–40 weeks' gestation: 44 normotensive pregnant women, 17 patients with pregnancy-induced hypertension, ten patients with mild or moderate preeclampsia, and 17 patients with severe preeclampsia.

All patients with pregnancy-induced hypertension or preeclampsia were seen consecutively over a 6-month period and were selected from women referred to the antenatal clinics and obstetric units of the Departments of Gynecology and Obstetrics, University Hospitals of Turin and Modena, Italy.

Blood samples were obtained at the time of diagnosis, and controls were matched for gestational age at the time of sampling. Only one blood sample was taken from each patient. No patient was in labor at the time of blood sampling. The study was approved by the Local Ethical Committee. Informed consent was obtained from each patient before inclusion in the study.

We assigned patients to the pregnancy-induced hypertension group or the preeclampsia group by using the criteria reported by Davey and MacGillivray.[22] According to these criteria, pregnancy-induced hypertension was diagnosed when two consecutive measurements (obtained 4 hours or more apart) of diastolic BP equal 90 mmHg or greater were found after the 20th week of pregnancy in a previously normotensive woman. Preeclampsia was diagnosed when two consecutive measurements of diastolic BP 90 mmHg or greater and proteinuria 300 mg or greater in one 24-hour urine collection were found after the 20th week of pregnancy in a previously normotensive and nonproteinuric woman. Severe preeclampsia was diagnosed in the presence of at least two of the following findings: diastolic BP 110 mmHg or greater, proteinuria more than 1 g/24 hours, fetal growth restriction, birthweight less than the tenth centile, or delivery before 34 weeks. According to the criteria currently in use in our departments, preeclampsia was considered to be severe also in the presence of the hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome, disseminated intravascular coagulation, or intrauterine fetal death, even if diastolic BP was less than 110 mmHg. The assignment of patients to each group was reevaluated at the end of pregnancy to confirm the diagnosis.

Blood was obtained by venipuncture and centrifuged; serum was stored at −80C until assay. One mL of serum was added to 30 mg of sulfosalicylic acid powder (Sigma Chemical Co., St. Louis, MO), vortexed, and centrifuged at 2000*g* for 20 minutes at 4C. A fraction of clear supernatant was mixed with an equal volume of ophtaldialdeide for 1 minute. This fluorescent derivative was then injected into a high-performance liquid chromatography apparatus (Waters Inc., Millipore, MN) equipped with an RP C-18 column. Flow rate was adjusted to 1 mL/min. The mobile phase was constituted by a mixture of 0.012 M phosphate buffer with acetonitrile and methanol (91:4:5), pH 5.9. Citrulline and arginine have a retention time of 6.8 and 10.5 minutes, respectively.

A pool of serum from pregnant women served as external standard and was tested over 6 months. Citrulline levels were 32.7 μmol/L and arginine levels were 97.1 μmol/L. Interassay coefficients of variation were 5.4% and 5.8%, respectively.

Statistical analysis was performed by using the Statistical Package for Social Science software (SPSS Inc., Chicago, IL), and data were analyzed for significant differences by using the Student–Newman-Keuls test. Differences were considered significant if $P < .05$. The linear regression equation was applied to correlate the plasma levels of L-citrulline and L-arginine with BP values, proteinuria, and birth weight.

Results

Age, parity, and pregnancy outcome of the study patients are shown in Table 1. Mean (± SE) levels of L-citrulline and L-arginine in each group of pregnant women are shown in Table 2. No differences were observed in mean gestational age at the time of blood sampling (controls, 30 ± 0.9 weeks; pregnancy-induced hypertension group, 31 ± 1.3 weeks; mild or moderate preeclampsia group, 30 ± 1.6 weeks; severe preeclampsia group, 28 ± 1.4 weeks).

The mean concentrations of L-citrulline were significantly higher in patients with severe preeclampsia than in controls, and patients with pregnancy-induced hypertension, or those with mild or moderate preeclampsia ($P < .05$). In contrast, concentrations of L-arginine were lower, although not significantly so, in patients with severe preeclampsia than in the other three groups. Therefore, the mean L-citrulline/L-arginine ratio, which reflects NO production, was significantly ($P < .05$) higher in patients with severe preeclampsia (0.59 ± 0.08) than in controls (0.30 ± 0.02), patients with pregnancy-induced hypertension (0.31 ± 0.06), or those with mild or moderate preeclampsia (0.30 ± 0.02). Individual values of the L-citrulline/L-arginine ratio for each group are reported in Figure 1.

No correlations were found between plasma levels of L-citrulline or L-arginine or the L-citrulline/L-arginine ratio and maternal BP values, proteinuria at 24 hours, or birth weight within each group.

Table 1. Age, Parity, and Pregnancy Outcome

	Age (y, mean ± SEM) (range)	Nulliparas *n* (%)	Weeks at delivery (mean ± SEM) (range)	Birth weight (g, mean ± SEM) (range)
Controls (n = 44)	30.2 ± 0.5 (24–37)	17 (39%)	38.9 ± 0.2 (36–41)	3213 ± 47 2370–3860
Pregnancy-induced hypertension (n = 17)	30.8 ± 1.0 (22–38)	8 (47%)	38.1 ± 0.4 (34–40)	2954 ± 135 1685–3910
Mild or moderate preeclampsia (n = 10)	29.3 ± 1.2 (23–36)	9 (90%)	35.9 ± 0.7* (31–39)	2448 ± 172* 1340–3150
Severe preeclampsia (n = 17)	30.8 ± 1.3 (24–42)	15 (88%)	30.9 ± 1.0† (25–39)	1342 ± 159† 580–3050

SEM = standard error of the mean.
* $P < .05$ compared with controls and patients with pregnancy-induced hypertension.
† $P < .05$ compared with controls, patients with pregnancy-induced hypertension, and patients with mild or moderate preeclampsia.

Discussion

At present, no consensus exists on the role of NO production in normotensive and hypertensive pregnant women, because the concentrations of NO metabolites or second messenger cGMP in plasma and urine have been reported to be increased, reduced, or unchanged in preeclamptic subjects when compared with controls.[10–19]

Our results show that plasma levels of L-citrulline and the L-citrulline/L-arginine ratio values, which reflect NO production, are significantly higher in women with severe preeclampsia than in normotensive pregnant women, patients with pregnancy-induced hypertension, or patients with mild or moderate preeclampsia. Because the decrease in L-arginine plasma levels mirrors the increase in L-citrulline, we can exclude the possibility that L-citrulline may be derived from other sources, such as the urea cycle. It should also be considered that L-citrulline could be reconverted to L-arginine, although the reduced production of L-citrulline that occurs with L-arginine loading in less severe cases of preeclampsia limits such a possibility.[21]

Of note, however, a trend toward reduced L-arginine levels in preeclamptic patients was recently reported (Jerath V, Awoniyi L, Leslie KK. L-Arginine and nitric oxide levels in pregnancy: Possible correlation with preterm labor and preeclampsia [abstract]. J Soc Gynecol Investig 1999;6(Suppl 1):120). These observations suggest that NO production is enhanced in the most severe forms of preeclampsia, and they do not support the data of Seligman et al,[11] who found a reduction in nitrite plasma levels in preeclampsia and proposed that diminished NO synthesis may play a key role in the pathogenesis of the disease.

To reconcile the above-reported discrepancies, a stepwise hypothesis was made[23]: in preeclamptic women, reduced endothelial and platelet activity of NO occur early in pregnancy, representing one among several predisposing factors; vascular resistance increases in uteroplacental circulation and platelet aggregability is enhanced because of targeted reduction of NO release; and an increased NO production seems to be stimulated when overt preeclampsia develops. Such a finding could be interpreted as a compensatory phenomenon,

Table 2. Mean Values (± Standard Error of the Mean) of L-Citrulline and L-Arginine for Study Group

	L-Citrulline level μmol/L (mean ± SEM)	L-Arginine level μmol/L (mean ± SEM)
Controls (n = 44)	28.6 ± 1.2	107.9 ± 7.0
Pregnancy-induced hypertension (n = 17)	26.4 ± 1.8	105.5 ± 9.6
Mild or moderate preeclampsia (n = 10)	29.8 ± 2.4	102.3 ± 7.8
Severe preeclampsia (n = 17)	44.1 ± 4.1*	83.6 ± 7.5

SEM = standard error of the mean.
* $P < .05$ compared with controls, patients with pregnancy-induced hypertension, and patients with mild or moderate preeclampsia.

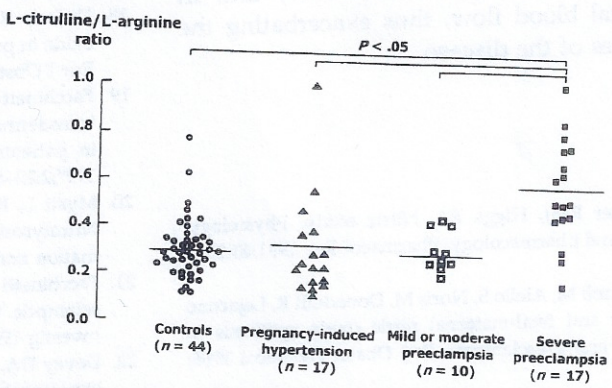

Figure 1. Individual values of L-citrulline/L-arginine ratio for each study group.

although it does not necessarily represent improvement in the clinical condition. Indeed, the abundance of NO in the presence of superoxide produces peroxynitrite, which further impairs vascular function.[20,24] Another possibility is that the NO pathway is upregulated by other metabolic systems, activated by hypoxia or endothelium damage, that are involved in the control of the maternal and fetoplacental circulation. For instance, hypoxia complicating preeclampsia may directly induce the expression and activity of endothelial NO synthetase in placental tissues, as is the case in cerebral endothelial cells during focal ischemia.[25] Furthermore, placental corticotropin-releasing hormone, levels of which are increased in the plasma of preeclamptic patients compared with controls,[26] has been demonstrated to stimulate NO synthesis.[27] For these reasons, the enhanced NO production in preeclampsia may be regarded as a secondary mechanism to maintain BP and peripheral perfusion within normal ranges, probably through interactions with other vasodilatory systems.

A more interesting hypothesis may be formulated if one considers the metabolic processing of NO after synthesis and release. Nitric oxide rapidly interacts with oxygen and is oxidized to the biologically inactive nitrite and nitrate.[1] Under physiologic conditions, this is the main pathway of NO inactivation. However, NO may also interact with superoxide to form peroxynitrate, which is a major cytotoxic agent produced during inflammation, sepsis, and ischemic injuries.[1] It has been suggested that the vascular endothelium can regulate the effects of NO by generating superoxide. An increase in superoxide production[28] and the vascular formation of peroxynitrite have been demonstrated in preeclampsia[29]; moreover, the generation of superoxide and peroxynitrite may be enhanced when neutrophils and macrophages are activated, as in the HELLP syndrome.[30] Therefore, it is likely that in the most severe forms of preeclampsia, the higher NO production, together with the increased levels of superoxide, may generate greater amounts of peroxynitrite, potentially leading to alterations in vascular reactivity and in systemic and local blood flow, thus exacerbating the pathologic features of the disease.

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Address reprint requests to:
Chiara Benedetto, MD, PhD
Department of Obstetrics and Gynecology
University of Turin
Via Baiardi 43
Turin 10126
Italy
E-mail: chbened@tin.it

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