System $y^+$ Arginine Transport and NO Production in Peripheral Blood Mononuclear Cells in Pregnancy and Preeclampsia

Nicola McCord, Paul Ayuk, Melanie McMahon, Richard C.A. Boyd, Ian Sargent, Christopher Redman

Abstract—Systemic inflammation and oxidative stress are features of normal pregnancy and, in excess, contribute to the pathogenesis of preeclampsia. Inflammatory cell activation stimulates uptake of arginine (the precursor for nitric oxide) by transport system $y^+$, expression of one of its genes (CAT-2) together with inducible nitric oxide synthase, leading to nitric oxide production. We investigated whether these changes occur in peripheral blood mononuclear cells in normal pregnancy and are exaggerated in preeclampsia. Samples from matched trios of nonpregnant, normal pregnant, and preeclamptic women were studied. Arginine transport was characterized, and the expression of inducible nitric oxide synthase and cell-specific nitric oxide production were measured. Arginine uptake by system $y^+$ was significantly increased ($P<0.001$) in peripheral blood mononuclear cells in normal pregnancy but not in preeclampsia. CAT-2 mRNA was not detected in cells from nonpregnant women but was detected in 3 of 10 normal pregnant and 8 of 10 of preeclamptic women ($P<0.001$). Inducible nitric oxide synthase protein expression was significantly increased in normal pregnant women ($P<0.05$) but not preeclamptic women. No significant differences in cell-specific nitric oxide production were observed. These changes confirm the predictions for normal pregnancy but not for preeclampsia in which, despite increases in CAT-2 expression, arginine uptake is not additionally increased. This may create a relative deficiency of arginine in PBMCs favoring superoxide and peroxynitrite production and contribute to oxidative and nitrosative stress in preeclampsia. (Hypertension. 2006;47:109-115.)

Key Words: arginine  nitric oxide  preeclampsia  pregnancy

Normal human pregnancy is associated with a maternal systemic inflammatory response.1,2 The response is exaggerated in preeclampsia,2 a potentially serious complication of pregnancy characterized by hypertension and proteinuria, which affects ≈3% to 4% of pregnant women. Activated inflammatory leukocytes produce free radicals including nitric oxide (NO) and superoxide ($O_2^-$).3,4 To what extent these can contribute to the systemic oxidative stress of both normal pregnancy or preeclampsia5 or the associated nitrosative stress (aberrant production of reactive nitrogen species that compromise the function of biomolecules via the nitration of critical amine and thiol residues) that is evident in preeclampsia6–8 is not known.

L-Arginine is the sole substrate for NO synthase (NOS), such that its availability governs the cellular production of NO.9 Although NOS enzymes are, in theory, saturated with arginine (the precursor for NO production),10 three genes (CAT-1, CAT-2, and CAT-3) encode for system $y^+$, of which CAT-1 and CAT-2 are well characterized in humans. CAT-1 is constitutively expressed, whereas CAT-2 is inducible and limited to activated inflammatory cells and the liver.10,11 Inflammatory stimulation induces both CAT-2 and inducible NOS (iNOS) in macrophages13 and endothelial cells,14 as well as other cell types in a coordinated response. In peripheral blood mononuclear cells (PBMCs) in nonpregnant subjects, transport systems $y^+$ and $y^+_L$ have been identified, contributing 13.7% and 86.3%, respectively, to total cationic amino acid transport.15 In sepsis, cationic amino acid (L-lysine) uptake has been dem-

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onstrated to be significantly increased in association with increased NO production. This has been demonstrated to be almost entirely because of increase in the activity of the \( y^- \) transporter (contribution to total uptake increased from 13.7% to 49.5%) with a corresponding induction of CAT-2 mRNA expression and no significant change in uptake by system \( y^- L \). In patients with chronic renal failure and uremia (a condition associated with increased circulation of proinflammatory cytokines), alterations in the \( L-\)arginine–NO pathway in PBMCs are characterized by a significant increase in system \( y^- \) activity with no change in system \( y^- L \) activity. Although a role for system \( y^- L \) in the regulation of the \( L-\)arginine–NO pathway in inflammatory cells in general, and PBMC in particular, has not been excluded, the available evidence is that inflammatory activation is associated with significant upregulation of system \( y^- \) activity with little or no change in system \( y^- L \) activity.

Given the evidence for inflammatory activation in normal pregnancy and preeclampsia, we hypothesized that normal pregnancy would be associated with activation of system \( y^- \) arginine transport, increased CAT-2 mRNA expression, increased iNOS expression, and NO production in maternal inflammatory cells, with an exaggeration of these responses in preeclampsia.

To test this hypothesis, we identified and characterized the system \( y^- \) transporter in PBMCs (inflammatory cells with the potential to produce free radicals including NO) in nonpregnant and normal pregnant women using kinetic and substrate inhibition studies. We then examined the activity of the \( y^- \) transporter, the expression of CAT-1 and CAT-2 mRNA and iNOS in PBMCs from nonpregnant women, normal pregnant women, and women with preeclampsia. Our data show evidence for inflammatory activation in normal third-trimester pregnancy. In preeclampsia, however, we report a disjunction between the activity of the system \( y^- \) transporter and the expression of CAT-1 and CAT-2 mRNA.

**Methods**

**Patients**

This study was approved by the Central Oxfordshire Research Ethics Committee, and all of the patients gave informed consent. Preeclampsia was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy. Hypertension was defined as new hypertension in the second half of pregnancy, with an exaggeration of these responses in preeclampsia.

**Preparation of PBMCs**

PBMCs are a mixed population of leukocytes comprising lymphocytes, monocytes and a few dendritic cells. They were isolated from 20 mL of heparinized blood using density gradient centrifugation. Cells were resuspended in Hanks’ balanced salt solution without Ca\(^{2+}\)/Mg\(^{2+}\)/phenol red at 15×10\(^6\) cells/mL. All of the reagents used were endotoxin-free.

**Transport Assays**

\( H^-\)arginine (final concentration 0.2 \( \mu \)mol/L, 53.4 Ci/mmol, NEN) uptake by PBMCs was measured by the method of rapid filtration. At time \( t=0 \), 100 \( \mu \)L of \( H^-\)arginine in Hanks’ balanced salt solution without Ca\(^{2+}\)/Mg\(^{2+}\)/phenol red at 37°C was added to 20-\( \mu \)L cell suspension. The mixture was incubated at 37°C for the desired time period after which uptake was terminated by the addition of 2 mL of ice-cold PBS. Cells were harvested by filtration through a 0.45-\( \mu \)m filter (Millipore) under vacuum and washed with 20 mL of ice-cold PBS. The filters were dissolved, and the number of disintegrations per minute counted over 5 minutes using a liquid scintillation counter (LS 5000CE, Beckman).

**Kinetic Analysis**

Kinetic studies were undertaken to identify the number of arginine transport systems that were functional and to characterize these systems based on their Michaelis constant (\( K_m \)) as described previously. The uptake of \( H^-\)arginine (0.2 \( \mu \)mol/L) was determined at 5 minutes (initial rate conditions), and the concentration of unlabeled L-arginine was 0.1 \( \mu \)mol/L to 1 mmol/L. Kinetic constants (\( K_m \) and maximum velocity (\( V_{max} \))) and uptake by passive diffusion (\( C_{iso} \)) were then determined.

**Identification of System \( y^- \)**

System \( y^- \) was identified from the \( K_m \) above and the presence of an \( L-\)glutamine-insensitive arginine transport system. \( H^-\)arginine (0.2 \( \mu \)mol/L) uptake was determined at 5 minutes in the presence of increasing concentrations of \( L-\)glutamine (0.1 \( \mu \)mol/L to 20 mmol/L). Data were analyzed using nonlinear regression with a 1-transport system model to detect a component of \( H^-\)arginine uptake that could not be inhibited by \( L-\)glutamine (\( C_{iso} \)). This component was compared with \( H^-\)arginine uptake by passive diffusion (\( C_{iso} \)) determined above. The concentration of \( L-\)glutamine that caused maximal inhibition of the glutamine-sensitive component was determined.

**System \( y^- \) 3H-Arginine Uptake in Pregnancy and Preeclampsia**

PBMCs were isolated from nonpregnant women, normal pregnant women in the second (13 to 24 weeks) and third (24 weeks to term) trimester, and from women with preeclampsia and matched controls.
Expression of CAT-1 and CAT-2 mRNA

Total RNA was extracted from PBMCs using the QIAmap RNA blood minikit (Qiagen) and treated with DNase. Two micrograms of RNA were reverse transcribed using random hexamer primers and MultiScribe reverse transcriptase (TaqMan Gold RT kit, Applied Biosystems). Real-time RT-PCR was performed in duplicate or triplicate using primers specifically designed for use in the TaqMan real-time PCR reaction (Table 2). Small variations in the starting quantity of cDNA in each sample were standardized by reference to the amplification of cDNA by primers and probes for the 18S component of ribosomal RNA (the expression of 11 housekeeping genes had been examined in preliminary experiments). The identity of the PCR product was additionally confirmed by visualization on a 3% agarose gel.

Expression of iNOS and endothelial NOS and NO Production

Real time RT-PCR was performed as above with primers and probes for endothelial NOS (eNOS) and iNOS as shown in Table 2. To quantify iNOS protein expression, PBMCs from nonpregnant women, normal pregnant women, and women with preeclampsia were fixed in 4% paraformaldehyde for 10 minutes at 4°C followed by permeabilization with PBS/0.1% BSA/0.1% saponin for 20 minutes at 4°C. Cells were then incubated in FITC-conjugated anti-iNOS mouse monoclonal antibody (BD Transduction Laboratories; 1:50 dilution) or FITC-conjugated mouse anti-human immunoglobulin isotype 2a (negative control; Dako) for 1 hour on ice. NO production was measured using the DAF-FM DA fluorescent dye in medium containing PBS/0.1% BSA/1 mM-L-arginine (10 μmol/L)+ DAF-FM DA (10 μmol/L) and superoxide dismutase (SOD, 1000 U/mL). The NO inhibitor Nω-nitro-L-arginine (5 mM L-arginine) was added to negative controls. Analysis was performed by flow cytometry with EXPO 32 software (Beckman Coulter) with gating on the negative controls. Data are expressed as the mean channel brightness of the positively labeled cells.

Statistical Analysis

Comparisons among PBMCs from nonpregnant women, normal pregnant women, and women with preeclampsia were made by ANOVA with Dunn’s post-test correction (GraphPad Prism 2.01; *P<0.05, **P<0.01, ***P<0.001) with the exception of CAT-2 expression, where the χ² test was used. Data are presented as mean±SEM.

Results

Kinetic Analysis

This analysis enabled an assessment of the number of transport systems and their characterization based on the $K_m$. In PBMCs from non-pregnant women, data were consistent with a 1 transport-system model with $K_m$ at 2.8 μmol/L and $V_{max}$ at 30.2 pmol/10⁷ cells per 5 minutes (please see online at http://hyper.ahajournals.org). In samples from normal pregnant women in the third trimester, data were consistent with 2 transport systems being active ($P=0.0003$, F test). One system had kinetic parameters similar to the system identified in PBMCs from nonpregnant women: $K_m$ at 2.1 μmol/L and $V_{max}$ at 33.9 pmol/10⁷ cells per 5 minutes. The high affinity of this system is suggestive of systems y’L or b⁰⁺. The second system had a higher $K_m$ consistent with system y⁺ activity: $K_m$ at 46 μmol/L and $V_{max}$ at 81.8 pmol/10⁷ cells per 5 minutes (please see online at http://hyper.ahajournals.org). L-arginine uptake by passive diffusion $C_{diff}$ was not significantly different in PBMCs from nonpregnant (0.064±0.029 pmol/10⁷ cells per 5 minutes) and normal pregnant women (0.01±0.015 pmol/10⁷ cells per 5 minutes).

Identification of System y⁺

Having provisionally identified the transport systems based on the $K_m$, we used substrate inhibition studies to confirm their identity. System y⁺ is not sensitive to glutamine inhibition, whereas systems B⁰⁺, y’L, and b⁰⁺ are inhibited. In nonpregnant women, glutamine-insensitive L-arginine uptake ($C_{arg}=0.079±0.023$ pmol/10⁷ cells per 5 minutes; Figure 1) was not significantly different from uptake by passive diffusion ($C_{diff}=0.064±0.029$ pmol/10⁷ cells per 5 minutes) indicating that system y⁺ was not a major contributor to arginine uptake. In third-trimester normal pregnant women, $C_{arg}$ was significantly greater than $C_{diff}$ (0.54±0.026 pmol/10⁷).
cells per 5 minutes and 0.01±0.015 pmol/10^7 cells per 5 minutes, respectively; P<0.001), demonstrating that ≥2 transport systems were active, one of which was glutamine insensitive (system y⁺). PBMCs of third-trimester pregnant women, therefore, differed from those of nonpregnant women by the presence of a transporter with characteristics of system y⁺. The time course of the acquisition of system y⁺ was investigated by examining normal pregnant women in the second trimester. Unlike the activity of system y⁺ in third-trimester samples, second-trimester samples had similar activity to samples from nonpregnant women (Figure 2A).

**System y⁺ ³H-Arginine Uptake in Pregnancy and Preeclampsia**

We investigated the hypothesis that system y⁺ activity was additionally increased in preeclampsia. In third-trimester pregnancies complicated by preeclampsia, system y⁺ arginine uptake was lower than in normal pregnancy and not significantly increased when compared with nonpregnant controls (Figure 2B).

**Expression of CAT-1 and CAT-2 mRNA**

From our hypothesis, we expected CAT-2 mRNA expression to be increased in third-trimester pregnancy and more so in preeclampsia. CAT-1 mRNA was detected in all of the samples studied. The ratio of CAT-1 mRNA to 18S mRNA was not significantly different in nonpregnant (1.23±0.18), preeclamptic (0.92±0.06), or normal pregnant controls (1.01±0.10; P>0.05; n=10). In contrast, CAT-2 mRNA was not detected in any of the samples from nonpregnant women but was detected in 8 of 10 samples from preeclamptic women and 3 of 10 normal pregnant controls (P=0.0009; χ² test). The ratio of CAT-2 mRNA to 18S mRNA was, however, small (0.0004±6×10⁻⁵) in preeclamptic women in comparison to CAT-1 mRNA.

**Expression of iNOS, eNOS, and NO Production**

We tested whether iNOS mRNA and protein expression and NO production were increased in PBMCs in preeclampsia. There were no significant differences in iNOS or eNOS mRNA expression in PBMCs in normal pregnancy and preeclampsia (Figure 3A and 3B). iNOS protein expression was significantly increased in PBMCs in normal pregnancy (P<0.05) but not in preeclampsia (Figure 3C). There was, however, no significant difference in NO production (data supplement, available online).

**Discussion**

In inflammatory cells, synthesis of NO via the L-arginine–iNOS pathway depends entirely on de novo L-arginine uptake by system y⁺, and is not dependent on transport by any of the other cationic amino acid transport systems, such as y⁺L or b⁰⁰⁰⁰. Therefore, in this study, we focused on the activity and expression of the glutamine-insensitive system y⁺ transporter.

The transport system identified in PBMCs from nonpregnant women had a lower Kₘ (2.8 µmol/L) than that reported for system y⁺ and was glutamine sensitive. This is, therefore, not system y⁺. However, in normal third-trimester pregnancy, we detected 2 transport systems, 1 with a Kₘ similar to that in nonpregnant women and a second with the Kₘ consistent with system y⁺ (46 µmol/L). Part of this activity was glutamine insensitive, characteristic of system y⁺, whereas the component that was glutamine sensitive is likely to be y⁺L. We
conclude that normal third-trimester pregnancy is associated with induction of system y’ activity in maternal PBMCs, consistent with inflammatory activation. In preeclampsia, however, the increase in system y’ activity was less pronounced than in normal pregnancy, contrary to our initial hypothesis.

CAT 1 mRNA expression was unchanged in PBMCs from all 3 of the groups studied, but there was no detectable system y’ activity in PBMCs from nonpregnant women. This suggests that the mRNA may not be translated in nonpregnant women or, if it is, the functional protein is not localized to the cell membrane. The changes in system y’ activity that were seen in normal pregnancy are, therefore, a result of posttranscriptional mechanisms or alterations in other CAT genes. When inflammatory cells are activated and CAT 2 mRNA expression induced, the uptake of arginine increases.20 In this respect, the relationship between system y’ arginine uptake and CAT 2 mRNA expression observed in normal third-trimester pregnancy is consistent with inflammatory activation and confirms our initial hypothesis.

In preeclampsia, however, although CAT 2 mRNA expression was greater than in normal pregnancy whereas CAT 1 expression was unchanged, system y’ activity did not increase in parallel. Possible explanations for this observation include defects in the translation of CAT mRNA, in translocation of the protein to the cell membrane, or alteration to these proteins with impairment of transport function. Without suitable CAT antibodies, correlation of protein expression and localization with the real-time PCR data are not currently possible.

Normal third-trimester pregnancy, but not preeclampsia, was associated with increased iNOS protein expression in PBMCs as expected with inflammatory activation. However, NO production was unaffected. The experimental conditions used (100 μmol/L L-arginine, 1000 iu/mL SOD) allowed maximal NO production to be measured. SOD was included in the assay to scavenge O2− that would interfere with detection of NO so that any possible increase in O2 production in preeclampsia (eg, as a result of relative arginine deficiency caused by lower system y’ activity) would not have occurred. The increase in arginine availability in PBMCs in normal pregnancy may increase NO bioavailability by decreasing O2− production rather than increasing NO production per se.

Our data indicate an apparent dysregulation in the L-arginine–NO system of circulating leukocytes of preeclamptic women. Whereas CAT-2 is induced, the activity of the y’ transporter does not increase in parallel, and neither is iNOS protein increased to the same extent as in normal pregnancy or even to a greater extent, as would be expected. We speculate that these alterations in arginine transport would predispose to relative L-arginine deficiency and favor the production of O2− and peroxynitrite (ONOO−) by NOS, which are 2 potentially harmful free radicals.21,22 In endothelial cells, similar changes have been observed. Hypoxia inhibits L-arginine transport with no significant effect on CAT-1 mRNA expression or membrane protein levels.23,24 This effect is not reversible after a return to normoxia for 24 hours.24 There is evidence that the placental bed in preeclampsia is hypoxic,25 where inflammatory leukocytes in the hypoxic intervillous space may be exposed to damage of membrane CAT proteins that impairs function. Such leukocytes would also be exposed to oxidative stress, which could alter membrane transport proteins, by the NO derivative ONOO− (which reacts strongly with thiol and tyrosine residues). Thiol and tyrosine reagents impair the function of amino acid transporters26,27 as has been observed in pulmonary artery endothelial cells.27 In previous work, we have demonstrated that ONOO− impairs arginine uptake in the human placenta.28 Increased ONOO− production in the maternal compartment in preeclampsia29 is, therefore, a possible explanation for the changes in system y’ activity in PBMCs in preeclampsia. There is evidence for a defect in arginine metabolism in maternal platelets in preeclampsia, which is not corrected by extracellular L-arginine,29,30 suggesting alterations in L-arginine transport or metabolism by NOS. Total L-arginine uptake has, however, been reported to be increased in erythrocytes in preeclampsia.31 Platelets have system y’, whereas erythrocytes do not, so the latter finding is probably less relevant than the former, which is more in line with our own observations.
Our data also show an increase in glutamine-inhibitable arginine uptake in normal third-trimester pregnancy compared with nonpregnant controls, most likely because of system y‘L. Although this is of potential interest, it was not the focus of this study, because such transport does not deliver arginine to iNOS in inflammatory cells. It is of interest that such an increase was not observed in human sepsis, indicating differences that are apparently pregnancy specific. The substrate specificity for y‘L includes cationic amino acids and neutral amino acids in the presence of sodium. Dual-label efflux experiments have shown that this transporter mediates an exchange of neutral and cationic amino acids. It is also sodium sensitive, and transport of other amino acids will compete with arginine so that its net effect on arginine uptake is hard to predict. In these experiments, only arginine with or without glutamine was included in the uptake medium. The presence of other amino acids in vivo would change the effects of y‘L but not so much of y‘.

A role for system y‘ activity in the regulation of the l-arginine–NO pathway in PBMCs will have to be established before its impact on disease processes is investigated. We also cannot comment to what extent other potential sources of NO in the vascular compartment (maternal endothelium and placenta) contribute to total NO production or, indeed, to the oxidative stress discussed above. It should be noted that, in human sepsis, there is evidence that endothelium is a minor source, and it has been speculated that margined leukocytes may be more important.

PERSPECTIVES

The balance between NO, and O2·− and ONOO− production by NOS is determined by arginine availability and is shifted toward O2·− and ONOO− production when there is arginine deficiency. We report that normal third-trimester pregnancy is associated with an increase in system y‘ arginine uptake and iNOS protein expression in PBMCs, which is characteristic of inflammatory activation. In preeclampsia, however, although the changes in CAT-2 mRNA expression are consistent with enhanced inflammatory activation, system y‘ arginine uptake and iNOS protein expression are not increased as they are in normal pregnancy. We hypothesize that the changes in system y‘ activity in preeclampsia are secondary to damage to membrane protein by placental-bed hypoxia. The resultant relative deficiency of arginine would favor formation of O2·− and ONOO−. These data are consistent with the hypothesis that the features of preeclampsia can be explained by the consequences of relative deficiency of available NO (secondary to oxidative degradation) and an excess of peroxynitrite.

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I. Kinetic characteristics of $^3$H arginine uptake by PBMC from non-pregnant and normal third trimester pregnant women. In non-pregnant women, the best fit regression line was consistent with one transport system. In third trimester normal pregnancy, however, there was statistical evidence for two transport systems (the two transport system model fitted the data significantly better than the one system model, $p = 0.0003$).
II. NO production by PBMC in normal pregnancy and pre-eclampsia. There was no significant difference in NO production by PBMC from non-pregnant (n = 10), normal pregnant (n = 8) or pre-eclamptic (n = 8) pregnancies as assessed by the % of positively labelled cells or the mean channel brightness of the positively labelled cells (data not shown).