NADPH Oxidase Activity in Preeclampsia With Immortalized Lymphoblasts Used as Models

Virginia M. Lee, Paulene A. Quinn, Sonja C. Jennings, Leong L. Ng

Abstract—Upon activation, neutrophils release reactive oxygen species that are believed to contribute to the widespread manifestation of preeclampsia. Neutrophils have an NADPH oxidase enzyme that catalyzes the production of reactive oxygen species. Little is known about the manifestations of the activated response and the upstream signaling pathways that regulate this process in preeclampsia. It is hypothesized that genetic factors may contribute to the release of reactive oxygen species and consequently the pathophysiology of the disease. We used Epstein-Barr virus–immortalized lymphoblasts from third-trimester, preeclamptic, postpartum preeclamptic women and their respective control subjects to assess NADPH oxidase–mediated reactive oxygen species production by using luminol-derived chemiluminescence and dihydrorhodamine-123 fluorescence. There was no effect of pregnancy status on the lymphoblast phorbol ester–stimulated luminol chemiluminescence area under the curve. However, lymphoblasts from preeclamptic patients had significant elevation of the lymphoblast phorbol ester–stimulated luminol area under the curve (F statistic 10.922, P<0.002). Similar findings were evident with dihydrorhodamine-123. No differences were revealed between preeclamptic and control cells when measuring the abundance of the phox proteins using Western blotting. Studies with genistein and tyrphostin implicated tyrosine kinase–dependent mechanisms in the control of NADPH oxidase–associated increased reactive oxygen species production in preeclampsia. These data show that preeclampsia is associated with a predisposition to increased agonist-stimulated NADPH oxidase–mediated reactive oxygen species production. The enhancement of reactive oxygen species generation may be important in mediating the endothelial dysfunction seen in preeclampsia. (Hypertension. 2003;41:925-931.)

Key Words: preeclampsia ■ oxidative stress ■ lymphocytes ■ genetics ■ signal transduction

Activated neutrophils have been implicated in the pathophysiology of preeclampsia and may play a significant role in the diverse manifestations of the disease. Recent studies have demonstrated increased production of reactive oxygen species (ROS) associated with neutrophil activation in preeclampsia. According to one study, agonist-induced ROS production in neutrophils was significantly higher in preeclamptic subjects compared with normal nonpregnant and pregnant subjects. However, other studies have produced conflicting results concerning neutrophil ROS production in preeclampsia. Increased neutrophil ROS production may play a role in the vascular endothelial damage and dysfunction associated with preeclampsia. In addition to damaging endothelium directly through lipid peroxidation, ROS inhibit vascular relaxation in vivo and in vitro by nitric oxide. It is possible that ROS contributes to the prostaglandin imbalance characteristic of preeclampsia, since oxidative stress increases prostaglandin H synthase activity.

The mechanisms leading to increased ROS production is unclear. In support of plasma factors being implicated in neutrophil activation, preeclamptic serum potentiates the production of ROS in nonpregnant neutrophils, whereas serum from normal or nonpregnant women does not. However, other studies have been unable to identify any evidence of such a factor in serum from preeclamptic women. Alternatively, a genetic predisposition may be implicated in this neutrophil activation and subsequent ROS production. The NADPH oxidase enzyme is an important source of ROS and consists of membrane-bound flavocytochrome b as well as cytosolic components. NADPH oxidase is activated by a variety of agents including n-formyl-Met-Leu-Phe (fMLP) and phorbol-12-myristate-13-acetate (PMA). Regulation of this process is mediated by specific signal transduction pathways that initiate the translocation of several cytosolic proteins to the membrane, where the active NADPH oxidase is formed. The signal transduction pathways controlling NADPH oxidase activation are still poorly documented. There are protein kinase C (PKC) dependent and independent pathways of activation that are either calcium dependent or independent. There is now increasing evidence implicating the role of tyrosine phosphorylation as an integral part of the signaling pathway as tyrosine phosphorylation of several polypeptides is increased in neutrophils stimulated with fMLP and PMA.
Like neutrophils, Epstein-Barr virus (EBV)-immortalized lymphoblasts express all constituents of the NADPH oxidase complex necessary to generate ROS and may therefore be a useful model for study of NADPH oxidase activity and subsequent production of ROS in preeclampsia. Consequently, EBV-immortalized lymphoblasts may provide a robust and stable cell line model free of the in vivo environmental influences.

The current study aimed to examine NADPH oxidase–mediated ROS production in EBV-immortalized lymphoblasts from both third-trimester preeclamptic and postpartum preeclamptic women and their respective control subjects. This may be a first step toward the identification of genetic influences on NADPH oxidase–mediated ROS production in preeclampsia, since cells are removed from the influences of plasma factors in vivo. Furthermore, we examined the basis for differences or upregulation in the signal transduction pathways upstream of NADPH oxidase–mediated ROS production.

Methods

Patients

The study group consisted of 11 third-trimester preeclamptic women, 11 third-trimester normotensive pregnant control subjects, 12 postpartum preeclamptic women, and 8 postpartum normotensive control subjects. Power calculations suggested that we needed 11 pregnant subjects and 8 postpartum subjects from each group to demonstrate a standardized difference of 1.2 between the lymphoblast NADPH oxidase activity in preeclamptic patients compared with the normotensive control subjects, with a power of 90% at P<0.05. Eleven subjects in each group were needed to demonstrate a standardized difference of 1.2 between lymphoblasts phox subunit abundance in preeclamptic versus control subjects, with a power of 90% at P<0.05. The preeclamptic women were diagnosed according to the internationally accepted criteria of Davey and MacGillivray, which states a blood pressure of >140/90 and >300 mg of protein in a 24-hour collection of urine, and none had a family history of hypertension because this may have influenced the results. Control subjects were normotensive throughout gestation and none had a family history of preeclampsia, hypertension, or diabetes mellitus. The study was approved by the Leicester Ethics Committee, and the subjects used gave informed consent.

Materials

The horseradish peroxidase (HRP)-linked donkey anti-rabbit IgG, enhanced chemiluminescence reagent, and Hybond–C–supported nitrocellulose membrane were all obtained from Amersham International. Fetal calf serum was from Globepharm Ltd; cyclosporin A was from Sandoz Inc; SB203580 was from Smith Kline Beecham Pharmaceuticals; and Ro318220 was from Roche Ltd. The tissue culture medium TC199 and all other chemicals were obtained from Sigma Chemical Co. Antibodies to the phox components p22, p47, p67, and gp91 were raised in-house, and IgG fractions were used for Western blotting.

Lymphoblast Culture

Immortalized lymphoblast cell lines were established as described previously. Cell lines were fed every 2 to 3 days with RPMI supplemented with 10% FCS, 2 mmol/L glutamine, penicillin (200 U/L), and streptomycin (0.2 mg/L).

Luminol-Derived Chemiluminescence

Measurement of ROS production was performed in buffer A consisting of (in mmol/L) NaCl 140, KCl 5, MgSO4 0.8, CaCl2 1.8, glucose 5, and HEPES 15, pH 7.4. ROS production was measured according to the method of Allen and Loose. Briefly, 400 000 neutrophils were washed in luminescence medium containing 5 μmol/L luminol (5-amino-2,3-dihydro 1,4-phthalazinedione) and 6 U/mL HRP. The cells were then resuspended in 500 μL of luminescence solution, and the baseline was measured in a thermostatically controlled EG&G Berthold LB953 tube luminometer at 37°C. The respiratory burst was then stimulated with the use of 1 μmol/L PMA. The response was recorded over a period of 30 minutes. The luminescence was measured as the light emission of a single sample over a time period. The area under the curve (AUC) was taken to represent ROS when the oxidase was stimulated. Simpson’s rule was used to calculate the AUC in relative light units (RLU/sec). Coefficients of variation for the same individual on different days for PMA-stimulated luminol-derived chemiluminescence (LDCL) was 12.4%.

Dihydrorhodamine-123 Fluorescence

ROS was also determined with the use of fluorescence of rhodamine-123 produced from oxidation of dihydrorhodamine-123, as previously described. Briefly, 105 cells were incubated at 37°C in 200 μL buffer A within ELISA plate wells, and dihydrorhodamine-123 (final concentration, 1 μmol/L) was added. Fluorescence was measured on a plate reader with excitation set at 505 nm (emission, 534 nm). Cells were stimulated with 1 μmol/L PMA and incubated for 60 minutes at 37°C. The fluorescence of rhodamine-123 was determined after subtracion of signals from wells without PMA addition to correct for auto-oxidation of dihydrorhodamine-123.

NADPH Oxidase Protein Abundance

Lymphoblast NADPH oxidase protein abundance was analyzed with the use of Western blotting. Fifty micrograms of protein extract was resolved on SDS-polyacrylamide gels (between 7.5 and 15%) and transferred to supported nitrocellulose. Proteins were detected by incubating blots with 1 μg/mL of p22, p47, p67, or gp91 antibody in 5% Marvel in PBS–0.1% Tween-20 for 1 hour, as previously described. HRP-linked donkey anti-rabbit IgG (1:1500) was added to the blots for 1 hour. The bands of interest were visualized with the use of enhanced chemiluminescence methodology. Densities of bands were scanned with the use of a Bio-Rad densitometer.

Assessment of Signaling Pathways in NADPH Oxidase Activation

Lymphoblasts were incubated in buffer A with various inhibitors of signal transduction pathways to elucidate control mechanisms of enhanced NADPH oxidase activity. A table that is available as an online supplement (at http://www.hypertensionaha.org) illustrates the inhibitors, their concentrations, duration of incubation, and effects. Inhibitors were added and incubated at 37°C for the stated times before assaying the cells for LDCL as above.

Statistical Analysis

Differences in lymphoblast LDCL and rhodamine-123 fluorescence were tested with the use of the Univariate General Linear Model (GLM) procedure in SPSS, Version 11. Other comparisons were performed using the Student t test. Box-and-whisker plots were produced, with median and boxes representing the interquartile range. All data were tested for normality by means of the Anderson-Darling test. In the protein abundance experiments, all data were normalized to a mean value of 1.0 in the control group. Statistical analysis was then performed accordingly. Probability values <0.05 were considered significant.

Results

The clinical characteristics of the women are presented in the Table shown here. Figure 1A shows a typical luminol-derived trace showing both preeclamptic and normotensive cell lines. Figure 1B illustrates the results obtained from both the third-trimester and postpartum cell lines of normal and
preeclamptic women. The median preeclamptic measurement \((10^6)\) was 34.19 RLU.sec, compared with a median value of 13.58 RLU.sec for the normal control cells. Baseline measurements were assessed, and no significant differences were present between the 2 groups. The median value obtained for the postpartum preeclamptic group was 31.19 RLU.sec, compared with 21.69 RLU.sec obtained for the postpartum normotensive group. There were no differences in baseline measurements. With the use of the univariate GLM procedure, there was again no significant effect of pregnancy status on lymphoblast ROS production \((F_{statistic}, 1.945; P = 0.002)\). The pre-

### Clinical Data for the Third Trimester Preeclamptics, Third Trimester Normotensive Controls, Postpartum Preeclamptics, and Postpartum Normotensive Controls

<table>
<thead>
<tr>
<th>Clinical Data</th>
<th>PET (n=11)</th>
<th>NT (n=11)</th>
<th>ppPET (n=12)</th>
<th>ppNT (n=8)</th>
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<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mean</td>
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<td>30 (±1.7)</td>
<td>36 (±2.0)</td>
<td>36 (±1.6)</td>
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<td>Gestation at delivery, weeks(^{diss})</td>
<td>(34^{13} (±1.6))</td>
<td>(40^{1} (±0.3)^{*})</td>
<td>(34^{15} (±1.5))</td>
<td>(40^{12} (±0.3)^{†})</td>
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<td>(39–41^{14})</td>
<td>(28–40^{13})</td>
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<tr>
<td>Mean</td>
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<td>109/65 (±2.8)</td>
<td>116/71 (±2.0)</td>
<td>115/64 (±2.1)</td>
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<tr>
<td>Mean</td>
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<td>114/66 (±2.2)^{†}</td>
<td>174/114 (±5.1)^{‡}</td>
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<td>119/71 (±3.5)</td>
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<tr>
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<tr>
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<td>27–39^{11}</td>
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<tr>
<td>Months postpartum at time of sampling</td>
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<td>5</td>
<td>6</td>
<td>5</td>
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Values are presented as mean (±SEM) and range.

\(^{*}P=0.011, †P=0.0001\) for the normotensive (NT) vs preeclamptics (PET) by Student t test; \(‡P=0.002, §P=0.0001\) for postpartum normotensive (ppNT) vs postpartum preeclamptics (ppPET) by Student t test.

However, 10 \(\mu\)mol/L rotenone, a mitochondrial oxidase inhibitor, had no effect on lymphoblast LDCL AUC, suggesting that NADPH oxidase is actually the enzymatic source of the ROS measured \((P=NS, Figure 1C)\). Scavengers were used to confirm the specificity of the techniques. Superoxide dismutase (SOD), 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron), and catalase have been included to verify that luminal was measuring ROS production. The pharmacological scavenger of superoxide Tiron (10 mmol/L) completely abolished the response produced on stimulation with PMA \((P<0.001, Figure 1C)\). Treatment with SOD (100 U/mL) only reduced the response to PMA by \(\approx90\%\) \((P<0.001, Figure 1C)\). The luminal response was \(\approx70\%\) inhibited by catalase \((P<0.001, Figure 1C)\). This suggested that luminal was not completely specific for hydrogen peroxide and measured other reactive oxygen species in addition to hydrogen peroxide.

These LDCL results were confirmed with a different assay for ROS, fluorescence of rhodamine-123, which measures other reactive species in addition to hydrogen peroxide. However, with the use of the Univariate GLM procedure, there was again no significant effect of pregnancy status on lymphoblast ROS production \((F^{1C})\).
However, lymphoblasts from pre-eclamptic women had higher ROS production than normal women (Figure 1D; F statistic, 27.55; \( P < 0.001 \)).

We assessed whether any differences existed in the abundance of the membrane bound and cytosolic subcomponents of the oxidase enzyme. Figure 2A illustrates typical Western blots probed with antibodies against the different phox components. Figure 2B illustrates the results of the densitometric analysis of the blots. Values were normalized to a mean of 1.0 in the control group. No significant differences were seen in abundance of any of the phox components of the oxidase enzyme.

These blots were obtained from unstimulated cells. We also investigated the effect of PMA stimulation (1 \( \mu \text{mol/L} \)) on abundance of phox subunits before and after 30 minutes of stimulation at 37°C in buffer A. All data on Western blots were normalized to 1 in the control group (n=8). There was no significant change in abundance of any of the subunits investigated (densitometry for control resting cells, PMA-stimulated control cells, preeclamptic resting cells, PMA-stimulated preeclamptic cells, respectively for the different subunits:p47 phox: 1.00±0.24, 1.14±0.11, 1.19±0.24, 1.33±0.11; p67 phox: 1.00±0.16, 1.11±0.22, 1.04±0.16, 1.23±0.23; p22 phox: 1.00±0.23, 1.18±0.24, 1.04±0.23, 1.30±0.24; \( P = \text{N/S} \) for all comparisons using the repeated-measures GLM procedure).

Because the higher ROS production in cell lines derived from preeclamptic patients was not due to differences in phox component abundance, we investigated the upstream signaling pathways involved in NADPH oxidase regulation. Inhibitors and concentrations used are reported in the table published as an online supplement. Ro318220, a PKC inhib-
itor, inhibited NADPH oxidase–mediated ROS production completely after stimulation with PMA in both preeclamptic (10.03 ± 1.99 versus 0.001 ± 0.0007 10⁷ RLU/sec) and normotensive control subject (3.97 ± 1.56 versus 0.0007 ± 0.0002 10⁷ RLU/sec) cell lines. The effect of genistein on inhibition of NADPH oxidase activation with PMA is illustrated in Figure 3. Genistein inhibited NADPH oxidase mediated ROS production in both the preeclamptic (10.30 ± 2.82 versus 4.05 ± 1.24 10⁷ RLU/sec) and normotensive control group (2.77 ± 1.28 versus 0.77 ± 0.36 10⁷ RLU/sec). The difference between mean uninhibited and inhibited ROS values was only significant in the preeclamptic group using a paired Student’s t test (P = 0.020). The effect of tyrphostin A20 on inhibition of NADPH oxidase activation with PMA is illustrated in Figure 4. Mean uninhibited (control) values were compared with mean inhibited values in both groups by means of a paired Student’s t test. Tyrphostin A20 inhibited NADPH oxidase activation in both the preeclamptic (8.92 ± 2.59 versus 5.875 ± 2.257 10⁷ RLU/sec) and normotensive (2.03 ± 1.10 versus 0.604 ± 0.38 10⁷ RLU/sec) control groups. The difference between mean uninhibited and inhibited ROS values was only significant in the preeclamptic group (P = 0.007). The effect of MEK, p38 MAPK, and phosphatidylinositol-3-kinase inhibitors (PD98059, SB203580, and wortmannin respectively) on NADPH oxidase activation was analyzed. None had significant inhibitory effects on PMA-stimulated ROS production in either preeclamptic or normotensive cell lines (data not shown).

**Discussion**

There is increasing evidence to suggest endothelial cell damage and dysfunction in the pathogenesis of preeclampsia. The actual cause of this endothelial damage is unknown, although neutrophils, through their ability to produce ROS, have been implicated as likely candidates. Superoxide anions have been shown to inactivate nitric oxide and reduce the release of prostacyclin, thus favoring vasoconstriction. High concentrations of superoxide have been found to reorient the arachidonic acid pathway in cells toward the production of thromboxane A₂, which is a potent stimulator of vasoconstriction and platelet aggregation. The imbalance between prostacyclin and thromboxane A₂ is a well-documented observation in preeclampsia. Furthermore, superoxide anions can initiate lipid peroxidation, resulting in endothelial cell lysis. It seems plausible that the increased neutrophil ROS may be important in mediating the endothelial damage seen in preeclampsia.

EBV-immortalized lymphoblasts were used to examine the role of NADPH oxidase activity and ROS production as an intermediate phenotype in preeclampsia. Luminol was incor-
eclamptic cells are condition rapidly regresses after parturition. of a genetic influence, since preeclampsia as a clinical independent of any effect of pregnancy status, is suggestive on the NADPH oxidase of isolated neutrophils because they measured by 2 different techniques. This increased sensitivity may have been influenced by genetic factors, since culture of these lines in vitro eliminates the influence of environmental (plasma) factors. Such experiments could not be performed on the NADPH oxidase of isolated neutrophils because they do not remain viable with prolonged culture. In addition, the presence of this increased sensitivity of the NADPH oxidase, independent of any effect of pregnancy status, is suggestive of a genetic influence, since preeclampsia as a clinical condition rapidly regresses after parturition.

There is evidence in the literature to suggest that preeclamptic cells are “primed” by some factor in the plasma, rendering them more sensitive to agonist stimulation with increased ROS production. TNF-α, platelet-activating factor, and syncytiotrophoblast microvesicles are among the factors that may contribute. However, the results presented do not favor this hypothesis because the increased sensitivity persisted in EBV-immortalized lymphoblasts when the environmental (plasma) factors were removed. This suggests that women with preeclampsia may be genetically predisposed to have an NADPH oxidase that is more sensitive to agonist stimulation, similar to that of a “primed” cell. It is therefore postulated that the preeclamptic environment may contain the actual stimulus for this activation, but a predisposition exists. A recent study by Barden et al. supports this theory by suggesting that there may be some underlying genetic predisposition to increased oxidative stress. Their study does not suggest that a stable circulating factor causes cell activation in preeclampsia. However, they showed that lipid peroxidation is indeed elevated before and after delivery in women with preeclampsia.

Underlying mechanisms for this increased ROS production are unclear. There is an increased abundance of p22 phox in hypertensive lymphoblasts, which may account for the increased ROS production on PMA stimulation. However, we found no differences in any of the phox subunits between preeclamptic and normal lines. None of our patients had a family history of hypertension. Upstream signaling pathways were examined to elucidate upregulation that may have contributed to this increased ROS production. Using the inhibitor Ro318220, we confirmed that PMA-mediated stimulation of the respiratory burst was dependent on PKC activation. In contrast to findings in neutrophils, p38 MAPK was not implicated in the signal transduction pathways leading to NADPH oxidase activation in these lymphoblasts, since the inhibitor SB203580 did not have any effect on ROS production in either group of cell lines. The minor role of ERK 1/2 in PMA-stimulated activation as the respiratory burst was also confirmed in the current study, since the MEK inhibitor PD98059 had no significant effect on PMA-stimulated ROS production of lymphoblasts. A role for phosphatidylinositol-3-kinase was also excluded because the inhibitor wortmannin had no effect on the response. However, both genistein and tyrphostin, inhibitors of tyrosine kinases, partially blocked the production of ROS on activation with PMA in both sets of cell lines but to a significant degree only in the preeclamptic group. These findings suggested the importance of a tyrosine kinase regulatory pathway involved in enhanced PMA-stimulated NADPH oxidase activity associated with the preeclamptic phenotype. Similar findings have been reported in the priming of neutrophils by TNF-α. Utsumi et al. suggested that tyrosine kinase activity was involved in the “priming-linked” generation of superoxide. However, the role for a tyrosine phosphorylation step in the increased sensitivity and activation of the NADPH oxidase of cells from preeclamptic patients remains elusive. Tyrosine phosphorylation of several polypeptides is increased in neutrophils stimulated with fMLP and PMA.

In conclusion, this is the first report of an increased sensitivity of the NADPH oxidase enzyme to stimulation with PMA in preeclampsia with EBV-immortalized lymphoblasts used as models. Results were confirmed by using 2 different ROS measuring techniques. This increased sensitivity persisted in the postpartum preeclamptic cell lines and may have been due to genetic factors, since environmental (plasma) factors were removed. The increased sensitivity may contribute to the neutrophil activation in preeclampsia through its ability to produce substantially more ROS on agonist stimulation. The enhancement of ROS generation may be important in mediating the endothelial dysfunction seen in preeclampsia. In addition, tyrosine kinase–dependent mechanisms may be implicated in control of the NADPH oxidase–associated increased ROS production with preeclampsia.

Perspectives
There is an increased sensitivity of NADPH oxidase enzyme on agonist stimulation with PMA in EBV-immortalized lymphoblasts isolated from preeclamptic and postpartum preeclamptic women, a novel cell culture model for further investigation of this preeclamptic intermediate phenotype. This phenomenon is tyrosine kinase dependent, and its persistence in cultured cells suggests a genetic origin. However, some caution must be exercised when generalizing these findings to the majority of patients with preeclampsia. Critical areas for further investigation concern the identification of low abundance–specific proteins that could be tyrosine phosphorylated, the involvement of these proteins in the signal transduction pathways in preeclampsia, and their investigation as targets for pharmacological manipulation. Moreover, the predictive value of such an intermediate phenotype could be examined in the future by prospective clinical studies in pregnancy.

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