NADPH Oxidase Activity in Preeclampsia With Immortalized Lymphoblasts Used as Models

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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.

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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 in preeclamptic versus control subjects, with a power of 90% at a standardized difference of 1.2 between the lymphoblast NADPH oxidase activity and 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 chemiluminescence tracing 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 and their respective control subjects.
preeclamptic women. The median preeclamptic measurement (10⁶) 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 controls. There were no differences in baseline measurements. With the use of the univariate GLM procedure with the lymphoblast LDCL AUC as the dependent variable and presence of preeclampsia and pregnancy status as the between-subject factors, there was no significant effect of pregnancy status on the lymphoblast LDCL AUC (\(F\) statistic, 1.945; \(P=NS\)). However, lymphoblasts from preeclamptic women had a significant elevation of the LDCL AUC (\(F\) statistic, 10.922; \(P<0.002\)). The preeclamptic cells (third trimester and postpartum) produced significantly more ROS in response to PMA stimulation than the normal control cells. The lymphoblast LDCL AUC was abolished with 20 μmol/L diphenylene iodonium (DPI), a flavin protein inhibitor (\(P<0.001\), Figure 1C). However, 10 μmol/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 luminol 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 ≈90% (\(P<0.001\), Figure 1C). The luminol response was ≈70% inhibited by catalase (\(P<0.001\), Figure 1C). This suggested that luminol 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 both superoxide and hydrogen peroxide. 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; \(P=NS\)). However, lymphoblasts from preeclamptic women had a significant elevation of the LDCL AUC (\(F\) statistic, 10.922; \(P<0.002\)). The preeclamptic cells (third trimester and postpartum) produced significantly more ROS in response to PMA stimulation than the normal control cells. The lymphoblast LDCL AUC was abolished with 20 μmol/L diphenylene iodonium (DPI), a flavin protein inhibitor (\(P<0.001\), Figure 1C). However, 10 μmol/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 luminol 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 ≈90% (\(P<0.001\), Figure 1C). The luminol response was ≈70% inhibited by catalase (\(P<0.001\), Figure 1C). This suggested that luminol was not completely specific for hydrogen peroxide and measured other reactive oxygen species in addition to hydrogen peroxide.

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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 \(\mu\)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; \(P=N/S\) 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 10^7 RLU.sec) and normotensive control subject (3.97±1.56 versus 0.0007±0.0002 10^7 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 10^7 RLU.sec) and normotensive control group (2.77±1.28 versus 0.77±0.36 10^7 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 10^7 RLU.sec) and normotensive (2.03±1.10 versus 0.604±0.38 10^7 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.18 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.5,19 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.20 The imbalance between prostacyclin and thromboxane A2 is a well-documented observation in preeclampsia.21 Furthermore, superoxide anions can initiate lipid peroxidation, resulting in endothelial cell lysis.22 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-

**Figure 2.** A, Western blots of EBV-immortalized lymphoblast extracts obtained by loading equal amounts (50 μg) of protein and probed with specific antibodies. No difference was evident in the abundance of any of the phox proteins when comparing PET with NT controls (n=12). B, Protein abundance of NADPH oxidase subunits from EBV lymphoblasts of NT and PET subjects. Results represent mean values of 3 separate experiments on each cell line; SEM are plotted. No significant differences exist (unpaired Student t test, P–NS, n=12).

**Figure 3.** Effect of genistein (100 μmol/L) on ROS production. Results represent mean values of 3 separate experiments; SEM are plotted. Difference between mean uninhibited and inhibited ROS values was only significant in the preeclamptic group (P<0.02 by paired Student t test, n=11).

**Figure 4.** Effect of tyrphostin (100 μmol/L) on ROS production. Results represent mean values of 3 separate experiments; SEM are plotted. Difference between mean uninhibited and inhibited ROS values was only significant in the preeclamptic group (P<0.007 by paired Student t test, n=11).
porated as the chemiluminogenic probe with which to measure the response because lucigenin responses were not sensitive enough to measure the ROS produced. Responses were corroborated with another ROS-sensitive probe, rhodamine-123 fluorescence. Lymphoblasts do not have fMLP receptors, so PMA was chosen as the agonist.23 This study revealed increased agonist-induced ROS production in both preeclamptic and postpartum preeclamptic lymphoblast lines compared with respective normal controls. Pregnancy had no significant effect on lymphoblast ROS production 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.24 TNF-α, platelet-activating factor, and syncytiotrophoblast microvesicles are among the factors that may contribute.7,25,26 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 al27 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.27

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.17 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,28 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.10 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 al29 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 in cells from preeclamptic patients remains elusive. Tyrosine phosphorylation of several polypeptides is increased in neutrophils stimulated with fMLP and PMA.11,30

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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