Study of Plasma Factors Associated With Neutrophil Activation and Lipid Peroxidation in Preeclampsia

Anne Barden, Jackie Ritchie, Barry Walters, Constantine Michael, Jennifer Rivera, Trevor Mori, Kevin Croft, Lawrie Beilin

Abstract—Neutrophil activation occurs in women with preeclampsia and is resolved after delivery. The present study examined whether circulating factors in plasma of women with preeclampsia caused neutrophil activation and lipid peroxidation. Twenty-one women with proteinuric preeclampsia were matched for age and gestational age with 19 normal pregnant women. Plasma was collected from all subjects before delivery and at 6 weeks postpartum and incubated with autologous white-cell buffy coat collected at the postpartum visit. Neutrophil activation was assessed by level of CD11b and CD18 expression after incubation with autologous antepartum or postpartum plasma. Lipid peroxidation was assessed by measurement of F2-isoprostanes in plasma, plasma–white cell incubates, and urine. Neutrophil CD11b and CD18 expression was not differentially altered by incubation with plasma from either women with preeclampsia or normal pregnant women and was similar between groups when incubation was performed with plasma collected after delivery. In preeclampsia, plasma F2-isoprostanes were significantly increased before and after delivery compared with controls. Plasma F2-isoprostanes were increased 2-fold after incubation of plasma withuffy coat, but preeclamptic women had higher levels compared with those of controls when either pregnant or postpartum plasma was used. In pregnant preeclamptics, plasma F2-isoprostanes were positively correlated with lymphocyte count. Six weeks after delivery, plasma F2-isoprostanes in the preeclamptic women were significantly positively associated with lymphocyte count and cholesterol and negatively associated with albumin. In conclusion, the present study does not suggest that a stable circulating factor causes neutrophil activation in preeclampsia. However, lipid peroxidation is elevated before and after delivery in women with preeclampsia, which suggests that these women may have an underlying predisposition to increased oxidative stress that may be driven by or contribute to a persistent low-grade inflammatory response. (Hypertension. 2001;38:803-808.)

Key Words: preeclampsia ■ oxidative stress ■ hypertension, gestational ■ isoprostanes ■ proteinuria ■ inflammation ■ pregnancy

Preeclampsia is associated with abnormal placentation and placental ischemia and is characterized by maternal hypertension and abnormalities of renal, hepatic, neural, coagulatory, and endothelial function. Researchers have postulated that an immune maladaptation may link placental abnormalities with the maternal syndrome. Immune abnormality has been speculated to lead to cellular activation and release of substances that alter maternal endothelial function in a direction that causes vascular leakage, coagulopathy, and hypertension. Evidence suggests that neutrophils are activated in both the placental bed and maternal circulation of women with preeclampsia and that this activation resolves after delivery. Neutrophil activation is associated with free radical release that either can affect endothelial function directly or contribute indirectly through production of lipid peroxides. Evidence also suggests that lipid peroxidation is exacerbated in preeclampsia and that this effect is not a result of hypertension per se. F2-isoprostanes are considered to be good markers of in vivo lipid peroxidation, and we have shown that plasma levels of free 8-isoprostane are raised in preeclampsia. Whether a circulating factor is responsible for the neutrophil activation and any subsequent lipid peroxidation observed in preeclampsia is not known. The present study aimed to examine whether a stable plasma factor could cause neutrophil activation and lipid peroxidation in preeclampsia. To test this hypothesis, autologous neutrophils were collected postpartum and neutrophil activation was assessed by examining the level of neutrophil CD11b and CD18 expression after incubation with plasma collected during pregnancy and at 6 weeks postpartum. Autologous neutrophils were used to eliminate the possibility of neutro-
phil activation due to an immune reaction of the plasma with foreign donor cells. If the hypothesis were be proven, we expected that neutrophils incubated with plasma from pregnant preeclamptics would have increased expression of CD11b and CD18 compared with neutrophils from normal pregnant subjects incubated under the same conditions. We also expected that incubation of neutrophils obtained from preeclamptic women with their plasma obtained at 6 weeks postpartum would result in reduced expression of CD11b and CD18 compared with incubation with plasma obtained during pregnancy and that this expression would not differ from that of neutrophils of normal pregnant women incubated under the same conditions. F$_2$-isoprostanes were measured in plasma, urine, and plasma-neutrophil incubates to assess the possible contribution of neutrophil activation to lipoperoxidation.

Methods

Two groups of women were recruited for the study. Twenty-one women with preeclampsia were selected after admission to King Edward Memorial Hospital for Women. Preeclampsia was defined as development of blood pressure $>$140/90 mm Hg after 20 weeks’ gestation and proteinuria of $>2$ as confirmed by 24-hour urine collection, in women with no known history of hypertension or renal disease, and whose blood pressure returned to normal levels by 6 months postpartum. The preeclamptic women had an average blood pressure at admission of 157 $\pm$4/103 $\pm$2 mm Hg as measured by standard mercury sphygmomanometer, and urinary protein averaged 2 mm Hg as measured by 24-hour urine collection. None of the women was in labor when samples were

Biochemical and Hematological Variables

Plasma electrolytes, creatinine, uric acid, albumin and urinary creatinine, and protein were measured by use of a COBAS MIRA analyzer in the Biochemistry Department at Royal Perth Hospital. A lipid profile that included levels of serum cholesterol and triglycerides was determined enzymatically with Abbott reagents on a COBAS MIRA analyzer in the Department of Biochemistry at Royal Perth Hospital. A full blood screen and white blood cell (WBC) differential were measured in the Department of Hematology at Royal Perth Hospital with a H1 Technicon automatic hematological analyzer.

Incubation Studies of Plasma and Buffy Coat

To test the hypothesis that a circulating factor in pregnancy causes neutrophil activation in preeclampsia, we examined the effect of incubating autologous antepartum or postpartum plasma on expression of neutrophil CD11b and CD18 by use of WBCs collected at 6 weeks postpartum. Postpartum WBCs were chosen for the experiment because neutrophil CD11b and CD18 expression have been shown to be similar at 6 weeks postpartum in women who had normal pregnancies compared with women who had preeclampsia. WBC buffy coat was chosen because it most closely resembled our previous whole-blood neutrophil activation studies. An incubation time of 20 minutes was chosen because it is within the time frame in which substances such as platelet-activating factor, thrombin, endothelin-1, and oxidized LDL have been shown to activate neutrophils.

Preparation of WBC Buffy Coat

At 6 weeks postpartum, whole blood (18 mL) was collected into EDTA and centrifuged at 500g for 21°C for 20 minutes. White cells were removed and washed twice with 0.154 mol/L PBS, pH 7.4, plus 0.6% citrate, to remove plasma proteins, and resuspended in the same buffer at $5 \times 10^7$ WBC/mL.

Incubation of Plasma and WBCs and Flow Cytometry of Neutrophil CD18 and CD11b

Plasma (1 mL) collected at antepartum and 6-week postpartum visits was incubated with each patient’s postpartum WBC buffy coat ($5 \times 10^7$ WBCs). The mixture was incubated for 20 minutes in a 37°C water bath with gentle agitation. An aliquot (100 $\mu$L) of the WBC-plasma incubate ($5 \times 10^7$ WBCs) was mixed with 10 $\mu$L of fluorescein isothiocyanate-labeled CD18 antibody and 10 $\mu$L of phycoerythrin-labeled CD11b antibody and incubated for an additional 15 minutes at room temperature. WBCs were fixed by adding 1 mL of Immunoprep reagent mixture (Coulter), and neutrophils were identified by forward and side light-scattering properties by use of flow cytometry. Fluorescence of CD18 and CD11b was measured on a Coulter Profile II flow cytometer calibrated with Immunochek fluorescent beads and standardized for linearity with Immunobrite fluorescent beads. Fluorescence was measured on a log scale, and mean fluorescence index was calculated by multiplying mean log fluorescence by percentage of neutrophils positive for fluorescent antibody. The remaining 900 $\mu$L of WBC-plasma incubate was protected from further oxidation by immediate addition of butylated hydroxytoluene 200 $\mu$g and centrifuged at 13 000 rpm for 2 minutes, and the supernatant was stored at $-80^\circ$C until measurement.

Measurement of F$_2$-Isoprostanes

Blood was collected into ice-cold tubes containing 1 mg/mL EDTA and reduced glutathione 1 mg/mL, centrifuged at 1500g for 10 minutes at 4°C, and protected from oxidation by addition of butylated hydroxytoluene 200 $\mu$g/mL before storage at $-80^\circ$C. A 24-hour sample of urine was collected into indomethacin 15 mg to prevent in vitro formation of prostanooids by contaminating leukocytes.

Measurement of F$_2$-Isoprostanes in Urine, Plasma, and Plasma-WBC Incubates

F$_2$-isoprostanes were measured by use of electron-capture negative-ion gas chromatography–mass spectrometry as previously described. Briefly, a mixture of urine (2 mL) and 8-iso-PGF$_2$-d$_5$ (5 ng) was acidified to pH 3 with 2 mol/L HCl, applied to a preconditioned C$_18$ Sep-Pak cartridge; and washed with water (pH 3), acetonitrile/water (15%/85%), and petroleum spirit and the F$_2$-
isoprostanes were eluted with ethyl acetate/petroleum spirit (50%/50%; 10 mL). Eluate was applied to a silica Sep-Pak cartridge and washed with ethyl acetate, and F₂-isoprostanes were eluted with ethyl acetate/methanol (50%/50%; 5 mL). Samples were purified by use of reverse-phase high-performance liquid chromatography. F₂-isoprostanes were treated with pentafluorobenzyl bromide (10% vol/vol in acetonitrile) and N,N-diisopropyllethylamine (10% vol/vol in acetonitrile) at room temperature for 30 minutes and with N,O-bis(trimethylsilyl)trifluoroacetamide plus trimethylchlorosilane (99%/1%) and anhydrous pyridine at 45°C for 20 minutes. Samples were analyzed on a HP 5890 series II plus gas chromatograph coupled to a HP 5989B mass spectrometer (Hewlett-Packard) operated in selective ion-monitoring mode at a 569 and 573 mass-to-charge ratio (m/z) range for 8-iso-PGF₂α-d₄.

Esterified F₂-isoprostanes in plasma and WBC incubates (2 mL) were hydrolyzed with 1 mol/L KOH in methanol at 40°C for 30 minutes and then treated with the same procedure as for urine. Intra-assay and interassay variation was 7% and 4%, respectively, for urine and 8% and 6%, respectively, for plasma.

Statistics

Results are expressed as mean±SEM. Between-group comparisons before delivery were performed by use of unpaired t tests or, where data were not distributed normally, a Mann-Whitney test. Multiple-regression analysis was used to formulate a model that best explained the variance in plasma F₂-isoprostanes in the preeclamptic group postpartum.

Results

At admission, women with preeclampsia had significantly higher blood pressures (158/104±3.6/2.1 mm Hg, preeclampsia; 106/61±2.3/1.7 mm Hg, normal pregnancy) and proteinuria (1.8±0.3 g per 24 hours preeclampsia; 0.2±0.03 g per 24 hours, normal pregnancy). Age (30.6±1.6 years, preeclampsia; 31.3±1.5 years normal pregnancy) and gestation at sampling (32.0±1.0 weeks, preeclampsia; 32.9±0.9 weeks, normal pregnancy) was similar. Babies born to women with preeclampsia were delivered earlier (32.4±1.0 versus 39.5±0.4 weeks) and had lower birth weights (1872±215 versus 3469±96 g) than those born to normal pregnant women. Plasma uric acid, creatinine, and triglycerides were elevated in the preeclamptic women antepartum, whereas plasma albumin was significantly reduced (Table). Leukocyte counts were significantly elevated in the women with preeclampsia, mainly as a result of an increase in neutrophil counts (Table). At 6 weeks postpartum, women who had preeclampsia had levels of blood pressure (114±2.9/72±1.6 mm Hg) and proteinuria (0.15±0.02 g per 24 hours) within the normal range.

Neutrophil Activation in Response to Preeclamptic or Normal Pregnant Plasma

Incubation of postpartum WBCs with autologous pregnant plasma did not result in different expression of neutrophil CD11b and CD18 between the groups. Similarly, neutrophil CD11b and CD18 expression was not different between groups when WBCs were incubated with autologous postpartum plasma. However, in normal pregnant women, CD11b expression in response to postpartum plasma was significantly less than CD11b expression in response to autologous pregnant plasma (P<0.04). This was not the case for preeclamptic women, whose CD11b expression was similar after incubation with either plasma (Figure 1).

Biochemical Measurements, Including Markers of Severity of Preeclampsia and Lipids Before Delivery and at 6 Weeks Postpartum

<table>
<thead>
<tr>
<th>Biochemical and Hematological Measurements</th>
<th>Normal Pregnancy (n=19)</th>
<th>Preeclampsia (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma albumin, mmol/L</td>
<td>34.0±0.4</td>
<td>29.3±0.4</td>
</tr>
<tr>
<td>Antepartum*</td>
<td></td>
<td></td>
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<tr>
<td>6 weeks postpartum*</td>
<td>44.5±0.4</td>
<td>42.5±0.6</td>
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<tr>
<td>Plasma uric acid, mmol/L</td>
<td>0.25±0.01</td>
<td>0.37±0.01</td>
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<tr>
<td>Antepartum*</td>
<td></td>
<td></td>
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<tr>
<td>6 weeks postpartum</td>
<td>0.32±0.01</td>
<td>0.32±0.01</td>
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<tr>
<td>Plasma creatinine, mmol/L</td>
<td>57.5±1.8</td>
<td>64.6±2.5</td>
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<tr>
<td>Antepartum*</td>
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<tr>
<td>6 weeks postpartum</td>
<td>76.8±2.5</td>
<td>72.2±2.5</td>
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<tr>
<td>Total cholesterol, mmol/L</td>
<td>1.75±0.09</td>
<td>1.57±0.08</td>
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<tr>
<td>Antepartum*</td>
<td></td>
<td></td>
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<tr>
<td>6 weeks postpartum*</td>
<td>1.66±0.09</td>
<td>1.30±0.08</td>
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<tr>
<td>Triglycerides, mol/L</td>
<td>2.56±0.15</td>
<td>3.65±0.23</td>
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<tr>
<td>Antepartum*</td>
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<tr>
<td>6 weeks postpartum</td>
<td>1.65±0.46</td>
<td>1.83±0.30</td>
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<td>Leukocyte count, 10⁶/L</td>
<td>9.67±0.42</td>
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<td>Antepartum*</td>
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<tr>
<td>6 weeks postpartum</td>
<td>6.51±0.30</td>
<td>6.89±0.37</td>
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<tr>
<td>Neutrophils, 10⁶/L</td>
<td>7.13±0.38</td>
<td>9.57±0.67</td>
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<tr>
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<tr>
<td>6 weeks postpartum</td>
<td>3.60±0.23</td>
<td>4.05±0.25</td>
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<tr>
<td>Lymphocytes, 10⁶/L</td>
<td>1.75±0.08</td>
<td>1.92±0.19</td>
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<tr>
<td>Antepartum*</td>
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</tr>
<tr>
<td>6 weeks postpartum</td>
<td>2.17±0.16</td>
<td>2.17±0.16</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*P<0.05 preeclampsia vs normal pregnancy.

Plasma F₂-Isoprostanes

Compared with normal pregnant women, plasma F₂-isoprostanes were significantly elevated in women with preeclampsia during pregnancy and remained so after delivery. Pregnancy was not associated with higher levels of plasma F₂-isoprostanes in either group (Figure 2). Exclusion of multiparous women from the analysis did not alter the result.

In pregnancy, the only significant correlation with plasma F₂-isoprostane levels was for lymphocyte counts (r=0.42; P=0.059) in women with preeclampsia. At 6 weeks postpartum, albumin, total cholesterol, and lymphocyte count were all correlated with plasma F₂-isoprostane levels in women with preeclampsia. Multiple-regression analysis was used to examine predictors of plasma F₂-isoprostanes measured at 6 weeks postpartum in women with preeclampsia. Plasma F₂-isoprostanes were significantly positively associated, with total cholesterol (B=705.9±214.8; P=0.005) and lymphocyte count...
and negatively associated with serum albumin (B = -246.6 ± 78.1; P = 0.006). This model accounted for 58% of the variance in plasma F2-isoprostanes in preeclampsia. The same model did not predict plasma F2-isoprostanes measured at 6 weeks postpartum in women who had normal pregnancies: total cholesterol (B = 300.8 ± 304.1; P = 0.339) lymphocyte count (B = 166.4 ± 656.1; P = 0.803), and serum albumin (B = -51.3 ± 173.8; P = 0.772).

**F2-Isoprostanes Generated in Plasma-WBC Incubates**

Incubation of plasma and WBCs increased levels of F2-isoprostanes 2-fold over those observed in plasma alone, regardless of whether the women had preeclampsia. F2-isoprostanes generated during incubation were significantly higher in women with preeclampsia compared with those having normal pregnancy, regardless of whether incubation was with plasma collected during pregnancy (Figure 2). The additional contribution to F2-isoprostane levels made by incubating WBCs with pregnant plasma was not different between the normal pregnant group 2698 ± 651 pmol/L and women with preeclampsia 3704 ± 1001 pmol/L. However, after incubation with postpartum plasma, the contribution to F2-isoprostane levels made by WBCs was significantly reduced in the normal pregnant group (968 ± 349 pmol/L) compared with the preeclampsics (3712 ± 903 pmol/L; P = 0.011). In normal pregnant women, the contribution to F2-isoprostane levels made by WBCs in response to postpartum plasma was significantly less than that contributed by incubation with autologous pregnant plasma (P < 0.040). This was not the case for preeclamptic women. Exclusion of smokers from the analysis did not affect the results; the preeclampsics still had significantly raised levels.

**Urinary F2-Isoprostanes**

Urinary F2-isoprostanes were significantly elevated in normal pregnant women during pregnancy compared with women with preeclampsia and decreased postpartum to be significantly lower than for women in the preeclamptic group. Levels of urinary F2-isoprostanes in women with preeclampsia were not affected by pregnancy itself (Figure 2).
Discussion
The present study aimed to test the hypothesis that a stable circulating factor present in plasma of women with preeclampsia causes neutrophil activation. To examine this hypothesis, neutrophils were collected from each subject at 6 weeks postpartum, and neutrophil activation was assessed after incubation with autologous plasma taken during pregnancy or postpartum from women who had preeclampsia or normal pregnancies. We did not find that plasma taken during pregnancy from women with preeclampsia activated their neutrophils to a greater extent than plasma taken during pregnancy from normal pregnant women studied under the same conditions. However, we did find that neutrophils from normal pregnant women were activated relatively less by postpartum plasma than antepartum plasma. This was not the case for women with preeclampsia.

The lack of difference in neutrophil activation between groups in response to plasma taken during pregnancy does not support a role for a stable circulating factor that causes neutrophil activation in preeclampsias. Therefore, neutrophil activation in women with preeclampsia could be due to exposure to hypoxic conditions or local inflammatory agents encountered during passage of neutrophils through the placental circulation. An alternative explanation is that neutrophils obtained from preeclamptic women who are pregnant are sensitized to circulating serum factors, whereas neutrophils obtained in the nonpregnant state are not. In normal pregnant women, less neutrophil CD11b expression was seen after incubation with postpartum plasma compared with incubation with plasma taken during pregnancy. This was not the case in women with preeclampsia, which suggests that plasma components affect neutrophil CD18 and CD11b expression to a similar extent regardless of whether plasma was collected when the preeclamptic women were pregnant.

These results confirm previous findings that women with preeclampsia are under increased oxidative stress. However, we also have demonstrated that levels of plasma F2-isoprostanes are raised in women with preeclampsia after delivery when other biochemical markers of preeclampsia have resolved. This suggests that women who develop preeclampsia may be predisposed to oxidative stress. Although we cannot exclude an undiagnosed underlying metabolic abnormality specific to multiparous preeclampsias that might affect lipid peroxidation, we believe it to be unlikely, given that blood pressure and proteinuria were within normal levels at 6 weeks postpartum and exclusion of multipara from the analysis did not alter the difference in plasma F2-isoprostane levels.

When plasma was incubated with autologous buffy coat, levels of total F2-isoprostanes were elevated compared with those of plasma alone, which suggests that free radical production by leukocytes can contribute to plasma F2-isoprostanes. In preeclampsias, incubation of WBCs with plasma led to levels of F2-isoprostanes that were significantly higher than those in incubations from normal pregnant women, regardless of whether incubation was performed with antepartum or postpartum plasma. In normal pregnant women, the contribution of WBCs to the level of F2-isoprostanes in the incubate was significantly less when postpartum plasma was used. This probably reflects reduced neutrophil activation in the presence of postpartum plasma from normal pregnant women.

In a previous study in which we examined free 8-isoprostane in women with preeclampsia, we found elevated levels compared with normal pregnancy. In contrast, plasma total 8-isoprostane was not different in preeclamptics during pregnancy, but a relative decrease was seen in postpartum levels of women with preeclampsia that was not seen in normal pregnancy. Activity of phospholipase-A2 was thought in part to determine levels of free isoprostanes in plasma, whereas total isoprostanes (free plus phospholipid-bound isoprostane) might be more representative of net oxidative stress. The difference in our findings for total isoprostanes for the 2 studies could be explained by differences in our methodology. Our initial study used an enzyme immunoassay that is reasonably specific for 8-isoprostane. The method in the present study uses a gas chromatograph-mass spectrometer and measures a number of F2-isoprostanes that coelute with deuterated 8-iso-PGF2α, internal standard. Potentially 32 enantiomers of 8-isoprostane exist that can be produced by free radical oxidation. Therefore, measurement of a group of F2-isoprostanes by use of gas chromatograph-mass spectrometer may reflect better the extent of lipid peroxidation than measurement of an individual isoprostane. Theoretically a small but insignificant contribution to F2-isoprostanes levels in plasma may be due to platelet and monocyte cyclooxygenase. Urinary F2-isoprostane excretion was depressed during pregnancy in preeclampsia, which confirmed our previous finding. In contrast, isoprostanes in urine are excreted in the unesterified form and may represent systemic or renal synthesis. The depressed excretion in preeclampsias is likely to reflect a more-global impairment of renal function in preeclampsia. The kidney can contribute to F2-isoprostane synthesis, and further studies that use more-specific urinary metabolites are required to confirm these observations.

In pregnant women with preeclampsia, the only significant predictor of plasma F2-isoprostanes was lymphocyte count. Increased levels of intracellular reactive oxygen species in lymphocytes have been reported in women with preeclampsia and could contribute to increased lipid peroxidation. Researchers have postulated that preeclampsia represents the extreme of a continuum of a maternal inflammatory response that occurs in pregnancy, and it is possible that the correlation between lymphocyte count and plasma F2-isoprostanes is a part of a more-general mild inflammatory response that is related to lipid peroxidation in preeclampsia. The correlations observed in women with preeclampsia at 6 weeks postpartum suggest that the mild inflammatory response may not be confined to pregnancy. Postpartum F2-isoprostanes in preeclampsias were positively correlated with lymphocyte count and cholesterol level and were negatively correlated with plasma albumin. Negative correlation of plasma F2-isoprostanes with albumin supports a connection between lipid peroxidation and inflammation in 2 ways. First, albumin is a negative acute-phase protein that decreases in concentration in inflammatory states. Second, because albumin is known to protect against oxidation, reduced albumin levels also would render these women susceptible to oxidative stress. The correlation between cholesterol and F2-
isoprostanes was observed in women who were relatively dyslipidemic, with increased levels of total and LDL cholesterol and triglycerides and significantly reduced levels of HDL cholesterol compared with normal pregnant women studied at the same time. The persistence of a postpartum dyslipidemic state confirms findings of an earlier study reported by us\(^3\) and suggests that these women may be predisposed to the metabolic syndrome and oxidative stress.

In conclusion, we have no evidence of a stable circulating factor in women with preeclampsia that causes neutrophil activation in the short term. However, plasma F\(_2\)-isoprostanes are elevated persistently both before and after delivery in women with preeclampsia, which suggests that they are under persistent oxidative stress that may be driven by or contribute to a low-grade inflammatory response.

Acknowledgment
The present work was funded by a grant from the National Heart Foundation of Australia.

References
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Hypertension. 2001;38:803-808
doi: 10.1161/hy1101.092969

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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