Inositol Phosphoglycan P-Type in Preeclampsia
A Novel Marker?

Philip J. Williams, Khalid Gumaa, Marco Scioscia, Christopher W. Redman, Thomas W. Rademacher

Abstract—A state of insulin resistance has been demonstrated in active preeclampsia, and women with clinical evidence of insulin resistance are at higher risk to develop this syndrome during pregnancy. Recently, inositol phosphoglycan P-type, a putative second messenger of insulin action, has been implicated in the pathophysiology of preeclampsia and is increased in the placenta, amniotic fluid, and maternal urine of preeclamptic women compared with normal pregnant women. We report here a case–control study to assess the potential of urinary levels of inositol phosphoglycan P-type as a screening test for preeclampsia. Twenty-seven preeclamptic women and 47 healthy pregnant women were recruited. A polyclonal antibody-based ELISA was developed to detect levels of inositol phosphoglycan P-type in urine. Its content in urine specimens was found to be 30-fold higher in preeclamptic subjects than control subjects (329.1 ± 21.8 versus 9.2 ± 1.5; P < 0.001), with a higher level in all of the preeclamptic cases. For 6 women who developed preeclampsia, > 1 gestational date sample of urine was available, and retrospective analysis showed a significant time-related increase of the urinary level of inositol phosphoglycan P-type ≤ 7 weeks before clinical diagnosis of preeclampsia. Urinary level of inositol phosphoglycan P-type increased after diagnosis indicating a possible pathophysiological threshold level and steeply decreased after delivery. (Hypertension. 2007;49:1-6.)

Key Words: preeclampsia ■ screening test ■ inositol phosphoglycan ■ biological marker ■ insulin resistance

Preeclampsia (PE) represents the most severe form of hypertensive disorder of human pregnancy being associated with a substantial maternal and perinatal morbidity and mortality.1 It affects ≈5% of all pregnancies, although its causes remain unclear. A state of insulin resistance has been demonstrated in active PE,2,3 and women with clinical evidence of insulin resistance are at higher risk of developing this syndrome during pregnancy.4

Recently, a family of putative insulin mediators, inositol phosphoglycans (IPGs), have been implicated in the pathophysiology of PE. A high concentration of bioactive IPG P-type (P-IPG) was found in human preeclamptic placenta,5 urine, and amniotic fluid.6 Further studies have provided evidence of insulin resistance in human placentas from preeclamptic pregnancies and demonstrated an association between P-IPG and the insulin signaling cascade.7

IPGs have been demonstrated to exert insulin-mimetic activity in both glucose and lipid metabolism mainly through the activation of pyruvate dehydrogenase phosphatase, glycogen synthase phosphatase, and glycerol-3-phosphate acyltransferase.8,9 The elevated concentration of glycogen in terminal villi from preeclamptic placentas, because of an increased activity of glycogen synthase,10 may be a consequence of the abnormal content of P-IPG in the placentas of preeclamptic patients.

In this report, we focus our attention on the urinary excretion of IPGs using a novel assay specifically developed to analyze urinary P-IPG. This assay was initially tested in 16 pairs of urine samples from preeclamptic and healthy pregnant women. Subsequently, we carried out a larger case-controlled study to confirm these findings. Furthermore, we report some pivotal results aiming to establish the potential of urinary excretion of P-IPG as a screening test to identify women at risk of developing PE.

Methods

This collaborative study was carried out at University College London and at the John Radcliffe Hospital. Approval of the local ethical committees and informed consent from all of the patients was obtained. A midstream urine specimen was collected from all of the women recruited for the study and stored at −20°C until required for assay. No significant change in the content of P-IPG was observed during storage of the samples for ≤ 1 year under different conditions (−20°C and −80°C). The study was divided into 2 steps. Initially, samples were assessed using stored urine samples from 16 preeclamptic and 16 healthy pregnant women matched for maternal age (± 3 years), weeks of gestation (± 1 week), and parity (nulliparous or multiparous). PE was diagnosed as the presence of new hypertension ≥ 90 mm Hg diastolic or ≥ 20 mm Hg above baseline reading (in ≥ 2

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From the Department of Molecular Pathology (P.J.W., M.S., T.W.R.), Molecular Medicine Unit, Royal Free and University College Medical School, London, United Kingdom; the College of Medicine and Medical Sciences (K.G.), Arabian Gulf University, Manama, Kingdom of Bahrain; the Department of Obstetrics and Gynaecology (M.S.), University of Bari, Bari, Italy; and the Nuffield Department of Obstetrics and Gynaecology (C.W.R.), University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom.

Correspondence to Marco Scioscia, Department of Obstetrics and Gynaecology, University of Bari–Policlinico, Piazza Giulio Cesare 11, 70125 Bari, Italy. E-mail marcoscioscia@gmail.com

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P-IPG Assay

A polyclonal antibody-based ELISA was developed to detect P-IPG activity in urine. Rabbit polyclonal IgG antibodies were generated using P-IPG prepared from a single urinary specimen from a patient with a very severe case of PE as described previously. For the polyclonal antibody production, 2 rabbits were immunized. Each rabbit was administered 1 mL of P-IPG in Freund’s complete adjuvant by intradermal injection to 5 sites (equivalent to total IPG-P from 22.5 mL of PE urine). Booster injections were given on days 30 and 62 in Freund’s incomplete adjuvant (equivalent to P-IPG from 17 mL of PE again urine). Test bleed were taken and monitored at regular intervals, rabbits were exsanguinated on day 70, and sera were collected, aliquoted, and stored at -20°C before use. The ELISA procedure has been described previously. PE samples were assessed blind in triplicate using a dilution of 1:20, and, initially, results were reported as absorbance at 450 nm. Positive control composed of a PE patient urine sample was used as a standard at dilution 1:5000, and the results were reported as percentage of this standard corrected by creatinine (1 arbitrary unit was defined as 1% of the plate positive control). The accuracy of the assay was evaluated by the recovery study and by the assessment of the coefficient of variation (CV). The recoveries were 100.2 ± 8.2 (mean ± SD; n = 15). We found interplate and interassay CVs of 7.3% and 12.1%, respectively.

Statistical Analysis

Continuous variables were assessed using a t test if a normal distribution was confirmed by the method of Kolmogorov–Smirnov (>0.1). Mann–Whitney U test was carried out to assess nonparametric continuous variables of P-IPG activity if raw data failed the Kolmogorov–Smirnov test in ≥ 1 group. Data with Gaussian distribution are presented as mean ± SD, whereas data not normally distributed are presented as median ± SE and 95% CI.

The P-IPG values were assessed for significant linear relationship with the onset (week of gestation) and severity (systolic, diastolic, and mean arterial pressure and platelet count) of PE using simple linear regression. Mean arterial pressure was calculated as systolic plus doubled diastolic divided by 3. Initially, sample size calculations were not estimated, because the difference found between the 2 groups on stored samples was very evident. On hindsight, power analysis of the P-IPG data from 27 versus 47 samples indicated that the power was 1.00 with a type I error rate (α) of 0.05 and a population SD of 10. The critical value of t for α set at 0.05 and at 0.01 was 1.99 and 2.65, respectively. Data were analyzed using the STATISTICA data analysis software system (version 6.0, StatSoft, Inc) with a significant difference set at P < 0.05.

Results

The ELISA assessment for P-IPG of stored urinary samples from preeclamptic and healthy pregnant women showed far higher readings in the PE groups (absorbance at 450 nm was 2.70 ± 0.94 versus 0.05 ± 0.03 in PE and control group, respectively; P < 0.001; Figure 1). These samples were from homogeneous populations as reported in Methods with a gestational age of 32.0 ± 4.5 and 31.4 ± 3.5 for PE and control subjects, respectively.

A larger case-control study included urinary samples from 27 preeclamptic and 47 healthy pregnant control subjects. Characteristics of the patients are reported in Table 1. Using the positive control urine as standard, the P-IPG content in

TABLE 1. Study Population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PE (n=27)</th>
<th>Controls (n=47)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>32.0 ± 4.5</td>
<td>31.7 ± 4.6</td>
<td>0.79</td>
</tr>
<tr>
<td>Gestational age at collection, wk</td>
<td>31.2 ± 4.3</td>
<td>31.4 ± 3.5</td>
<td>0.83</td>
</tr>
<tr>
<td>Nulliparous, n, %</td>
<td>8 (29.6)</td>
<td>14 (29.8)</td>
<td>1.00</td>
</tr>
<tr>
<td>Delivery at &lt; 37 weeks, n, %</td>
<td>24 (88.9)</td>
<td>40 (85.1)</td>
<td>0.74</td>
</tr>
<tr>
<td>P-IPG, arbitrary units, corrected by mmol/L of creatinine</td>
<td>61.0 ± 21.8</td>
<td>6.6 ± 1.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Patient characteristics (mean ± SD) were assessed by t test, whereas Mann–Whitney U test was performed to assess differences in urinary excretion of P-IPG (1 arbitrary unit was defined as 1% of the plate positive control) corrected by urinary creatinine (median ± SE and 95% CI).
urinary specimens was 30-fold higher in PE subjects than control subjects (361.0 ± 21.8% versus 6.6 ± 1.5%; P < 0.001; Figure 2) with higher readings in all of the PE cases. Because the assay was not linear above 150%, dilutions were required to get true comparative values into the linear region (Figure 3). Bioactivity of P-IPG was then normalized on urinary creatinine as we reported previously a direct correlation of urinary P-IPG levels (expressed as units of P-IPG per micromole of urinary creatinine) and units of P-IPG in 24-hour collections of urine (93.5 ± 64.6% versus 37.6 ± 20.2%; P < 0.001; Table 1).

P-IPG release in maternal urine was negatively correlated to gestational age (r = -0.40; P < 0.05), and a borderline positive relationship was found with blood pressure (Table 2). Urinary protein content was assessed and plotted against P-IPG activity (reported as absorbance). A reference limit was calculated (mean ± 2 SD) from a control group made of women without overt PE (control subjects plus women who developed PE later on). P-IPG did not correlate with protein content in samples from control pregnant patients (r² = 0.02; Figure 4A), whereas PE samples showed a direct correlation (r² = 0.14; P < 0.05; Figure 4B).

For 6 women who developed PE, >1 sample of urine was collected (urine specimens ranged between 2 and 6), and these patients were taking part in other research protocols. These samples were assessed retrospectively and not considered in the previous analysis (either comparison with control group or correlation with clinical parameters). They revealed a significant increase of urinary P-IPG excretion ≤ 6 weeks before clinical diagnosis of PE (Figure 5). In 3 cases (study cases 1.2, 1.11, and 1.22) multiple samples were available 6 to 8 weeks before PE diagnosis, and a curve was plotted showing a time-related increase of urinary P-IPG. Urinary P-IPG content was higher than the reference limit (27.96% of the standard positive control) of the control population, respectively, 3, 6, and 5 weeks before the clinical diagnosis of PE. In another 2 cases (study cases 1.16 and 2.3) only 1 sample was available before the onset of PE (3 weeks and 1 week before diagnosis, respectively) and had P-IPG values within the reference range (Figure 3). Urinary excretion of P-IPG kept increasing after diagnosis (study cases 2.3 and 2.6), then dropped sharply after delivery (cases 1.11 and 1.22; data not shown). For the study case 1.22, a few samples were retrospectively recovered and revealed a 3-fold increase of P-IPG excretion from week 34 of gestation (28.3% versus 9.2%, which is the mean value for the control group), 1 month before the clinical onset of PE. Unfortunately, no samples were collected in that month, but the excretion of P-IPG decreased 2 days after delivery compared with the last value available.

**Discussion**

We have demonstrated an increase of P-IPG urinary excretion in all of the cases of active PE recruited for this study. The P-IPG content of maternal urine was remarkably high in PE patients when compared with control subjects, representing a reliable marker of this syndrome. Furthermore, P-IPG seems to increase a few weeks before the onset of clinically diagnosed PE and to decrease after delivery.
Pregnancy is physiologically associated with a state of insulin resistance, which is further aggravated in hypertensive disorders during pregnancy. Both inflammation and insulin resistance have been reported to be associated with PE. An insulin resistant state before pregnancy is a clear risk factor that may lead to the development of this complex maternal disorder, although a direct association between the systemic inflammatory activation, which is a feature of PE, and insulin resistance is still debated.

IPGs are phospholipid-derived putative second messengers of insulin and can stimulate glucose and lipid metabolism. P-IPG seems to play an important role in mediating the insulin effects on peripheral glucose use under physiological conditions, whereas a lack in generation/release of this molecule has been suggested to underlie insulin resistance in noninsulin-dependent diabetic men. We have reported recently an increased urinary excretion of this molecule during pregnancy compared with nonpregnant women with a particularly high concentration in the amniotic fluid in healthy pregnancies. P-IPG production was enhanced by active PE as demonstrated by an increased content or release of this molecule in placentas or fluids (amniotic fluid and urine) of women with this syndrome. The presence of a systemic inflammation during PE with circulating inflammatory leukocytes and subsequent endothelial damage is well documented. Poor placentation is often associated with this maternal syndrome, although this does not always lead to overt PE. An increased amount of placental debris and circulating factors derived from the placenta may contribute to the clinical syndrome. Moreover, this systemic inflammatory response has complex effects on metabolism, and the enhanced insulin resistance represents an additive insult ending up in clinical PE.

A variety of biomarkers have been proposed over the years as candidates for prediction of this syndrome. Recent investigations found a strict correlation between soluble fms-like tyrosine kinase-1 (sFlt-1), placental growth factor (PIGF), and PE. Elevated levels of circulating sFlt-1 and reduced excretion of PIGF in maternal urine have been reported in PE women. These molecules are interrelated as parts of the vascular endothelial growth factor system, and increased levels of sFlt-1 are able to lower the PIGF level.
Only within 5 weeks of onset of clinical PE were urinary PI GF levels\textsuperscript{26} and serum sFlt-1 levels\textsuperscript{25} significantly changed (decreased and increased, respectively). In our study, urinary P-IPG increases 5 to 6 weeks before the onset of disease, similar to the change in sFlt-1 reported above. From a diagnostic standpoint, the use of independent biomarkers in combination may result in an increased sensitivity and specificity.

A limitation of this study is its own setting, because case–control studies tend to overestimate diagnostic accuracy and do not permit estimating the screening effectiveness in the general population.\textsuperscript{27} Interpreting these data with associated severity of the disease (early onset and blood pressure) would have been perhaps too ambitious because of the limited number of cases (27).

In women with PE, P-IPG seems to correlate with proteinuria, although the reported \( r^2 \) value is low. This is likely to be related to the severity of the disease, but the small number of cases longitudinally assessed does not allow us to draw any conclusion. Ferrazzani et al\textsuperscript{28} reported a direct correlation among proteinuria, severity of PE, and outcomes. Thus, it is plausible that the more severe the PE the more P-IPG release can be expected. Some samples with low protein content showed higher P-IPG activity than others with higher proteinuria. A larger study is under way to properly assess these associations.

In the longitudinal assessment of PE subjects presented here, a decrease in urinary excretion of P-IPG before delivery was found in 2 cases. Whether these inconsistent findings are related to antihypertensive treatment and decreased blood pressure or other factors is still debatable.

The accuracy of a screening test is determined by its specificity and sensitivity. In light of our results, P-IPG urinary excretion represents a potentially excellent screening test. In fact, all of the cases of PE assessed showed a P-IPG urinary content well above 2 SD (calculated as percentage of standard), and none of the controls were higher than this reference. Moreover, this marker increased quickly in urine, doubling or trebling within a week, allowing for confirmatory tests in the following days, further increasing the detection/confidence rate. The development of an ELISA-based assay to quantify P-IPG in urine and amniotic fluid instead of the spectrophotometric pyruvate-dehydrogenase activity assay\textsuperscript{29} used previously to measure P-IPG greatly simplifies and reduces the time taken and cost of the procedure. In the spectrophotometric method, IPGs have to be extracted before the assay in a 3-day procedure.\textsuperscript{30} Unfortunately, the spectrophotometric procedure remains the only reliable method for the assessment of P-IPG in serum samples, because serum contains unspecified components that interfere with the ELISA assay. On the other hand, the ELISA assay can be performed directly on crude urine samples and provides reliable results as confirmed by a low CV (reported above). Moreover, this assay is not invasive and is, therefore, suitable for routine use in clinical practice, because it can be performed on midstream urine specimens.

\textbf{Perspectives}

Our findings suggest that P-IPG may play a role in disease evolution, possibly being directly related to the underlying disease process. Here we present P-IPG as a biological marker of PE in early pregnancy, although the causes for its increase in these cases are yet to be clarified (primary or secondary to inflammation and insulin resistance?). Although it remains uncertain to what extent these factors are pathognomonic in PE, the possibility of having a reliable marker for this syndrome is of key importance. From a clinical point of view, it is particularly useful to detect women who are likely to develop PE to begin a close monitoring of their blood pressure and proteinuria. In addition, the potential for massive savings on patient hospitalization exists by targeting it only to those patients likely to develop PE.

Larger prospective studies to better characterize the relationship among P-IPG, insulin resistance, and PE are needed. Pivotal data on the possible use of this marker in gestational...
diabetes mellitus seem to reflect the central role of these molecules in insulin resistance syndromes with a seemingly reactive role of the fetal/placental unit to maternal hyperglycemia (M Scioscia, unpublished data, 2006). Further research is under way to assess the potential of this molecule as an early marker of PE.

Disclosures
A patent of invention of the presented ELISA method has been filed in 2003 (No. EP1295122). None of the authors have a financial interest in the patent as it is currently owned by SRPharma.

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