Vascular dysfunction in preeclampsia
van Wijk, M.J.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 7

The phospholipid composition of microparticles and endothelial dysfunction in preeclampsia

Abstract

Objective: To determine whether the phospholipid composition of circulating microparticles from women with preeclampsia, normal pregnant and nonpregnant women and to establish their effect on endothelial function in isolated myometrial arteries from normal pregnant women.

Methods: Plasma samples were collected from women with preeclampsia (n=11), normal pregnant (n=10) and nonpregnant women (n=10), matched for age and gestational age. The phospholipid composition of the microparticles was determined by high performance thin layer chromatography. Myometrial resistance arteries were isolated from myometrial biopsies at cesarean delivery of normal pregnant women and mounted in a wire myograph after overnight incubation with isolated microparticles from women with preeclampsia, normal pregnant and nonpregnant women. BK-mediated relaxation was determined.

Results: Microparticles in all groups mainly consisted of PC and smaller amounts of SM, PEa, PI and lyso-phospholipids. The percentage lyso-PC was significantly decreased in pregnancy and preeclampsia. There were no major differences in phospholipid composition between women with preeclampsia and normal pregnant women. BK-mediated relaxation was significantly decreased in myometrial arteries after incubation with preeclamptic microparticles, in contrast to normal pregnant and nonpregnant microparticles.

Conclusions: Circulating microparticles from women with preeclampsia diminish endothelium-dependent relaxation after overnight incubation in isolated myometrial arteries. This difference does not seem to result from differences in the phospholipid composition of circulating microparticles.
Introduction

Circulating microparticles may play a role in the pathogenesis of many cardiovascular diseases, since they affect inflammation, coagulation and vascular function. Microparticles have been studied in various disease states, in which their numbers, cellular source and composition proved altered (see chapter 3). Previously, we determined numbers and cellular origin of circulating microparticles in women with preeclampsia, normal pregnant and nonpregnant women. Although the total number of circulating microparticles was similar in these three conditions, increased numbers of lymphocyte and granulocyte microparticles were found in women with preeclampsia (see chapter 5). Furthermore, we reported that isolated microparticles from women with preeclampsia diminished endothelium-dependent relaxation in isolated myometrial arteries from normal pregnant women, in contrast to microparticles from normal pregnant women (see chapter 6). The mechanism(s), however, through which these preeclamptic microparticles cause endothelial dysfunction are currently unknown, but may be related to their phospholipid composition.

There is only very limited information available on the phospholipid composition of microparticles in health and disease. Weerheim and coworkers determined the phospholipid composition of microparticles from venous blood of healthy humans by high performance thin layer chromatography (HPTLC) [185]. These microparticles proved to contain mainly PC (approximately 60%), and smaller amounts of SM, PEa and PS [185]. Although circulating microparticles in healthy humans are mainly platelet-derived, and thus may be expected to resemble the phospholipid composition of platelet plasma membranes, the phospholipid composition of these circulating microparticles clearly differs from that of platelet plasma membranes [270]. These apparent differences may be due to the presence of microparticles from non-platelet origin, such as erythrocytes and leukocytes, and/or from selective shedding of phospholipids into microparticles. This latter hypothesis is supported by the finding that major differences can occur in protein/antigen composition of microparticles and the composition of the “parent cell” membrane, indicating selective shedding of proteins/antigens into microparticles [162,189-192]. Fourcade and coworkers reported the phospholipid composition of microparticles from synovial fluids of inflamed joints of arthritic patients [165]. These microparticles contained PC, SM, PM, and lyso-phospholipids (all 20-25%), and small amounts of PS [165]. Clearly, this phospholipid composition differed significantly from that of microparticles in blood from healthy humans [185]. Possibly this is due to the different cellular origin of the microparticles: whereas microparticles in inflamed synovial fluids are mainly leukocyte-derived [187], microparticles in healthy humans are mainly platelet-derived. Alternatively, the high concentrations of lyso-phospholipids in synovial microparticles are due to the presence of phospholipases in the synovial fluid. Thus, the phospholipid composition of microparticles is likely to be dependent on their cellular origin, the mechanisms underlying their release, and the presence of environmental factors such as phospholipases.

Since circulating leukocyte microparticle numbers are increased in preeclampsia, and preeclampsia is characterized by an inflammatory state, the phospholipid composition of
microparticles may be changed in such a way that they induce endothelial dysfunction upon incubation of isolated arteries with microparticles. Therefore, in this pilot study we determined the phospholipid composition of circulating microparticles from women with preeclampsia, normal pregnant and nonpregnant women and established their effect on endothelial function in isolated myometrial arteries from normal pregnant women.

Methods

Subjects
Plasma samples were collected in the Academic Medical Center in Amsterdam after approval by the local medical ethical committee. Myometrial biopsies were collected in Huddinge University Hospital in Stockholm after approval by the local medical ethical committee. After obtaining informed consent, plasma samples were collected from women with preeclampsia, normal pregnant, and nonpregnant women, and myometrial biopsies were obtained at elective cesarean delivery from normal pregnant women. For collection of the plasma samples normal pregnant women were matched for age (± 5 yr) and gestational age (± 2 wk) with women with preeclampsia and nonpregnant women were matched for age (± 5 yr) with the women with preeclampsia.

Collection of blood samples and isolation of microparticles
Blood samples were taken from the antecubital vein without tourniquet through a 19G butterfly needle with a vacutainer system into a 4.5 mL tube containing 0.105 mol/L citrate (Becton Dickinson; San Jose, USA). Blood samples were processed immediately at room temperature to prevent any cell activation. Cells were removed by centrifugation for 20 minutes at 1550 g at room temperature to obtain plasma. Plasma samples were divided in aliquots of 1 mL, snap frozen in liquid nitrogen in order to preserve the microparticle structure, and stored at -80°C. Before the start of the experiments the frozen plasma aliquots were thawed on melting ice.

Myometrial biopsies
After obtaining informed consent from normal pregnant women undergoing an elective cesarean delivery at term, a full thickness myometrial biopsy was taken from the upper margin of the uterine incision following the delivery of the placenta. The site of the biopsy was never the site of the placenta location. The biopsies were collected into ice-cold PSS. Small arteries of about 200-600 μm in diameter and with a length of approximately 2 mm were immediately dissected from healthy myometrium in the biopsy. From each biopsy four or more arteries of comparable size and preferably from the same artery segment were dissected.

Wire myography
Plasma samples (250 μL) from a matched set of women were defrosted on melting ice for each experiment. Results of some of the women with preeclampsia and normal pregnant women (n=6
in each group) were previously reported (see chapter 6). Plasma samples were centrifuged at 17570 g for 30 minutes to pellet the microparticles. Subsequently, 225 μL of the microparticle-free supernatant was removed from each tube and the microparticle pellet was resuspended in the remaining 25 μL of the plasma and 225 μL of PSS. As previously confirmed, the fraction isolated from plasma according to the above-described protocol contains microparticles (see chapter 5). The microparticle solution was diluted to 5% (v/v) and heparin (1 IU/mL) was added to prevent clotting in the solutions. In each solution a freshly dissected myometrial artery was placed and left to incubate overnight at 4°C. Control incubation was done with PSS, with or without supplementation of 0.5% microparticle-free plasma from one of the patient groups.

After overnight incubation, the arteries were mounted on stainless steel wires, 40 μm in diameter, which were attached to a force transducer and a micrometer, respectively, in the organ baths of a 4-channel multi myograph (model 610, Danish Myo Technology; Aarhus, Denmark), which were filled with PSS. Vessel length was measured with a calibrated eyepiece micrometer under the microscope. After all arteries were mounted, they were allowed to equilibrate for 30 minutes at 37°C, while continuously being oxygenated with 5% CO₂ in O₂. All solutions were refreshed every 30 minutes. A standardized normalization procedure was then performed to allow for calculation of the artery diameter at which the in vivo transmural pressure of the relaxed artery would have been 100 mmHg. Arteries were then set at 0.9 times this diameter, since it is generally accepted that this is the diameter that enables optimal contractile ability for the arteries with a low resting tension. Myodac software was used for these calibrations and for data registration (version 2.1, Danish Myo Technology).

After the normalization procedure the arteries were left to equilibrate for 20 minutes and a reference constriction was then elicited with PSS containing 124 mmol/L potassium and 1 μmol/L NE (Sigma Aldrich; Stockholm, Sweden). Following a washout period of 15 minutes, constriction was induced with 10 nmol/L VP (Sigma Aldrich) until a stable constriction was achieved. A concentration-response curve with increasing concentrations of BK (1 nmol/L to 3 μmol/L, Sigma Aldrich) was then performed.

**Phospholipid composition of microparticles**

Microparticles were isolated from 4 × 250 μL of the plasma from a matched set of women by centrifugation, as described previously. To remove most plasma, the microparticles were resuspended in citrate-substituted PBS and centrifuged again. The supernatant was removed. Phospholipids were extracted from the microparticles by application of methanol/chloroform/acetic acid in various concentrations as described previously by Weerheim and coworkers [185]. The chloroform fractions were pooled and dried under nitrogen. After redissolving the phospholipids in methanol: chloroform (100 μL; 2: 1), paired samples were applied with a Camag Linomat IV (Merck, Darmstadt, Germany) to HPTLC glass plates with a thin silica layer (Merck) as narrow bands (3 mm). A mix of phospholipid standards, containing PC, lyso-PC, SM, PS, lyso-PS, PEa, lyso-PEa and PI (Sigma Aldrich, Zwijndrecht, The Netherlands), was also loaded on the HPTLC plate at increasing concentrations to enable identification and quantification of individual phospholipid spots. The plates were developed in a
Camag horizontal developing chamber (Merck) with dichloromethane: ethylacetate: acetone (80: 16: 4) for 70 mm to remove cholesterol, free fatty acids and triglycerides. After drying the plates at a heating plate (40°C, 10 minutes), the phospholipids were separated by chloroform: ethylacetate: acetone: isopropanol: ethanol: methanol: water: acetic acid (30: 6: 6: 16: 28: 6: 2) solution for 55 mm. After drying and cooling the plate, 10 mL charring reagent (7.5% Cu-acetate (w/v), 2.5% CuSO₄ (w/v) and 8% H₃PO₄ (v/v) in water) was applied for 60 seconds. The excess charring reagent was removed and the plate was allowed to complete charring at increasing temperatures (60-140°C), before being analyzed by photodensitometric scanning (GS800 Scanner plus Quantity One Software, version 3.2.2.; Bio-Rad, Veenendaal, The Netherlands). Concentrations of phospholipids were determined using the standard ranges, which were fitted to a nonlinear curve (equation $y = a x/(b + x)$) in GraphPad (GraphPad Prism version 3.0; San Diego, USA). The mean value of a duplicate determination was used.

Statistical analysis

ANOVA with a Bonferroni post hoc test was used to test for differences between groups, in patient characteristics, artery characteristics and phospholipid composition. For the concentration-response curves to BK RM-ANOVA was used to test for differences between the groups. $P \leq .05$ was considered statistically significant. Data are presented as means ± SEM, unless indicated otherwise.

Results

Subjects

Characteristics of the women included in this study from whom plasma samples were collected are presented in table I. In five women with preeclampsia perinatal death occurred. Data of the normal pregnant women from whom myometrial biopsies were obtained are also presented in table I.

Phospholipid composition of microparticles

The total concentration of phospholipids from microparticles did not significantly differ between the three groups (32.7 ± 6.8 μg/mL plasma in microparticles from women with preeclampsia vs 22.8 ± 3.4 μg/mL in microparticles from normal pregnant women and 19.9 ± 2.1 μg/mL in microparticles from nonpregnant women, $P = .16$). In table II the phospholipid composition of microparticles in the different groups is presented. Microparticles in all groups mainly consisted of PC and smaller amounts of SM, PEa, PI and lyso-phospholipids. There were no significant differences in phospholipid concentrations of microparticles between normal pregnant women and women with preeclampsia. Only the percentage lyso-PC was significantly decreased in normal pregnancy and preeclampsia as compared to nonpregnant women ($P < .001$ for both).
Table I. Patient characteristics.

<table>
<thead>
<tr>
<th>Sampling</th>
<th>PE</th>
<th>PR</th>
<th>NP</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>n =</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>30.0 ± 1.5</td>
<td>30.8 ± 1.6</td>
<td>29.3 ± 1.4</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>28.6 ± 1.2</td>
<td>28.7 ± 1.5</td>
<td>-</td>
<td>38.4 ± 0.6</td>
</tr>
<tr>
<td>Parity</td>
<td>0 (0-4)</td>
<td>0.5 (0-4)</td>
<td>0 (0-1)</td>
<td>2 (1-4)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>155 ± 6*</td>
<td>101 ± 3</td>
<td>113 ± 4</td>
<td>116 ± 3</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>99 ± 3*</td>
<td>55 ± 3*</td>
<td>73 ± 3</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>Proteinuria (g/24 h)</td>
<td>4.1 ± 1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Delivery**

<table>
<thead>
<tr>
<th></th>
<th>PE</th>
<th>PR</th>
<th>NP</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (wk)</td>
<td>30.5 ± 1.0*</td>
<td>39.0 ± 0.9</td>
<td>-</td>
<td>38.4 ± 0.6</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>986 ± 187*</td>
<td>3455 ± 180</td>
<td>-</td>
<td>3312 ± 187</td>
</tr>
</tbody>
</table>

Demographic data of the included women with preeclampsia (PE), normal pregnant (PR) and nonpregnant (NP) women that contributed plasma or myometrium. Data are mean ± SEM, except for parity, which are median (range).

* ANOVA, P <.001 vs normal pregnant women

**ANOVA, P =.001 vs nonpregnant women**

Table II. Phospholipid composition of membranes of microparticles.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Preeclampsia</th>
<th>Normal pregnant</th>
<th>Nonpregnant</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso-PC</td>
<td>1.7 ± 0.2*</td>
<td>1.4 ± 0.2*</td>
<td>3.7 ± 0.3</td>
<td>0.0-4.2</td>
</tr>
<tr>
<td>SM</td>
<td>17.7 ± 1.5</td>
<td>20.4 ± 2.8</td>
<td>20.6 ± 1.3</td>
<td>14.0-24.3</td>
</tr>
<tr>
<td>PC</td>
<td>59.3 ± 4.1</td>
<td>48.6 ± 4.5</td>
<td>46.4 ± 4.4</td>
<td>50.9-65.5</td>
</tr>
<tr>
<td>PI</td>
<td>3.0 ± 0.5</td>
<td>3.6 ± 0.5</td>
<td>4.1 ± 0.5</td>
<td>1.5-7.8</td>
</tr>
<tr>
<td>PE</td>
<td>10.8 ± 1.8</td>
<td>11.1 ± 1.9</td>
<td>12.0 ± 1.8</td>
<td>5.2-20.5</td>
</tr>
</tbody>
</table>

Phospholipid composition of circulating microparticles (mean percentage ± SEM) from venous blood of women with preeclampsia (n=8), normal pregnant (n=7) and nonpregnant women (n=7). For comparison the composition of microparticles from healthy controls (range) is also presented [185].

*ANOVA, P <0.001 vs nonpregnant women.

Wire myography

Diameters and contractile properties of the normal pregnant arteries after overnight incubation with microparticles from different patient groups are presented in table III. The mean diameter of
the arteries used for incubation with PSS was significantly lower than the diameter of arteries used for incubation with preeclamptic or normal pregnant plasma. The VP constriction, as a percentage of the reference constriction to NE and potassium, was significantly increased in arteries incubated with PSS compared with arteries incubated with preeclamptic or normal pregnant microparticles. There was no correlation between artery diameter and normalized VP constriction (P = .83).

Arteries incubated with PSS, normal pregnant or nonpregnant microparticles all relaxed to BK to a similar extent (normal pregnant vs PSS P = .39, normal pregnant vs nonpregnant P = .19 and nonpregnant vs PSS P = .72) (figure 1). There was no beneficial or deteriorating effect of 0.5% plasma of any of the women’s plasmas to the PSS incubation. BK-mediated relaxation was significantly impaired after incubation with preeclamptic microparticles compared with normal pregnant (P = .002) and nonpregnant microparticles (P = .05) and with PSS (P = .04) (figure 1).

Table III. Artery characteristics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PE</th>
<th>PR</th>
<th>NP</th>
<th>PSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>497 ± 73</td>
<td>516 ± 50</td>
<td>417 ± 61</td>
<td>283 ± 26*</td>
</tr>
<tr>
<td>Reference constriction (mN/mm)</td>
<td>3.7 ± 0.5</td>
<td>4.0 ± 0.6</td>
<td>3.5 ± 0.5</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Vasopressin constriction (%)</td>
<td>110 ± 2</td>
<td>109 ± 4</td>
<td>113 ± 4</td>
<td>127 ± 5*</td>
</tr>
</tbody>
</table>

Characteristics of the included arteries, incubated with microparticles from the blood of women with preeclampsia (PE), normal pregnant (PR) or nonpregnant (NP) women or with PSS.  
*ANOVA P < .05 vs arteries incubated with preeclamptic and normal pregnant microparticles.

Figure 1. BK-mediated relaxation in isolated myometrial arteries from normal pregnant women after overnight incubation with microparticles from women with preeclampsia (black diamonds), normal pregnant women (open circles) or nonpregnant women (black triangles) or with PSS (crosses).
Comments

We found that microparticles isolated from venous blood of women with preeclampsia significantly impair endothelium-dependent relaxation of isolated myometrial arteries from normal pregnant women after overnight incubation, in contrast to microparticles derived from normal pregnant or nonpregnant women. This difference did not seem to be due to an altered phospholipid composition of these microparticles, since there were no significant differences in phospholipid composition between microparticles from venous blood of women with preeclampsia and normal pregnant women. The concentration of lyso-PC, however, was significantly lower in microparticles from normal pregnant women as compared to nonpregnant women.

The phospholipid composition of circulating microparticles from women with preeclampsia, normal pregnant and nonpregnant women closely resembled the composition of microparticles in blood from healthy controls [185]. As already outlined in the introduction, the composition of these microparticles clearly differed from that of platelet plasma membranes [270], erythrocyte ghosts [185] and leukocyte microparticles in inflamed joints [165]. Previously, we established the cellular origin of circulating microparticles from preeclamptic patients. These microparticles predominantly originated from platelets (approximately 85%), erythrocytes (8%) and leukocytes (6%) (see chapter 5). The fact that microparticles from human plasma samples contain elevated concentrations of both PC and SM compared to platelet plasma membranes and erythrocyte ghosts, suggests that PC and SM may be selectively released into the microparticle membranes.

The microparticle fractions studied all contained detectable quantities of lyso-PC, with the highest level in nonpregnant women. Lyso-PC is formed by hydrolysis of PC by secretory phospholipase A₂. Since secretory phospholipase A₂ levels are known to be increased in blood from preeclamptic women [271,272], especially microparticles from these women were expected to contain elevated levels of lyso-PC. However, one has to bear in mind that also lysophospholipase activity is increased in preeclamptic plasma [273]. Thus, the overall action of phospholipase and lyso-phospholipase activities on microparticles is difficult to predict. Lysophospholipids, in particular lyso-PC, are of interest, since they affect endothelial function. In the present study, however, no relation was observed between the lyso-PC levels and endothelial dysfunction. Since we measure the overall phospholipid composition, there is still the possibility that large variations occur between individual microparticles (or subpopulations thereof) with regard to their, e.g. lyso-PC, contents. Therefore, the possibility cannot yet be excluded that particular subpopulations of microparticles, containing elevated concentrations of lyso-PC, affect endothelial function in vivo and in vitro.

It is important to realize that the fact that we found no differences in the phospholipid composition of circulating microparticles between normal pregnant and preeclamptic women does not warrant the conclusion that there are no differences. Due to the small sample size of this pilot study only major differences would probably be detected. Small differences may thus be present between the groups. Furthermore, we did not determine the concentrations of all phospholipids that could be present, but only of the ones that were most likely to be present and
we did not determine the exact fatty acid composition or the oxidation status of the detected phospholipids. Since we determined the phospholipid composition of all circulating microparticles, differences in the phospholipid composition of subgroups of microparticles of different cellular origin could not be detected.

An alternative pathway through which phospholipids in microparticles may be involved in vascular dysfunction in preeclampsia is through their oxidation status. Huber and coworkers recently reported the presence of oxidized phospholipids in microparticles released from endothelial cells exposed to an oxidative stress stimulus or undergoing apoptosis, as well as in microparticles exposed to oxidative stress after their formation. These oxidized phospholipids were absent in microparticles released from cells stimulated with a non-oxidative stimulus. The presence of these oxidized phospholipids in microparticles was related to attraction of monocytes to the endothelium [172]. Oxidized PC also causes impairment of endothelium-dependent relaxation in isolated bioassay arteries [238]. Since preeclampsia is accompanied by oxidative stress [274], and administration of antioxidants vitamins C and E to women at high risk of developing preeclampsia reduced the incidence of preeclampsia dramatically [77], oxidized phospholipids may be involved in the generation of vascular dysfunction in preeclampsia. Future research will hopefully clarify this issue.

We conclude that microparticles isolated from the blood of women with preeclampsia diminish endothelium-dependent relaxation in isolated myometrial arteries from normal pregnant women, in contrast to microparticles isolated from the blood of normal pregnant and nonpregnant women. This difference does not seem to result from differences between groups in the overall phospholipid composition of the microparticles, but more extensive studies are required to elucidate the role of phospholipids in the generation of vascular dysfunction in preeclampsia.