Vascular dysfunction in preeclampsia
van Wijk, M.J.

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Chapter 4

Endothelial function in myometrial resistance arteries of normal pregnant women perfused with syncytiotrophoblast microvillous membranes


Abstract

Objective: To investigate the effects of STBM in concentrations, found \textit{in vivo} in women with preeclampsia, on endothelial function in isolated resistance arteries.

Methods: Twenty-nine myometrial resistance arteries were isolated from biopsies of healthy term pregnant women obtained during cesarean delivery. The arteries were mounted in a pressure arteriograph and perfused intraluminally for three hours with STBM (20 to 2000 ng/mL) or with erythrocyte membranes or PSS as controls, all substituted with 0.5% bovine serum albumin (BSA). BK concentration-response curves were performed before and after perfusion. The BK concentration-response curves were fitted to the Hill equation and maximal dilatation and the pEC$_{50}$ values were determined from these fits. Differences within groups were analyzed with a paired Student's $t$ test. Electron microscopic evaluation of the endothelium was performed.

Results: Neither STBM nor erythrocyte membrane perfusion affected maximal dilatation or the pEC$_{50}$ values of the BK concentration-response curves at any concentration. Examination by electron microscopy showed no obvious damage to the endothelium after perfusion with STBM or erythrocyte membranes.

Conclusion: Perfusion with STBM in concentrations up to 100 times those reported in preeclampsia has no significant effect on BK-mediated dilatation in isolated myometrial arteries.
**Introduction**

Preeclampsia is a multifactorial disease that remains a leading cause of maternal and fetal morbidity and mortality. The etiology of this syndrome is still unclear. In early pregnancy, defective placentation appears to be the central feature of the disease. Multiple factors of fetal and maternal origin are likely to influence placentation, resulting in a reduced blood flow to the placenta, thereby causing a relative placental hypoxia, leading to the release of one or more, yet unidentified, factors into the maternal circulation. These circulating factors then cause the generalized maternal endothelial dysfunction observed in the late phase of preeclampsia, when clinical symptoms develop. Indeed, several studies have shown that the serum and plasma of women with preeclampsia can alter endothelial cell function, indicating the presence of such factors in the maternal blood [53, 57, 130, 243].

STBM have been proposed to be one of the factors linking the defective placentation to the endothelial dysfunction [65]. Morphologic evaluation of placentas of women with preeclampsia shows abnormally shaped syncytiotrophoblast microvilli and areas of focal necrosis, associated with a reduced number of microvilli [60]. These changes resemble those seen in placental villi cultured under hypoxic conditions [61]. *In vitro* experiments showed that STBM can interfere with endothelial cell growth in cultured endothelial cells by suppressing proliferation and disrupting the cell monolayer [65] and that STBM prepared from placentas of normal pregnant women and of women with preeclampsia had similar effects on cultured endothelial cells, indicating the effect of STBM to be a quantitative rather than a qualitative effect [65]. Furthermore, perfusion of isolated subcutaneous arteries from normal pregnant women with STBM abolished ACh-induced vasodilatation [66]. The concentration of STBM used in these experiments, however, far exceeded those determined by Knight et al, who studied the presence of STBM in the maternal circulation in pregnancy and preeclampsia [64]. They reported the concentrations of STBM to be significantly increased in the blood of women with preeclampsia, with the concentration in the uterine vein exceeding that in the peripheral venous circulation, confirming the placental origin of the STBM [64].

The aim of this study was to assess whether STBM at concentrations similar to those found *in vivo* in women with preeclampsia represent a pathogenic pathway for the impaired endothelial function, observed in the resistance vasculature in preeclampsia. We chose to study the effect of STBM on the myometrial resistance vasculature, not only since myometrium is readily available in normal pregnant women undergoing cesarean delivery, but also since uteroplacental blood flow has been reported to be impaired *in vivo* [244] in women with preeclampsia, and endothelium-dependent dilatation was found to be impaired *in vitro* in isolated myometrial arteries from women with preeclampsia [88, 129, 245, 246]. As a measure of endothelial dysfunction we used BK-mediated dilatation, since various studies have shown BK-mediated dilatation to be diminished in the myometrial vascular bed in preeclampsia [129, 245, 246].
Methods

Membrane preparation

STBM were prepared using a modified method of Smith et al. [247] described by Smarason et al. [65] from three placentas of healthy pregnant women who were delivered at term by elective cesarean delivery. After preparation, STBM were dissolved in PBS and kept frozen until required. Erythrocyte membranes were prepared according to the method described by Khalfoun et al. from the blood of three healthy men [248]. Erythrocyte membranes were also dissolved in PBS and kept frozen until required. STBM and erythrocyte membrane solutions were both prepared by one of the authors (A.K. Smarason) according to the same methods and with the same equipment used to prepare STBM and erythrocyte membranes for previous experiments [65,66]. When required, STBM and erythrocyte membranes were thawed, filtered through a 20 μm fabric filter in order to remove aggregates and then diluted 1:100 in PSS to form a stock solution. A sample was then taken for quantification of protein content of the STBM and erythrocyte membrane solutions, and assessed using a bicinchoninic acid protein assay (Pierce, Rockford, USA). The protein contents of the STBM and erythrocyte membrane solutions used in our experiments were similar to those used in previous experiments [65,66] (internal communications). In pilot experiments, in the absence of albumin, it became clear that STBM as well as erythrocyte membrane fragments aggregate and stick to the arteries and the cannulas, thereby interfering with the perfusion. Thus, obstruction of the upstream and downstream pipettes caused irregular and uncontrolled perfusion in our servo-controlled perfusion system. In three arteries in which perfusion with STBM 20 ng/mL (n=1) and 200 ng/mL (n=2) was attempted, the endothelium was visibly damaged and BK-mediated dilatation was reduced. A reduced BK-mediated dilatation was also observed after perfusion with erythrocyte membranes 20 ng/ml (n=1). To prevent this unphysiological aggregation of particles and the resulting failure of perfusion, 0.5% BSA was added to the stock solution after taking the sample for protein quantification. Stock solutions were kept, while continuously being stirred at 4°C. Before the start of the perfusion the stock solution was dissolved in 0.5% BSA-substituted PSS to obtain the desired concentration of STBM and erythrocyte membranes.

Subjects

This study was performed after approval by the Ethical Committee in Huddinge University Hospital, Sweden. Healthy pregnant women (n=18), 4 nulliparous and 14 multiparous were included in the study after obtaining informed consent. Their mean age was 32 years (range 20 to 40) and their mean gestational age was 38.5 weeks (range 37 to 41). The women were undergoing elective cesarean delivery for the indications breech presentation (n=4), previous cesarean delivery (n=6), cephalo-pelvic disproportion (n=3) and psychosocial indications (n=5). None of the women was on any medication except for iron supplementation. A biopsy of the myometrium was taken immediately following delivery from the upper edge of the transverse incision in the lower uterine segment and placed in ice-cold PSS at once. The biopsy site was
never the site of the placental bed and in case of a repeat cesarean delivery care was taken that the biopsy contained healthy myometrial tissue. From 10 biopsies more than one artery was used (in 2 cases 3 arteries and in 8 cases 2 arteries). In these cases one artery was perfused with STBM, another with erythrocyte membranes and if possible still another with PSS. From 1 biopsy no arteries could be obtained. Chemicals were prepared as stock solutions and all concentrations are expressed as final concentrations in the system.

**Experimental protocol**

Intramyometrial resistance arteries were identified and a 2.5-3 mm long segments were carefully dissected from the surrounding tissue. They were then mounted in a pressure arteriograph (Living Systems Instrumentation Inc, Burlington, USA). Special care was taken to ensure that the direction of flow *in vitro* was the same as the direction *in vivo*. The organ bath was perfused with PSS, kept at 37°C and gassed with 5% CO₂ in O₂. The intraluminal perfusion fluid consisted of 0.5% BSA-substituted PSS. The arteries were pressurized to 60 mmHg and stretched until they appeared straight. A servo-controlled pump maintained the required intraluminal pressure, using an ‘in line’ pressure transducer to monitor the proximal and distal pressure. The internal diameter of the artery was recorded continuously using a video dimension analyzer. Arteries were excluded when they failed to maintain pressure due to leakage or to demonstrate occlusion of the lumen in response to NE 1 µmol/L in potassium-substituted PSS (64 mmol/L KCl in PSS).

After an equilibration period of approximately 45 minutes NE 1 µmol/L was added to the superfusate for 15 minutes to achieve a stable constriction. Subsequently a BK concentration-response curve was constructed by addition of increasing concentrations of BK (final concentrations 1 nmol/L to 1 µmol/L) in NE-substituted PSS, application of each concentration lasting three minutes. After a wash out period of 15 minutes intraluminal flow was initiated, using a flow pump. The proximal and distal pressures were adjusted in order to keep the intraluminal pressure constant at 60 mmHg. The arteries were perfused intraluminally for three hours at a flow rate of 75 µL/min with STBM at concentrations of 20 ng/mL (n=4), 200 ng/mL (n=6) or 2000 ng/mL (n=6), or as controls with erythrocyte membranes at concentrations of 200 ng/mL (n=4) or 2000 ng/mL (n=3) or with BSA-substituted PSS (n=6). After a three-hour perfusion the intraluminal perfusate was washed out for 15 minutes with BSA-substituted PSS. Arteries were again preconstricted with NE 1 µmol/L, and the BK concentration-response curve was repeated. To obtain the passive diameter of the artery, the artery was superfused with a calcium-free PSS, containing 1 mmol/L EGTA and 0.1 mmol/L papaverine. After finishing the protocol the arteries were fixated in Karnovsky’s solution (2.5% glutaraldehyde and 2% paraformaldehyde in PBS, pH 7.2) at 60 mmHg in the pressure myograph and then stored for later electron microscopic evaluation.
Data analysis

Analysis of variance (ANOVA) was used to test for differences in passive diameter of the arteries between groups. BK-mediated dilatation was calculated as the dilatation to BK after preconstriction as percentage of the maximal possible dilatation by the formula: \( (D_{\text{BK}}-D_{\text{NE}})/(D_{p}-D_{\text{NE}})) \times 100\% \), where \( D_{\text{BK}} \) = diameter with BK, \( D_{\text{NE}} \) = diameter after preconstriction with NE and \( D_{p} \) = passive diameter. BK concentration-response curves were analyzed by non-linear regression to a Hill curve (GraphPad Prism, version 3.0; San Diego, USA). For each individual curve the maximal dilatation to BK and the pEC\(_{50}\) value, the -log molar concentration of BK needed to produce 50% of the maximal dilatation, were estimated. When the estimated maximal dilatation was greater than 100%, maximal dilatation was set at 100% and a new best fitting curve was calculated. Estimated maximal dilatations to BK and the pEC\(_{50}\) values before and after perfusion were compared with paired student’s t tests and the 95% confidence intervals of the mean differences were calculated (SPSS Statistics, version 8.0.2). Differences were assumed statistically significant when \( P < .05 \). All data are presented as the mean (SD), unless stated otherwise.

Results

There were no differences between the 6 experimental groups in the passive diameter of the arteries. Mean (range) diameters of arteries perfused with STBM 20, 200 and 2000 ng/mL were 390 (320-500), 346 (262-469) and 350 (187-430) \( \mu \)m respectively. Mean (range) diameters of arteries perfused with erythrocyte membranes 200 and 2000 ng/mL were 373 (188-506) and 381 (325-457) \( \mu \)m. Mean (range) diameters of arteries perfused with PSS were 477 (295-699) \( \mu \)m.

There was no visible aggregation of STBM or erythrocyte membranes on the surface of the arteries. Myometrial arteries showed a concentration-dependent dilatation to BK. This response to BK was reproducible after three hours of perfusion with BSA-substituted PSS (figure 1). BK concentration-response curves for STBM 20, 200 and 2000 ng/mL and control substances erythrocyte membranes 200 and 2000 ng/mL all showed BK-mediated dilatation before and after perfusion (figure 1). One artery in the STBM 200 ng/mL group showed no dilatation at all after perfusion. Since all other arteries perfused with STBM 200 and 2000 ng/mL (n=11) showed levels of BK-mediated dilatation after perfusion similar to the levels before (with maximal dilatation after perfusion in these arteries varying between 71.2% and 98.7%), this extremely outlying artery was removed from the data set.

Estimated maximal dilatation to BK and pEC\(_{50}\) values were not significantly different before and after perfusion with STBM in concentrations of 20, 200 and 2000 ng/mL, as well as after perfusion with control substances BSA-substituted PSS, erythrocyte membranes 200 and 2000 ng/mL (table I).
Figure 1. Percentage dilatation to BK in concentrations of 1 nmol/L to 1 μmol/L after preconstriction with NE 1 μmol/L before (black circles) and after (open circles) a three-hour perfusion with 0.5% BSA-substituted PSS, erythrocyte membranes (RBCM) 200 and 2000 ng/mL, and STBM 20, 200, and 2000 ng/mL. Data points represent the mean (SEM). The curves shown are the best fitting curves for the means, calculated by non-linear regression.
Table I. Endothelial function.

<table>
<thead>
<tr>
<th></th>
<th>Max Dil</th>
<th>95% CI</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Max Dil</td>
<td>Before</td>
</tr>
<tr>
<td>STBM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 ng/mL</td>
<td>93.4 (5.3)</td>
<td>91.4 (1.2)</td>
<td>-6.9 - 10.8</td>
<td>7.09 (0.59)</td>
</tr>
<tr>
<td></td>
<td>[87.1 – 100]</td>
<td>[90.4 – 93.1]</td>
<td></td>
<td>[6.28 – 7.62]</td>
</tr>
<tr>
<td>200 ng/mL</td>
<td>98.7 (2.2)</td>
<td>87.8 (10.0)</td>
<td>-3.0 - 24.9</td>
<td>7.30 (0.86)</td>
</tr>
<tr>
<td></td>
<td>[94.8 – 100]</td>
<td>[76.3 – 98.7]</td>
<td></td>
<td>[6.05 – 8.38]</td>
</tr>
<tr>
<td>2000 ng/mL</td>
<td>96.4 (5.5)</td>
<td>87.4 (10.4)</td>
<td>-6.0 - 23.8</td>
<td>7.44 (1.15)</td>
</tr>
<tr>
<td></td>
<td>[86.1 – 100]</td>
<td>[71.2 – 95.1]</td>
<td></td>
<td>[6.37 – 9.08]</td>
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<tr>
<td>RBCM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 ng/mL</td>
<td>89.7 (14.4)</td>
<td>98.1 (3.8)</td>
<td>-32.0 - 15.2</td>
<td>6.64 (0.89)</td>
</tr>
<tr>
<td></td>
<td>[69.5 – 100]</td>
<td>[92.5 – 100]</td>
<td></td>
<td>[5.45 – 7.50]</td>
</tr>
<tr>
<td>2000 ng/mL</td>
<td>70.7 (7.4)</td>
<td>95.2 (8.3)</td>
<td>-62.3 - 13.2</td>
<td>7.66 (0.54)</td>
</tr>
<tr>
<td></td>
<td>[63.3 – 78.2]</td>
<td>[85.7 – 100]</td>
<td></td>
<td>[7.27 – 8.28]</td>
</tr>
<tr>
<td>PSS</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>96.7 (5.9)</td>
<td>97.3 (5.8)</td>
<td>-2.3 - 1.0</td>
<td>7.16 (0.53)</td>
<td>7.39 (0.71)</td>
</tr>
<tr>
<td></td>
<td>[63.3 – 100]</td>
<td>[85.5 – 100]</td>
<td></td>
<td>[6.51 – 7.73]</td>
</tr>
</tbody>
</table>

Mean percentages of estimated maximal dilatation (Max Dil) to BK after NE-induced constriction (SD) [range] and mean pEC<sub>50</sub> values (-log molar BK concentration) (SD) [range] and 95% confidence intervals (95% CI) of the differences between the measurements before and after a three-hour perfusion with STBM, erythrocyte membranes (RBCM) and BSA-substituted PSS.

Electron microscopic examination of longitudinal sections of the arteries showed no differences in the aspect of the endothelium in arteries perfused with STBM 2000 ng/mL, erythrocyte membranes 2000 ng/mL and BSA-substituted PSS. The endothelial lining appeared intact and similar in all arteries and no adherence of STBM or erythrocyte membranes to the endothelial surface was seen (data not shown).

Comments

This study could not demonstrate any effect of STBM in concentrations found in women with preeclampsia on BK-mediated dilatation in isolated myometrial arteries. Therefore, if this finding applies to the entire maternal circulation, the endothelial dysfunction observed in preeclampsia would not be caused by a direct effect of STBM on the endothelial cells.

The STBM concentrations we chose to test were based on the findings of Knight et al. [64]. These authors found the STBM concentration in women with preeclampsia to be 28.3 ng/mL in
the uterine vein and 10.0 ng/mL in the peripheral venous circulation. Considering the possibility that a proportion of the deported STBM is trapped in the capillary bed, we estimated the STBM concentration in peripheral arteries to be 20 ng/mL. In addition the higher concentrations of 200 and 2000 ng/mL were tested.

One could argue that the three-hour incubation time was too short to induce endothelial dysfunction, since elevation of STBM in the maternal circulation of women with preeclampsia has been reported to be present already 14 days before delivery [64]. However, in the study on cultured endothelial cells by Smarason et al. [65] disruption of the cultured endothelial cell monolayer by STBM occurred as early as after two hours of incubation, and ACh-mediated dilatation in subcutaneous arteries was also reduced after a two-hour perfusion with STBM, although the STBM concentration used in this study was very high [66]. Recently, Hayman et al. [249] reported that incubation of myometrial arteries with 2% plasma from women with preeclampsia caused a reduced BK-mediated dilatation already after an incubation period of one hour. Thus, the fact that in the current study perfusion with STBM in concentrations up to 100 times the estimated concentrations in women with preeclampsia for three hours did not cause an altered BK-mediated dilatation indicates that it is unlikely that exposure to the pathophysiological concentration for a longer duration would have resulted in alteration of the responses.

Our choice of BK-mediated dilatation to assess endothelial function was based on the fact that several groups have shown BK-mediated dilatation to be impaired in various vascular beds in preeclampsia, including the myometrial vascular bed [87,129,245,246]. Therefore, the fact that we found no direct effect of STBM on BK-mediated dilatation in the myometrial vascular bed further weakens the arguments for the involvement of STBM in development of endothelial dysfunction in preeclampsia. The possibility that the myometrial circulation is relatively insensitive to the actions of STBM, because of their location in vivo in the proximity of syncytiotrophoblast tissue, is doubtful due to the fact that STBM are released into the uterine venous circulation and only reach the myometrial arterial circulation, after circulating through the lung capillary bed, in concentrations that are probably similar to those in the other resistance vascular beds. One could thus expect STBM to induce similar responses in myometrial and subcutaneous arteries.

In pilot experiments, without addition of albumin to the perfusate, we observed aggregation of particles as described in the Methods section. The particles stuck to the arteries and cannulas, thereby blocking perfusion and damaging the endothelium visibly, resulting in the reduction of BK-mediated dilatation in these arteries. Such aggregation is not pathophysiologically relevant, since luminal albumin prevents such clotting in vivo. Indeed, when we added a low concentration of albumin, all particles remained in suspension, and we were able to maintain adequate perfusion of the vessel in both the STBM and erythrocyte membrane groups. The albumin concentration in the maternal circulation in pregnancy usually is 3.1% to 4.6%. Although albumin concentrations in women with preeclampsia can decrease, especially in the presence of edema and proteinuria, levels remain far above the 0.5% that we used.
From the results of our study we may conclude that STBM per se play no major role in the development of endothelial dysfunction in the uterine circulation during preeclampsia. It is however possible that these particles do play a role in combination with other factors. When STBM are released into the maternal circulation they not only come in contact with the endothelium but also with maternal blood cells. Leucocytes have been reported to be activated in preeclampsia [250,251]. Knight showed that STBM-like material can be found on monocytes [252], which could form a mechanism for direct activation of monocytes and possibly other immune cells. Furthermore, Von Dadelszen et al. [76] demonstrated that when STBM are incubated with cultured endothelial cells, a substance is produced that activates peripheral leucocytes and primes peripheral monocytes to give greater responses after activation. Activation of leukocytes can induce production of inflammatory cytokines and other substances, resulting in an increase in oxidative stress, thereby creating an alternative pathway for induction of endothelial dysfunction [68]. Ongoing research in our laboratories will in the future hopefully elucidate the role of STBM-leukocyte interactions in the development of endothelial dysfunction in preeclampsia and help us to obtain a better understanding of preeclampsia and to develop new intervention strategies.

In conclusion, we could not show a direct effect of STBM on BK-mediated dilatation in isolated myometrial resistance arteries after a three-hour perfusion with STBM in concentrations up to 100 times higher than the concentration reported \textit{in vivo} in preeclampsia. If these results in myometrial arteries can be extrapolated to the general circulation, these data argue against a direct role for STBM in the etiology of endothelial dysfunction in preeclampsia.

We would like to thank B.M. van den Berg for his help with electron microscopy.