Decreased plasma hemopexin activity is associated with increased placental and monocyte AT-1 receptor expression and lower plasma AT-1 receptor in preeclampsia

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In preparation
Abstract

Introduction: Hemopexin (Hx) is a free heme scavenger, which also has protease activity. During healthy pregnancy, Hx activity increases, while in women with preeclampsia, Hx activity was decreased. This is likely due to high levels of its natural inhibitor, ATP. As active Hx induced AT-1R shedding in vitro, it was suggested that decreased Hx activity in preeclampsia leads to higher AT-1R expression and subsequently increased angiotensin II sensitivity. In the current study, we aimed to evaluate whether changes in Hx activity during pregnancy and in preeclampsia correlated to in vivo shedding of the AT-1R.

Methods: Hx activity, as well as plasma ATP levels and alkaline phosphatase (AP) activity, were measured in nonpregnant, healthy pregnant and early- and late-onset preeclamptic women. In addition, angiotensin II type 1 receptor (AT-1R) expression on peripheral blood monocytes and in the placenta was determined using flow cytometry and immunohistochemistry. Plasma AT-1R levels were measured with western blotting.

Results: Plasma Hx and AP activity increased in healthy pregnant women compared with nonpregnant women. This associated with lower monocyte AT-1R expression and higher plasma AT-1R levels. Plasma Hx activity decreased and ATP levels increased in women with preeclampsia, while placental and monocyte AT-1R expression was increased and plasma AT-1R levels were decreased in preeclamptic women.

Conclusions: Our data suggest that also in vivo active Hx activity sheds the AT-1R from tissue, such as monocytes and placenta. Therefore active Hx may play a role in the development of the decreased responsiveness to angiotensin II in healthy pregnancy, while the decreased Hx activity in preeclampsia may result in increased angiotensin II sensitivity.
Introduction

Hypertension and proteinuria in the second half of pregnancy are the main symptoms of preeclampsia, the most common pregnancy complication. Preeclampsia occurs in around 3-5% percent of all pregnancies [1] and, due to lack of knowledge about the exact pathophysiology, an adequate therapy for the disease is still unavailable. Symptoms of preeclampsia may develop early (before 34 weeks) and late (after 34 weeks) in pregnancy. Although similar maternal symptoms are observed in these two subtypes of preeclampsia, the pathophysiologies of early- and late-onset disease have been assumed to be different [2,3]. In early-onset preeclampsia, which has been mostly studied, a central role for the placenta in the pathogenesis has been recognized. Poor placentation in the first trimester is thought to lead to placental oxidative stress and hypoxia, which may result in the production of various stress-related factors such as anti-angiogenic factors, cytokines, microparticles and DAMPs (like extracellular ATP) [4,5] from the placenta. These are thought to contribute to systemic inflammation and endothelial dysfunction, which then leads to hypertension and proteinuria [4]. In contrast, less is known about the pathophysiology of late-onset disease, but it was shown to be associated with various maternal factors, such as diabetes mellitus, a BMI >25 and a family history of hypertension [3,6,7]. Signs of hypoxia have been shown in the placenta of late onset preeclamptic women [8]. It has recently been suggested that this may be due to poor placentation, but in a milder form [9]. Therefore problems with placental perfusion and symptoms of preeclampsia may not arise until the end of pregnancy in these women [9].

Hemopexin (Hx) is a plasma protein that is mainly known as a free heme scavenger [10]. However, Hx was recently found to have serine protease activity [11]. This activity could be inhibited by extracellular nucleotides, such as ATP [11], and increased by ATP hydrolysing enzymes such as alkaline phosphatase (AP) [11]. Interestingly, during healthy pregnancy, the protease activity of Hx was shown to be increased [12]. Hx activity started to rise early in pregnancy (week 10-15) and remained high until the end of pregnancy [13]. Although the physiological role of increased Hx activity during pregnancy is not exactly known, we have recently shown that active Hx can shed the Angiotensin II type 1 receptor (AT-1R) from endothelial cells and monocytes [13], suggesting that the increased Hx activity may contribute to decreased sensitivity to angiotensin II [14]. This suggestion is in line with the observation that the timing of the increase in Hx activity coincided with the timing of the decrease in angiotensin II sensitivity, i.e. both processes start around week 10-15 of pregnancy [15].

In women with early-onset preeclampsia, decreased Hx activity was found in plasma. This decreased Hx activity was suggested to be due to increased plasma levels of ATP, its natural inhibitor [13]. We hypothesized that the decreased Hx activity in preeclamptic women may lead to decreased shedding of the AT-1R from the vascular wall [13] and therefore result in
increased angiotensin II sensitivity, which is specific for this condition [14,15]. Increased angiotensin II sensitivity may play a role in the development of hypertension in preeclampsia [14,15].

Based on the above mentioned observations, we hypothesize that the increase in Hx activity in healthy pregnancy would result in increased shedding of the AT-1R in pregnant women. In contrast, in women with preeclampsia, the decreased plasma Hx activity would lead to decreased shedding of the AT-1R. Therefore, in the present study, we investigated plasma Hx activity, ATP levels, AP activity, AT-1R expression on peripheral blood monocytes and the placenta and plasma AT-1R levels, in nonpregnant, pregnant and early- and late-onset preeclamptic women.

**Methods**

**Patients**

The present study was approved by the medical ethics committee of the University Medical Center Groningen. Written informed consents were obtained from all patients. Patients with early-onset and late-onset preeclampsia, and healthy pregnant controls were recruited from the antenatal ward of the University Medical Center Groningen. Nonpregnant controls were recruited from the hospital staff. Preeclampsia was defined according to the standards of the International Society for the Study of Hypertension in Pregnancy (ISSHP): diastolic blood pressure of 90 mmHg or more on two or more consecutive occasions more than 4 hours apart and proteinuria of more than 300 mg/24 hours [16]. Early-onset preeclampsia was defined as the onset of preeclampsia before 34 weeks, while late-onset preeclampsia was defined as the onset of preeclampsia after 34 weeks.

**Blood samples:** From early and late-onset preeclamptic patients, blood samples were taken during routine sampling at the hospital. Blood samples from healthy pregnant controls were gestational age matched with the preeclampsia groups. Exclusion criteria for all groups were pre-existing hypertension, diabetes mellitus, vasculitis, renal disease, autoimmune disease, malignancies or women who had recent trauma or surgery. See Table 1 for patient characteristics. Blood samples were drawn from the antecubital vein into 10 mL tubes containing EDTA (BD Biosciences, Breda, the Netherlands) or heparin (BD Biosciences). After this, part of the heparin blood was used for flow cytometry to determine AT-1R expression on monocytes (see below). The remaining heparin blood and the EDTA blood samples were immediately centrifuged twice (1200 rpm, 10 min. at 4°C followed by 2500 rpm, 10 min. at 4°C), and plasma was frozen at -80°C until further analysis.

**Placental biopsies:** We collected placenta samples from early-onset preeclamptic women and late-onset preeclamptic women. As all women with early-onset preeclampsia delivered by caesarean section, for controls we collected placentas from women who delivered by caesarean section for other reasons than preeclampsia (for instance breech presentation). Most of the women with late-onset preeclampsia delivered vaginally, therefore for control we
collected placentas from healthy pregnant women who delivered vaginally. See Table 2 for patient characteristics.

Immediately following delivery, six tissue samples for immunohistochemistry (blocks of 1 by 1 cm) were randomly taken from the chorionic villi of each placenta after stripping the decidua, and were snap frozen in liquid nitrogen and stored in -80°C until further processing. For RNA isolation, 5 small biopsies were taken randomly from the chorionic villi (without decidua), pooled and snap frozen and stored in -80°C until further processing.

**Plasma Hx activity with Hx substrate**

Using a glomerular ECM stripping assay we have previously shown that the plasma Hx activity increased during pregnancy and is lower in preeclamptic patients [12]. More recently, based on studies in which we evaluated the site at which the protease activity of Hx clips proteins, we developed a substrate for measuring Hx activity, the fluorescent substrate Hx-MCA, which was synthesized by Pepscan (Pepscan, Lelystad, the Netherlands). With this specific fluorescent substrate Hx activity was measured in plasma from early and late pregnant women and in plasma from early and late-onset preeclamptic patients.

**Specificity of fluorescent Hx substrate**

To test the substrate, we first tested whether recombinant Hx (rHx) and various Hx fractions purified from plasma (as described previously [11,17]) were able to cut this substrate.
Therefore, rHx (0.5 mg/ml protein) and Hx313, Hx338, Hx23, HxF (2 mg/ml protein) were diluted 1:10 and incubated with 200 µl dilution buffer (0.2M Tris + 0.9% NaCl pH 7.6) with 80 µM substrate (diluted 1:50). The activities of these preparations were also tested using the standard Hx stripping assay as described previously [17].

To test the specificity of this novel substrate for Hx activity, other well-known proteases present in the plasma were incubated with the fluorescent substrate Hx-MCA. Kallikrein (diluted 1:100; Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands), factor X (diluted 1:100; Siemens Healthcare) and thrombin (diluted 1:7; Siemens Healthcare, Den Haag, the Netherlands) were diluted with 200 µl substrate solution (dilution buffer with 80 µM substrate for Hx; diluted 1:50) in a 96-wells plate and emission was measured at 460 nm on a Varioskan spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

As Hx has serine protease activity, we further tested whether Hx activity on the substrate was inhibited with protease inhibitors phenylmethanesulfonyl fluoride (PMSF; 2mM; Sigma-Aldrich), Leupeptin (20 µg/mL; Roche Nederland BV, Woerden, the Netherlands) and Aprotinin (0.5 U/mL; Sigma-Aldrich), and Matrix metalloproteinases (MMP) inhibitors BB3103 (8 µM, Britisch Biotech (Vernalis), Cambridge, UK) and Galardin (10 µM, Abmole Biosciences, Hong Kong). The inhibitors were pre-incubated with Hx (plasma derived fraction; diluted 1:10 in dilution buffer) in a 96 wells plate for 30 min. in a total volume of 100 µl. Then 100 µl substrate was added to each well (diluted 1:25 with dilution buffer) and emission was measured as described above.

Table 2. Patient characteristics of early and late-onset preeclamptic women and healthy pregnant control women from which placental tissue was obtained.

<table>
<thead>
<tr>
<th></th>
<th>Pregnant (controls for early PE)</th>
<th>Preeclampsia (early-onset)</th>
<th>Pregnant (controls for late PE)</th>
<th>Preeclampsia (late-onset)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of women</td>
<td>15</td>
<td>24</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>Maternal age (Y)</td>
<td>33.7 (±5.1)</td>
<td>32 (±5.5)</td>
<td>33.5 (±7.6)</td>
<td>32.8 (±4.1)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>127 (±11)</td>
<td>175 (±19)*</td>
<td>113 (±14)</td>
<td>158 (±18)*</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>81 (±5)</td>
<td>111 (±9)*</td>
<td>73 (±9)</td>
<td>99 (±12)*</td>
</tr>
<tr>
<td>Proteinuria (g/24 hr)</td>
<td>NA</td>
<td>3.53 (±3.5)*</td>
<td>NA</td>
<td>1.0 (±0.6)</td>
</tr>
<tr>
<td>Delivery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>39 (38-41)</td>
<td>29 (26-32)*</td>
<td>40 (37-41)</td>
<td>37 (32-40)*</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3759 (±650)</td>
<td>1057 (±360)*</td>
<td>3544 (±541)</td>
<td>2634 (±604)*</td>
</tr>
</tbody>
</table>

Means with max and min values are shown for the gestational age, means with standard deviations are shown for the other parameters. NA: not applicable. * p<0.05, significantly different compared with healthy pregnant group.
Measurement of plasma Hx activity
Plasma Hx activity was measured in EDTA plasma samples using the Hx-MCA substrate (Pepscan). The plasma samples were diluted 1:3 with the substrate solution (dissolved as described above) to a final volume of 200 µl (diluted 1:25, 0.16 mM) at 37°C. The emission was measured at 460 nm on a Varioskan spectrophotometer (Thermo Fisher) after 30 min. of incubation (at 37°C).

Measurement of plasma Hx concentration
To check whether the changes in Hx activity were not due to different plasma levels of Hx, we analysed Hx protein concentration in our plasma samples. The Hx concentration was measured in the plasma samples using a human Hemopexin ELISA (Sino Biological Inc., Beijing, China), according to the manufactures instructions.

Plasma ATP levels
Plasma ATP levels were measured as described previously [12].

Total and placental plasma AP activity
Total AP activity was analysed in heparin plasma samples. The plasma samples were diluted (1:40) with a mixture of buffer and substrate (Tris 0.1 M pH 9.8 with MgCl2 2 mM and para-nitrophenylphosphate (PNPP) substrate 1.25 mM) in a 96-wells plate. Directly after adding this substrate solution, absorbance at 405 nm was measured on a Varioskan spectrophotometer (Thermo Fisher) every minute for 30 min. The increase in absorbance every minute was calculated, and the average increase in the 30 minutes resembled the AP activity. A standard curve ranging from 500 to 0 U/L AP activity (bovine intestinal AP; AM-Pharma, Bunnik, the Netherlands) was used to calculate the results. As the placenta produces AP during pregnancy, placental AP activity was also analysed according to the same protocol. Since placental AP is the only AP isoform that is heat resistant and other isoforms are inactivated by heating [18], to specifically detect placental AP activity, part of the plasma samples were heated to 65°C for 1 hour preceding the above described protocol.

Staining and semi-quantitative analysis of placental AP activity
To visualize tissue AP activity in the placenta, placental cryostat sections (4 µm) were stained for AP activity using a histochemical enzyme assay according to Gomori method described by Van Goor [19]. The intensity of AP staining in placental sections was scored semi-quantitatively in a double blind manner by an independent observer. The intensity of AP activity scaled between 0 and 7, with 0 representing absence of staining and 7 demonstrating the highest staining intensity.
AT-1R expression on peripheral blood monocytes

AT-1R expression was measured on monocytes in peripheral blood, easily accessible cells. Therefore, whole blood samples were diluted 1:1 with RPMI (Sigma Aldrich) and incubated with PE-conjugated anti-CD14 antibodies (mouse-anti-human; diluted 1:38; IQ Products, Groningen, the Netherlands) first. Samples were incubated with BD FACSTM Lysing Solution (BD Biosciences) for 5 minutes to lyse the erythrocytes, centrifuged (1200 rpm, 5 min.) and incubated with 10% goat serum in phosphate-buffered saline (PBS) for 30 min. Thereafter, samples were washed with 1% BSA in PBS and incubated with unconjugated anti-AT-1R-antibodies (mouse-anti-human; diluted 1:18; Abcam, Cambridge, UK) for 30 min. After washing with 1% BSA in PBS twice, samples were incubated with FITC-conjugated goat-anti-mouse secondary antibodies (diluted 1:38; Dako, Heverlee, Belgium) for 30 min. Control samples were incubated without primary antibodies, but with secondary antibodies. Samples were washed twice with 1% BSA in PBS and resuspended in 2% paraformaldehyde in PBS and kept at 4 °C until FACS analysis. All steps were performed at room temperature.

Flow cytometry: Data were collected (at least 50.000 events per sample) on a BD FACS Calibur Flow Cytometer (BD Biosciences) and were analysed using FlowJo software (Tree star, Inc., Ashland, OR, USA). To analyse AT-1R expression on monocytes, first live cells were selected from the FCS/SCC plot and copied to a new plot. Here, a gate was drawn around the CD14-positive cells. The CD14-positive population was copied to a histogram for AT-1R expression. The gate for the percentage of AT-1R expressing monocytes was set using the control sample with only secondary FITC conjugated goat-anti-mouse antibodies. In this control sample the gate was set to 1% FITC-positive cells and copied to the CD14 population in the samples, which did contain the AT-1R antibodies. Data were expressed as the percentage of AT-1R expressing cells within the whole CD14-positive monocyte population (% of monocytes). Mean fluorescent intensity (MFI) was calculated on the AT-1R+ population, and in the control samples (stained with only secondary antibodies). To correct for day to day variation, the AT-1R MFI for the positive population was divided by the MFI of the control sample to gain an AT-1R MFI ratio.

Immunohistochemical staining and semi-quantitative analysis of placental AT-1R expression

Placental biopsies were stained and quantitatively scored for AT-1R expression. From one placental biopsy 4 µm sections were cut. Sections were stained for AT-1R expression (rabbit-anti-human, anti-AT-1, 1:200 diluted, Sigma Aldrich). After drying and fixation with acetone (10 min.), sections were let to dry and after that incubated with the primary antibody in PBS (overnight at 4°C). The next day sections were washed with PBS and exogenous peroxidase activity was blocked (0.25% H2O2 in PBS for 30 min.). After washing with PBS, sections were incubated with biotin conjugated swine-anti-rabbit (1:300 diluted in PBS, 60 min. Dako), washed again and incubated with peroxidase conjugated streptavidin (1:300 diluted in PBS, 30 min. Dako). Staining was visualized with 3-amino-9-ethyl-carbazole and haematoxylin. All
of the incubation steps were carried out at room temperature. Control sections were stained without primary antibodies and were consistently negative. The intensity of AT-1 receptor staining in placental sections was scored semi-quantitatively in a double blind manner by an independent observer. The intensity of AT-1 receptor scaled between 0 and 4, with 0 representing absence of staining and 4 demonstrating the highest staining intensity.

**Placental tissue RNA isolation**

RNA was isolated from placental biopsies using TRIzol (Life Technologies Europe, Bleiswijk, the Netherlands) according to the manufacturer’s protocol. After isolation of the RNA, RNA quality and concentration was measured on a Nanodrop device and samples were stored in -80°C until PCR.

**Placental AT-1R mRNA expression with real-time RT-PCR**

Placental AT-1R mRNA expression was analysed using real-time RT-PCR with predesigned gene expression assay Taqman primer-probe combinations. The expression of two splice variants of the AT-1R, variant A: hAT1E14 (HS01096942_m1, Life Technologies) and variant B: hAT1E124 (HS99999095_m1, Life Technologies), that have been shown to be expressed in the placenta [20], were analysed as compared to the reference gene PSMD-4 (Hs00356654_m1, Life Technologies). RNA was reverse transcribed into cDNA with Superscript II reverse transcriptase (Life Technologies). PCRs were performed in triples in a reaction volume of 10 µl with PCR master mix (Eurogentec, Maastricht, the Netherlands) and 40 cycles were run on an ABI Prism 7900HT Sequence Detection System (Life Technologies).

**Plasma AT-1R expression using Western blot**

To measure the AT-1R in the plasma, we used a western blot method. Plasma samples (diluted 100 times with SDS-PAGE sample buffer) were run over a 10% polyacrylamide gel using gel electrophoresis (PAGE) and subsequently processed for Western blotting, according to standard methods. In short, gels were blotted on a PVDF membrane in blotting buffer (60 min. at 100V). Membranes were blocked using 5% BSA in Tris-buffered saline (TBS) on a platform rocker for 45 min. and subsequently incubated overnight with a monoclonal anti-AT-1R antibody in Tris-buffered saline with 0.05% Tween-20 (TBS-T) with 1% BSA (mouse-anti-human-AGTR-1, diluted 1:2667; Abcam) on a roller mixer (overnight at 4°C). The next day, the membranes were washed for four times with TBS-T and incubated with secondary goat-anti-rabbit peroxidase conjugated antibodies in TBS-T with 1% ELK (diuted 1:1000; Dako) for 60 min. AT-1R bands were visualized with electrochemiluminescence according to standard procedures using Luminol and IPBA with a Molecular Imager Geldoc XR system (Biorad, Veenendaal, the Netherlands). Analysis of the size and intensity of the bands was performed using Image Lab 4.0.1 software (Biorad).
**Statistical analysis**

The effect of normal pregnancy, gestational age and late or early-onset preeclampsia were evaluated using Mann Whitney U tests. The correlation between Hx activity with the new substrate and Hx activity with the apyrase stripping assay was tested using linear regression. Data are presented as means with SEM. For all statistical tests, differences were considered to be significant if p<0.05 and a statistical trend if p<0.10.

**Results**

*Development of a new substrate for measuring Hx activity*

The new fluorescent substrate for Hx, Hx-MCA, was first tested on recombinant Hx (rHx) and various active Hx fractions, isolated from plasma (Figure 1A). As can be seen from Figure 1A, as compared with control (medium), rHx and all Hx fraction isolated from plasma, are able to clip the substrate, although activities were variable. The activity of rHx is lower is compared to most of the plasma fractions, which is due to lower Hx concentration of rHx.

Figure 1B shows the relation between Hx activity measured with the stripping assay and with the new substrate. The figure shows a clear positive correlation between Hx activity measured with the standard stripping assay and with the new Hx-MCA substrate (correlation coefficient R²=0.66).

*The Hx-MCA substrate is specific for Hx activity*

The test the specificity of this substrate for Hx activity, we incubated the substrate with other plasma serine proteases, i.e. kallikrein, factor X and thrombin. None of these plasma serine proteases were able to clip the substrate (results not shown).

![Figure 1](image.png)

**Figure 1.** Specificity of substrate Hx-MCA for measurement of Hx activity. With the novel Hx-MCA substrate, Hx activity was determined for recombinant Hx (rHx) and various active Hx fraction (Hx-313, Hx-338, Hx-23, Hx-F), isolated from plasma, and water as a control (A). Hx activity with the Hx-MCA substrate was compared with Hx activity as measured with the ‘apyrase stripping assay’ (B). Linear regression was performed to check the correlation between Hx activity with the Hx-MCA substrate and with the ‘apyrase stripping assay’; the correlation was significant, p<0.05; R²=0.66.
As Hx was shown to have protease activity [11], protease inhibitors (PMSF, Leupeptin and Aprotinin) were tested to see whether this would inhibit Hx activity. Moreover, MMP-inhibitors (BB3103 and Galardin) were also tested (Figure 2). Addition of protease inhibitors PMSF and Leupeptin indeed inhibited Hx activity with Leupeptin being the most efficient, while Aprotinin was less effective (Figure 2). The MMP-inhibitors BB3103 and Galardin did not affect Hx activity (Figure 2).

**Plasma Hx activity decreased and ATP levels increased in preeclamptic women**

Using the new Hx-MCA substrate, plasma Hx activity was increased in both groups of healthy pregnant women, compared to nonpregnant controls (p<0.05; Figure 3A). Furthermore, in accordance with previous results, in early-onset preeclamptic women the plasma Hx activity was decreased compared to healthy pregnant controls (p<0.05; Figure 3A). In addition, in late-onset preeclamptic women, plasma Hx activity was also lower compared with healthy pregnant women at the end of pregnancy (p<0.05; Figure 3A).

The plasma Hx protein concentration was not different in healthy pregnant women compared with nonpregnant women (Figure 3B). Compared with their healthy pregnant controls, plasma Hx protein concentration tended to be slightly lower in early-onset preeclamptic women (p<0.10). In contrast, in women with late-onset preeclampsia increased Hx protein concentrations were observed compared with their healthy pregnant controls (p<0.05; Figure 3B).

Plasma ATP levels were lower at week 36 of healthy pregnancy compared to nonpregnant women (p<0.05; Figure 3C). Both early- and late-onset preeclamptic women displayed increased ATP levels compared with their respective gestational age matched healthy pregnant controls (p<0.05; Figure 3C).
Plasma total and plasma placental AP activity increased in preeclamptic women.

AP can hydrolyse ATP and thereby reactivate Hx. We therefore measured plasma total and placental AP activity in pregnancy and preeclampsia. Compared to nonpregnant women, total plasma AP activity increased at 30 weeks and was even further increased at week 36 of healthy pregnancy (p<0.05; Figure 4A). Both in women with early and late-onset preeclampsia, total AP activity was significantly higher compared to healthy pregnant women at the same gestational age (p<0.05; Figure 4A). Plasma placental AP activity was also higher at week 30 in pregnant women and further increased at week 36 compared with nonpregnant women (p<0.05, Figure 4B). In both groups of preeclamptic women, plasma placental AP activity was significantly increased as compared with their respective healthy pregnant control women (p<0.05, Figure 4B).
No changes in placental AP activity in pregnant and preeclamptic women
As placental AP activity was raised in plasma, we analysed AP activity on placental tissue. In the placenta, AP activity was exclusively found on the villous syncytiotrophoblast layer. No changes in AP activity was observed in placentas from healthy pregnant compared with preeclamptic women, not in early-onset nor in late-onset preeclamptic women (Figure 5).

AT-1R expression is higher on monocytes of preeclamptic women
Figure 6 shows the AT-1R expression on monocytes from nonpregnant, pregnant and
preeclamptic women. As no differences in AT-1R expression was found between healthy pregnant women at week 30 and around term, these two control groups were taken together in this figure. AT-1R expression was decreased on monocytes from healthy pregnant women compared with nonpregnant controls (p<0.05; Figure 6A). In preeclamptic pregnancies, in which Hx activity was decreased, we hypothesized AT-1R expression would be higher as compared with healthy pregnancy. Indeed, on monocytes from both early and late-onset preeclamptic women, AT-1R expression was increased compared with monocytes from healthy pregnant women (p<0.05; Figure 6A). Similar differences were obtained for the intensity of AT-1R expression per monocyte (p<0.05, Figure 6B).

**Placental AT-1R expression is higher in preeclamptic women compared to healthy pregnancy**

Placental biopsies were stained and quantitatively scored for AT-1R expression, and representative stained samples are shown in Figure 7A-D. AT-1R expression was mainly found on the multinucleated syncytiotrophoblast cells lining the villi (black arrows) and on foetal endothelial cells in the villi (red arrows). No changes in the cell types expressing the AT-1R were observed in healthy pregnant compared to preeclamptic women. In both early-onset and late-onset preeclamptic women, placental AT-1R expression was higher compared with normal pregnancy (p<0.05; Figure 7E). This higher expression of the AT-1R in preeclamptic placentas was associated with a decreased expression of both AT-1R splice variants (A and B), i.e. increased delta Ct for both variants (p<0.05; Figure 7F+G).

Figure 6. AT-1R expression on peripheral blood monocytes. Using flow cytometry, AT-1R expression was determined on peripheral blood monocytes, the percentage of AT-1R-positive monocytes (A) as well as the mean fluorescent intensity (MFI) of AT-1R expression per monocyte (B) was analysed on monocytes from nonpregnant (NP, open bars) women, early and late-onset preeclamptic women (PE, striped bars) and gestational age matched healthy pregnant controls for both preeclampsia groups (taken together, P, solid bars). Means with SEM are shown. a:p<0.05, decreased in healthy pregnant women as compared with nonpregnant women, Mann-Whitney U test. *:p<0.05, increased in preeclamptic women as compared with healthy pregnant women, Mann-Whitney U test.
Decreased plasma AT-1R in women with early- and late-onset preeclampsia

As AT-1R expression was decreased in healthy pregnant women as compared to nonpregnant women and increased in preeclampsia as compared with healthy pregnancy, we evaluated whether increased levels of (parts of) the AT-1R were present in the plasma of healthy pregnant women and decreased levels in plasma of preeclamptic women. This is shown in Figure 8. As we found no differences in levels of the AT-1R between week 30 of pregnancy and week 36 of pregnancy, we pooled the data of all healthy pregnant women. In plasma...
from pregnant women, increased levels of AT-1R were found as compared to nonpregnant women (p<0.05; Figure 8). Lower levels of AT-1R were observed in plasma from early- and late-onset preeclamptic women compared to healthy pregnant women (p<0.05; Figure 8).

**Discussion**

The present study confirmed increased plasma Hx activity during healthy pregnancy and decreased plasma Hx activity in preeclampsia, as well as with increased plasma ATP levels during preeclampsia. This was associated with decreased monocyte AT-1R expression during healthy pregnancy and increased monocyte AT-1R expression during preeclampsia. Moreover, (parts of) the AT-1R are present in plasma and are increased during healthy pregnancy and decreased in preeclamptic patients. During healthy pregnancy, we also observed increased plasma AP activity. As AP activity is able to hydrolyse ATP [21] and thus activate Hx, it may be suggested that the increased plasma AP activity during healthy pregnancy is (partly) responsible for the increased Hx activity. During preeclampsia, plasma AP activity was even further increased, which may be a compensatory mechanism for the increased ATP levels.

We have previously measured Hx activity with two different methods: 1) the so-called ‘apyrase stripping assay’ [11]; and 2) using an artificial chromogenic substrate for serine proteases [11,22]. With the apyrase stripping assay, increased Hx activity was found in plasma from healthy pregnant women compared with nonpregnant women [12,13]. However, this method is very labour-intensive. In addition, the chromogenic substrate was developed to measure kallikrein activity and is for that reason not specific for Hx [11,22]. Therefore, the specific cleavage sites of active Hx were identified and a specific fluorescent substrate for Hx was designed. The present data showed that rHx and various batches of Hx isolated from plasma are able to clip this substrate. Moreover, the activity of Hx as measured with this substrate correlated well with the activity measured with the stripping assay. The substrate was not cleaved by other plasma serine proteases like kallikrein, factor X and thrombin, showing that the substrate was specific for Hx activity. Moreover, the breakdown of the substrate by
Hx was inhibited by protease inhibitors like PMSF and Leupeptin, but not by MMP-inhibitors. This is in line with our previous data showing that Hx has protease activity [11].

With the new substrate, we confirmed that plasma Hx activity was increased in healthy pregnant women compared with nonpregnant women. In the nonpregnant woman, we suggest existence of a homeostatic balance between Hx activity, plasma and vascular ATP hydrolysing enzymes such as AP and CD39, and circulating plasma ATP levels. Plasma Hx activity may be low in these women due to inhibition by physiological plasma levels of ATP (mean of 2 µM), as well as low ATP hydrolysing enzymes, such as AP. The increase in Hx activity during healthy pregnancy may have several causes. During pregnancy, the observed increased plasma AP activity, as well as the presence of an extra vascular bed (i.e. the placenta) covered with CD39 and AP [23], may contribute to breakdown of ATP and the reactivation of Hx. Indeed, ATP levels appear to be lower already at week 30 compared with nonpregnant women, while significantly decreased ATP levels were observed at term.

The decreased Hx activity in women with both types of preeclampsia is likely due to increased ATP levels, the natural inhibitor of Hx activity. It was not the result of decreased Hx protein concentrations, since we found only a minor trend to decreased Hx levels in early-onset preeclamptic women and even higher Hx protein levels in late-onset preeclamptic women compared with their healthy pregnant controls. The exact cause of increased ATP levels in early and late-onset preeclamptic women is unknown. However, since ATP is released by hypoxic and stressed cells [24], and both early- and late-onset preeclampsia is associated with placental stress [8,9], it seems likely that the stressed placenta may be the source of the increased plasma ATP levels. In addition, activated endothelial cells [25] and activated leukocytes [26] in women with preeclampsia may also release ATP into the peripheral circulation [27,28]. Lastly, decreased expression of the ATP-hydrolysing enzyme CD39 was found in the preeclamptic placenta compared with healthy pregnancy [8], which may lead to higher ATP levels.

Since active Hx was shown to shed the AT-1R in vitro [13], increased Hx activity during pregnancy was hypothesized to be associated with decreased AT-1R expression and increased levels of plasma AT-1R, compared with nonpregnant women. Indeed, lower AT-1R expression on monocytes and higher plasma AT-1R levels were found in healthy pregnant women compared with nonpregnant women. Further studies are needed to evaluate the vascular expression of the AT-1R in pregnant women, although decreased AT-1R expression was found in the uterine placental bed [29], and studies have shown that active Hx is able to decrease responsiveness to angiotensin II in rat aortic rings [13]. This may suggest that active Hx indeed sheds the AT-1R from vascular cells. Therefore, it is tempting to speculate that increasing Hx activity during pregnancy induced AT-1R shedding from tissues, including vascular cells, resulting
in decreased vascular angiotensin II responsiveness during pregnancy [15]. In line with this hypothesis, the decreased Hx activity in preeclamptic patients may result in decreased shedding of the AT-1R, and contribute to increased angiotensin II sensitivity in peripheral vasculature of women with preeclampsia.

This study for the first time shows that (parts of) the AT-1R can be found in plasma. AT-1R protein expression has always been thought to only be regulated at the gene level [30,31]. Downregulation of AT-1R mRNA is usually found in situations of decreased AT-1R expression [32]. Our study, however, showed decreased AT-1R mRNA expression in the placenta, in the face of increased protein expression. This study thus provides new insights into the regulation of the AT-1R, although regulation of receptor expression due to shedding of receptors has been observed before for other (serine) proteases, such as metalloproteases and elastase [33-36]. We measured the level of the AT-1R in the plasma using western blot. It remains to be established whether we are measuring the whole receptor or parts of the receptor. However, it may be speculated that the shed receptor or parts of the receptor also have a physiological function and is able to bind angiotensin II. Similar to the role of other soluble receptors [37], the circulating receptor may inhibit circulating angiotensin II to bind to vascular receptors.

The plasma AP activity was found highest at the end of pregnancy, which is in accordance with other studies [38,39]. The increase in AP activity in our samples from healthy pregnant women is mainly due to increased placental AP activity. The increase in AP activity during pregnancy can have other functional roles besides ATP hydrolysis and Hx reactivation. It may be needed for the prevention of (delivery associated) infections, since AP is known to detoxify LPS [40]. This is in accordance with the even higher AP activity at term compared with week 30 of pregnancy. Interestingly, in plasma from women with preeclampsia, AP activities were even further increased, due to increased placental AP. The increased plasma placental AP activity in women with preeclampsia may have a compensatory function, for instance induced by the increased ATP levels, since Sawada et al. showed that ATP may stimulate AP production in osteogenic ligament cells [41]. Moreover, disturbed placental perfusion, like in preeclampsia, also increases AP release [42]. Although the mechanism is unclear, the placental AP is expected to be shed from the syncytiotrophoblast. However, no changes in placental AP activity were observed in pregnant compared with preeclamptic women. Increased release of placental AP by syncytiotrophoblast cells in preeclamptic women may therefore be directly compensated, for instance by increased AP mRNA production in these cells, again possibly under the influence of high ATP levels.

The results presented in the current study are in accordance with the suggestion that active Hx plays a role in the decreased AT-1R expression and development of the decreased responsiveness to angiotensin II in healthy pregnancy, and that decreased Hx activity in
Preeclampsia may result in increased angiotensin II sensitivity. Therefore, increasing Hx activity during preeclampsia may result in decreased angiotensin II sensitivity and decreased blood pressure in preeclampsia. We suggest to increase Hx activity during preeclampsia using AP or other ATP-hydrolysing enzymes.

References
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Treatment of ATP-induced preeclampsia-like symptoms with alkaline phosphatase in pregnant rats

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