

Plasma Hemopexin Activity in Pregnancy and Preeclampsia

Winston W. Bakker,¹ Rogier B. Donker,^{5,2} Albertus Timmer,¹
Mariëlle G. van Pampus,² Willem J. van Son,³
Jan G. Aarnoudse,² Harry van Goor,¹ Klary E. Niezen-Koning,⁴
Gerjan Navis,³ Theo Borghuis,¹ Rianne M. Jongman,¹ and
Marijke M. Faas⁵

¹Departments of Pathology and Laboratory Medicine, University Medical Center of Groningen and University of Groningen, Groningen, The Netherlands

²Departments of Obstetrics and Gynecology, University Medical Center of Groningen and University of Groningen, Groningen, The Netherlands

³Department of Internal Medicine, University Medical Center of Groningen and University of Groningen, Groningen, The Netherlands

⁴Department of Pediatrics, University Medical Center of Groningen and University of Groningen, Groningen, The Netherlands

⁵Division of Medical Biology, Department of Pathology of Laboratory Medicine, University Medical Center of Groningen and University of Groningen, Groningen, The Netherlands

Objective: Plasma hemopexin activity, associated with increased vascular permeability, was evaluated in healthy pregnant and non-pregnant women and in pre-eclamptic women. **Methods:** Hemopexin activity and the hemopexin inhibitor, extracellular ATP, were assayed in plasma from pregnant (n = 10), preeclamptic (n = 9), and non-pregnant women (n = 10) using standard methods. Abdominal fascia tissue fragments from preeclamptic and pregnant women were immunohistochemically stained for vascular ecto-apyrase or ecto-5'nucleotidase. **Results:** The data show significantly enhanced Hx activity exclusively in plasma from pregnant women and significantly enhanced plasma ATP in pre-eclamptic women compared with the other groups. Dephosphorylation of preeclamptic plasma resulted in reactivation of Hx activity. Fascia tissue-samples from preeclamptic women showed reduced ecto-apyrase activity and enhanced ecto-5'nucleotidase activity compared to pregnant women. **Conclusion:** Enhanced hemopexin activity may be associated with normal pregnancy, but not with preeclampsia. Decreased hemopexin in pre-eclamptic patients may be due to enhanced plasma ATP, which is possibly promoted by diminished activity of vascular ecto-apyrase.

Keywords Hemopexin, Pregnancy, Preeclampsia.

Address correspondence to Winston W. Bakker, Dept. of Pathology and Laboratory Medicine, UMCG Hanzeplein 1 postbox 30001; 9700RB Groningen, The Netherlands. E-mail: w.w.bakker@path.umcg.nl

INTRODUCTION

The acute phase reactant hemopexin (Hx) is believed to bind and transport heme and therefore may serve as a scavenger of reactive oxygen metabolites (1, 2). Previously we have shown that Hx or an isoform of this glycoprotein shows protease activity *in vitro*, demonstrated for instance by hydrolysis of artificial substrates for serine proteases (3). In addition, Hx is able to affect extracellular matrix molecules in the glomerular microvasculature (4, 5). Intrarenal infusion of Hx into the rat kidney *in vivo* induces transient proteinuria concurrently with glomerular alterations that resemble those seen in minimal change nephrotic syndrome (MCNS) in relapse (6). Consistent with these observations is our recent demonstration of significantly enhanced plasma Hx activity in patients with MCNS in relapse in contrast to subjects in remission (7). Hx-associated protease activity can be inhibited by serine protease inhibitors such as antithrombin III, but also by nucleotides such as extracellular ATP (8). Extracellular ATP may be one of the "natural" inhibitors of Hx. It is conceivable that Hx occurs in the circulation of healthy individuals in its non-active form, possibly due to complexing with extra cellular ATP. Indeed, inactivated Hx, obtained after incubation with ATP, can be reactivated by treatment with either soluble apyrase, an ATP-hydrolyzing phosphatase, or endothelial cells *in vitro*, which express ecto-apyrase (8). Apparently, dephosphorylation of inhibited Hx *in vitro* restores its protease activity.

There are several reasons to suppose that Hx may be activated during normal or complicated pregnancy, like preeclampsia (PE). First, even in uncomplicated pregnancy, glomerular permeability for plasma proteins increases (9). Second, although no overt edema is common in normal pregnancy, in contrast to PE, there is a tendency to increased peripheral vascular permeability and edema which may lead to mild edema (10). Third, circulating blood comes into close contact with the ecto-apyrase coated syncytiotrophoblast villi which may activate Hx in a similar way as shown for ecto-apyrase coated endothelial cells *in vitro* (8).

PE is a major pregnancy complication of unknown etiology, clinically characterized by hypertension and proteinuria and frequently edema, occurring in the second half of pregnancy. Although no hypertension is observed in MCNS, whereas the glomerular lesion in this disorder clearly differs from that in PE, the latter complication and MCNS have some characteristics in common, *i.e.*, edema and proteinuria. Regarding the enhanced Hx activity in MCNS (7), we initially expected to observe enhanced Hx activity in normal pregnancy, and even higher plasma Hx activity in subjects with PE, as compared with controls.

The considerations mentioned above prompted us to investigate Hx activity in normal pregnant individuals and subjects with severe PE, showing clear-cut proteinuria, *versus* age matched non-pregnant control women. The

results show indeed enhanced Hx activity in plasma from normal pregnant women, compared with healthy non-pregnant individuals, but no detectable Hx activity in subjects with PE. Moreover, in plasma from subjects with PE, a relatively high level of extracellular ATP occurred, as compared to normal pregnant or non-pregnant women. To examine whether increased extracellular ATP in PE may be associated with decreased vascular ecto-apyrase activity in this condition, we performed immunohistochemical staining upon tissue fragments of abdominal fascia from these individuals. It appeared that exclusively subjects with PE showed decreased vascular ecto-apyrase activity as compared with normal pregnant women. Moreover, the vasculature of these PE patients also showed increased activity of 5' ecto-nucleotidase as compared with normal pregnant women. This staining pattern, *i.e.*, decreased ecto-apyrase expression and increased ecto-5' nucleotidase activity, is characteristic for ischemia (11), suggesting vascular ischemia in PE patients.

MATERIALS AND METHODS

Patients

The present study was performed after approval by the medical ethics committee of the University Medical Center Groningen. Written, informed consent was obtained from all patients. PE patients and healthy pregnant controls were recruited from the antenatal ward of the University Medical Center Groningen. We included PE patients (for both biopsies and blood samples) who delivered by cesarean section (indications for cesarean section were deteriorating fetal or maternal conditions, or both) and healthy pregnant control women with singleton pregnancies at term. For biopsies of healthy pregnant women, we included healthy pregnant women who delivered by cesarean section. Elective cesarean section in this group was performed for several reasons, *i.e.*, fetal breech presentation, contradicted pelvic outlet and previous obstructive labor. Cesarean sections of all women were performed under spinal anesthesia and none of the patients were in active labor before or during cesarean section. For blood sampling, we also included a group of non-pregnant women. They were recruited from hospital staff and students. Exclusion criteria for all groups were pre-existent hypertension, diabetes mellitus, vasculitis, renal disease, autoimmune disease, malignancy, or history of recent trauma or surgery.

PE was defined according to the standards of the International Society for the Study of Hypertension in Pregnancy (ISSHP): diastolic blood pressure of 90 mm Hg or more on two or more consecutive occasions more than 4 hours apart and proteinuria of more than 300 mg/24 hours. Both developed after 20 weeks gestation and returned to normal values within 3 months after delivery. Early-onset of disease was present in all patients, and all patients had cesarean deliveries between 26 and 34 weeks of gestation. PE of all

subjects in this study was severe, as defined by diastolic blood pressure of at least 110 mm Hg on two or more consecutive occasions and/or proteinuria of more than 5.0 g/24 hours.

Collection of Patient Material

Blood Samples

For PE patients ($n = 9$), blood samples (taken 1 to 5 days before cesarean section) were obtained during routine pre-operative blood sampling from the antecubital vein in lithium heparin tubes and in EDTA tubes (Venoject, Terumo Europe NV, Leuven, Belgium) and stored at 4°C. From healthy, pregnant control women ($n = 10$), blood sampling was matched for gestational age with the PE patients. Blood samples were also taken from a group of age-matched non-pregnant women ($n = 10$). For all samples, within 2 hours tubes were centrifuged at 900 g for 10 minutes. Plasma samples were frozen in aliquots at -80°C. Pilot studies have shown that this short (2-hour) delay in centrifuging and freezing of the blood samples does not affect Hx activity or ATP concentrations.

Biopsies

From both PE ($n = 6$, gestational age between 26 and 34 weeks) and normal pregnant patients ($n = 8$; gestational age between 37 and 40 weeks), during cesarean section by Pfannenstiel incision, tissue biopsies up to approximately 1.5 cm in diameter were resected from abdominal fascia from all participants. Tissue biopsies were, immediately after surgical resection, snap-frozen in liquid nitrogen and stored at -80°C until further analysis. We studied patients who suffered from severe and early-onset PE. Consequently, cesarean delivery and associated biopsy sampling in this PE group was necessarily carried out at an earlier gestational age as compared to the healthy pregnant control group.

Evaluation of Plasma Hx Activity

Protease activity of plasma Hx was evaluated by the “glomerular ECM stripping assay,” as described previously (5–7). This *in vitro* assay is based on the potential impairment by Hx of glomerular extracellular matrix molecules (ECM), such as glomerular ecto-apyrase expression.

Acetone fixed cryostat (rat) kidney tissue is incubated with either (1:8) diluted heparinized plasma samples (100 μ L/section for 60 minutes) from either pregnant women, subjects with PE, non-pregnant control women, or phosphate buffered saline (PBS). Following incubation, sections were washed and stained for glomerular ECM's using standard immunological methods (3) and examined microscopically. Glomerular ECM's such as sialoglycoproteins

or glomerular ecto-apyrase were routinely stained immunohistochemically as described elsewhere (3). Loss of reaction product after incubation is interpreted as Hx mediated protease activity provided that monoclonal anti-Hx antibody is able to inhibit this response. Thus, to check for the specificity of the effect, incubations were done with or without supplementation of monoclonal anti Hx IgG (150 $\mu\text{g}/\text{mL}$; kindly provided by Dr E. Hansen, Southwestern Medical Center, University of Texas, Dallas). If monoclonal anti-Hx IgG is able to inhibit enzymatic impairment of glomerular ECM by plasma *in vitro*, it is highly likely that Hx is involved rather than other plasma enzymes. Reaction product was semi quantitatively evaluated in a double blind fashion; approximately 20 representative glomeruli per section (4 sections per individual) were scored and the arithmetic mean of each group was expressed as arbitrary units. Scoring was done using an arbitrary scale as described elsewhere (7), reflecting the Hx associated protease activity (a relative low amount of reaction product reflects relative high Hx protease activity). Evaluation was done using an arbitrary scale: 0–2 abundant staining; 2–4 clearly detectable staining; 4–6 faint staining; 6–8 very faint staining; 8–9 very faint to undetectable staining.

Measurement of Plasma ATP

Blood samples decoagulated with EDTA were diluted (1:3) with PBS supplemented with EDTA (1.0 mM). Extracellular ATP was assayed according to Gorman et al. (12), with minor modifications according to the manufacturer's instructions (Promega, Madison, Wisconsin, USA). Diluted plasma (150 μL) was incubated with 150 μL substrate, *i.e.*, 87.0 $\mu\text{g}/\text{mL}$ luciferin (Beetle E 1602, Promega, USA) supplemented with MgCl_2 (10.0 mM), and luciferase (500,000 RLU/ml in PBS (Quantilum r-luciferase; Promega, Madison, Wisconsin, USA). The relative light units (RLU) were detected using a luminometer (Thermo Luminoskan Ascent, Thermo Fisher Scientific, Waltham, MA, USA) in 96-well ELISA trays. The results were calculated from standard curves using concentrations between 10^{-4} M till 10^{-10} M ATP in PBS with EDTA.

Dephosphorylation of Plasma from Subjects with PE

As we supposed that in subjects with PE extracellular ATP may inhibit plasma Hx activity, we treated plasma from subjects with PE with soluble apyrase to check for reactivation of their plasma Hx after dephosphorylation. Therefore diluted heparinized plasma samples (1:8) from individual subjects with PE ($n = 5$) were incubated with soluble apyrase (grade VII, Sigma, St Louis, MO) (5.0 units per ml PBS) supplemented with 0.1 mM MgCl_2 for 1.5 hours at room temperature upon cryostat kidney sections. For comparison control samples from the same subjects with PE were incubated with heat-inactivated soluble apyrase. Evaluation of Hx activity was done as described above using the glomerular ECM stripping assay.

Immunohistochemistry of Patient – or Control Tissue Samples

Cryostat sections of abdominal fascia from subjects with PE ($n = 6$), or from control pregnant women ($n = 8$) were fixed with acetone and stained for ecto-apyrase with monoclonal anti-apyrase IgG and goat anti-mouse IgM conjugated with horseradish peroxidase as a second step according to standard methods. Tissue samples were also stained using enzyme histochemistry for the activity of vascular ecto-5'nucleotidase according to standard methods (11, 13). Evaluation of the expression of both vascular ecto-apyrase or ecto-5'nucleotidase was done semi quantitatively using an arbitrary scale by two independent observers in a double blind fashion.

Statistics

Results are expressed as means \pm standard deviation (SD). PE samples and the respective controls (gestational age-matched healthy pregnant individuals and age-matched non-pregnant controls) were always done within the same experiments. To evaluate differences between data of non-pregnant, pregnant and PE women, Wilcoxon Signed Rank test was used. Differences were considered significant if $p < 0.05$.

RESULTS

From Figures 1 and 2 it can be seen that the mean plasma Hx activity in samples from normal pregnant individuals is significantly enhanced as compared to that of non-pregnant women or subjects with PE (8.64 ± 1.06 versus 1.92 ± 0.64 and versus 3.04 ± 1.32 respectively) (Figure 1). This is also illustrated in Figure 2, which shows representative glomeruli stained for glomerular ECM's after contact with plasma's from the various groups. This figure shows a decreased amount of reaction product in glomeruli exclusively after contact with plasma from pregnant individuals. (Figure 2).

From Figure 3 it can be seen that in subjects with PE the mean plasma titer of ATP is significantly enhanced ($4.7 \pm 2.9 \times 10^{-6}$ M), as compared with that of non-pregnant control women or control pregnant women. ($1.14. \pm 1.29 \times 10^{-6}$ M and $1.64. \pm 0.88 \times 10^{-6}$ M respectively). Treatment of plasma from subjects with PE, which show low Hx activity (mean 1.8 ± 1.6) with apyrase, resulted in significant activation of plasma Hx activity in these samples (mean 5.0 ± 0.8) (Figure 4).

Immunostaining of cryostat sections of fascia showed in five out of six cases clearly diminished staining of vascular ecto-apyrase of the subjects with PE studied ($n = 6$), as compared with fascia obtained from control pregnant women ($n = 8$), who underwent cesarean section for other reasons than PE (Figure 5). Figure 5a and b shows representative vasculature stained for ecto-apyrase of five out of six PE patients and of eight normal pregnant patients.

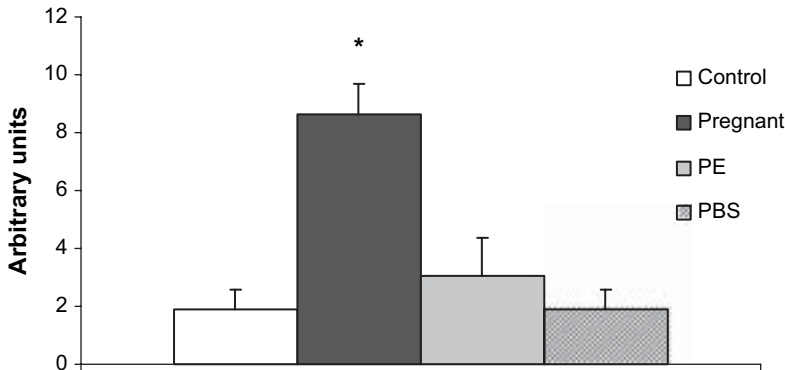


Figure 1: Mean Hx activity of plasma samples from non pregnant control women (*open column*; $n = 10$), pregnant women (*solid column*; $n = 10$) or subjects with PE (*gray column*; $n = 9$). Hatched column refers to background staining after incubation of kidney tissue with PBS ($n = 10$). Columns represent mean levels of protease activity of plasma Hx, expressed as arbitrary units (\pm SD). The Hx activity was calculated from the mean amounts of reaction product of kidney sections following staining for glomerular ecto-apyrase after incubation with either plasma samples or PBS. Low stainability reflects relatively high Hx activity. Evaluation was done using an arbitrary scale: 0–2 abundant staining; 2–4 clearly detectable staining; 4–6 faint staining; 6–8 very faint staining; 8–9 very faint to undetectable staining. It can be seen that enhanced protease activity of plasma Hx occurs exclusively after incubation of the sections with plasma from pregnant subjects. * $p \leq 0,01$, pregnant versus non pregnant; Wilcoxon).

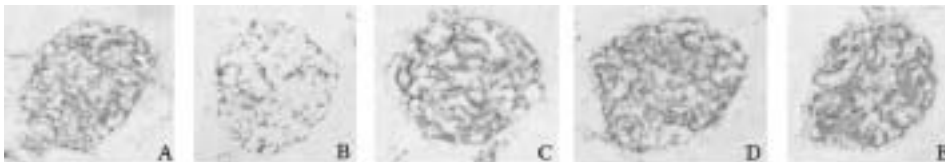


Figure 2: Representative micrographs of glomeruli following immunostaining for glomerular ECM's after incubation with plasma samples from either a non-pregnant control donor (A); a pregnant donor (B); the same pregnant donor as in B supplemented with monoclonal anti Hx IgG (0,15 mg/mL PBS) (C); a donor with PE (D) or after incubation with PBS alone (E). Immunostaining was done with monoclonal anti apyrase IgG. It can be seen that reduced stainability due to plasma Hx occurs exclusively following incubation with plasma from the pregnant individual (B) (final magnification $\times 300$).

The biopsy from one patient with PE showed decreased staining of vascular ecto-apyrase; this was, however, less prominent. In particular endothelial sites show lack of reaction product (Figure 5b vs 5a). Vessel walls of similar tissue fragments stained for ecto-5' nucleotidase activity showed enhanced stainability in samples from subjects with PE in all cases in contrast to controls showing negative staining of their vessel walls (Figure 5c vs. 5d, showing representative examples).

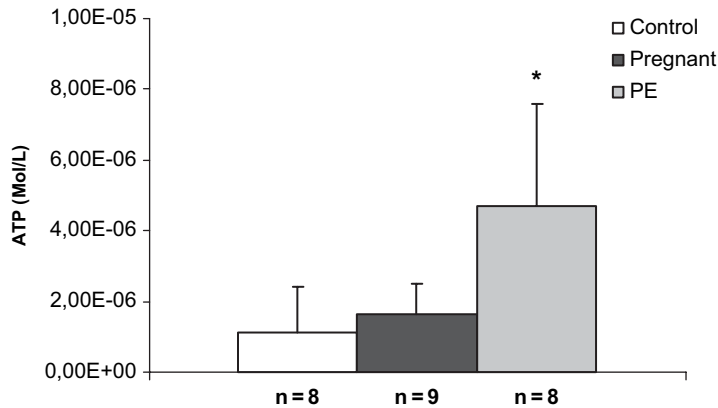


Figure 3: Plasma titer of extracellular ATP assayed using the luciferine/luciferase method. Columns represent mean levels (\pm SD) of ATP (Mol/L) in plasma from non pregnant control donors (*open column*; n = 8), pregnant donors (*solid column*; n = 9) or subjects with PE (*gray column*; n = 8). It can be seen that the mean titer of ATP of subjects with PE is significantly higher as compared with that of non pregnant women or pregnant individuals. * $p \leq 0,01$; PE versus pregnant donors and versus non pregnant control donors, Wilcoxon.

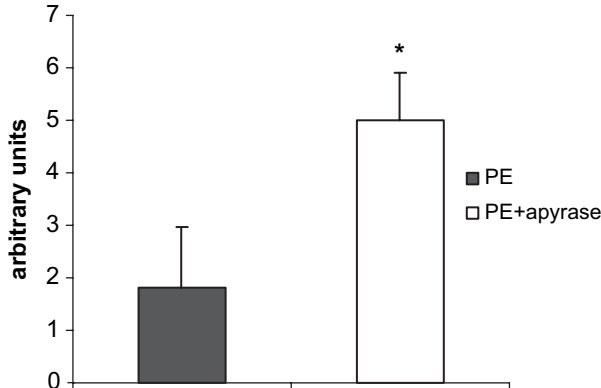


Figure 4: Dephosphorylation of PE plasma. Mean Hx activity of plasma samples from subjects with PE treated with heat inactivated soluble apyrase (*solid column*; n = 5) or from subjects with PE after treatment with active soluble apyrase (5.0 units /mL) (*open column*; n = 5). Columns represent mean levels of protease activity of plasma Hx expressed as arbitrary units (\pm SD). Evaluation was done using an arbitrary scale as described under Figure 1. It is shown that a significant increase of Hx activity occurs exclusively after treatment of PE plasma with soluble apyrase (*open column*). (* $p \leq 0,01$; Wilcoxon)

DISCUSSION

Plasma samples from pregnant individuals showed enhanced Hx activity in all samples studied (Figures 1 and 2). Apparently enhanced plasma Hx activity

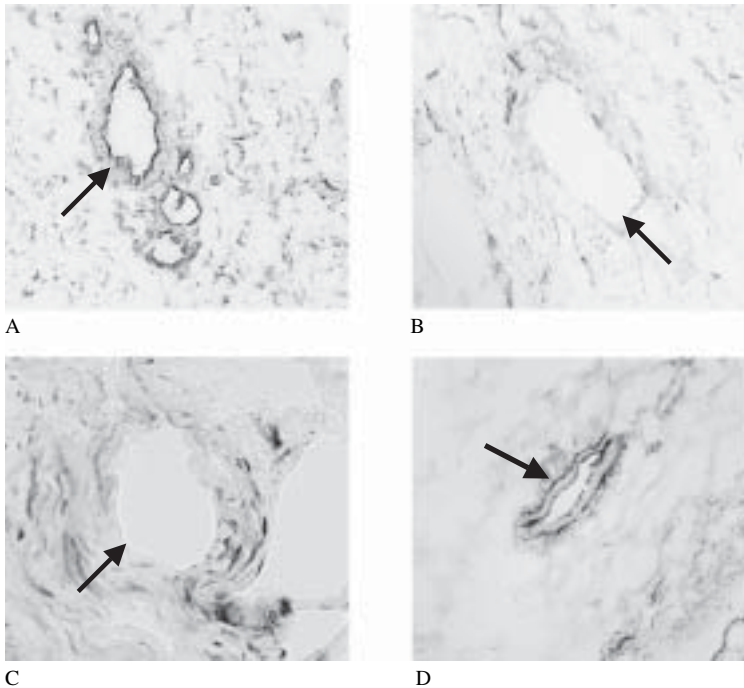


Figure 5: Representative micrographs of cryostat sections from abdominal fascia from a pregnant control individual (A and C) or from a subject with PE (B and D), stained for ecto-apyrase (A and B) or ecto-5' nucleotidase (C and D). It is shown that positive vascular immunostaining for ecto-apyrase is present mainly at the endothelial sites (*arrow*), in vessels of the control pregnant individual (A), whereas loss of vascular stainability occurs in the vessels of the tissue obtained from the subjects with PE (B, *arrow*). Histochemical staining for ecto-5' nucleotidase is negative in the endothelium of the vessel from the pregnant control individual (C, *arrow*) whereas vessel walls from the subject with PE stain positive for this enzyme activity (D, *arrow*).

is associated with uncomplicated pregnancy in the third trimester. This situation resembles the enhanced Hx activity observed in subjects with MCNS (7). As we have observed approximately the same level of increase of plasma Hx activity in subjects with MCNS in relapse as compared with the pregnant women in the present study, the question arises why no overt proteinuria is seen in normal pregnancy. However, the many adaptation mechanisms occurring in normal pregnancy may counteract proteinuria due to enhanced Hx activity. For instance, in normal pregnancy, enhanced resorption of filtered protein by proximal tubules occurs (14).

As we observed in previous studies the occurrence of activated plasma Hx in association with proteinuria (7), we now expected to find an even further enhanced Hx activity in patients with severe PE, all showing proteinuria, compared to control pregnant individuals. However, as shown in Figures 1 and 2, no enhanced Hx activity was shown in the PE patients studied

compared to non-pregnant subjects. As plasma Hx is produced mainly by the liver, it cannot be excluded that decreased Hx activity is due to liver dysfunction occurring often in severe PE. However, it is more likely that the presence of a potent Hx inhibitor, *i.e.*, extracellular ATP, may cause down-regulation of Hx activity in subjects with PE. (Figure 3). This putative inhibition of activated Hx by extracellular ATP is supported by our observation that dephosphorylation of plasma from subjects with PE using apyrase *in vitro*, is able to restore the Hx activity in these plasma samples a great deal (Figure 4). The relatively low plasma ATP levels measured in control donors are in agreement with those reported by other authors (15). Plasma samples from non-pregnant individuals ($n = 12$) did not show significant reactivation following treatment with apyrase (results not shown). The origin of the relatively increased level of extracellular ATP in subjects with PE, however, is unclear but may come from significant regions of ischemia in the placentas of these patients (16–18). It is conceivable that decreased activity of vascular ecto-apyrase occurring in PE in peripheral blood vessels (Figure 5a and b), may contribute to the enhanced plasma titer of extracellular ATP, as ischemic vessel walls lacking sufficient ecto-apyrase activity are not able to hydrolyze this nucleotide properly.

The mechanism of Hx activation in pregnancy is unclear. In view of previous findings *in vitro*, showing that endothelial ecto-apyrase is able to activate non-active Hx to its active isoform (Hxa) (8), we feel that *in vivo* the placental circulation may be involved in Hx activation in a similar manner. Thus, in pregnant individuals the circulating blood comes into close contact with a relative huge surface of placental syncytiotrophoblast lined with ecto-apyrase (19–21). It is conceivable that this promotes the conversion of non-active Hx to Hxa. If this is true one might also expect lower plasma ATP titers in the pregnant versus the non-pregnant condition due to additional hydrolysis of extracellular ATP by the placental ecto-apyrase. The reason why this was not detected is obscure, but it cannot be excluded that some ATP is released from the placenta of normal pregnant women accounting for the similar plasma ATP levels in both groups of individuals.

The relevance of activated Hx in normal pregnancy remains to be elucidated. It is clear that an expanded vascular bed insensitive for vasoconstrictors like angiotensin II (AngII) promotes proper perfusion of the placenta with maternal blood (22, 23). It has recently been shown that this insensitivity to AngII may be due to decreased expression of the AngII receptor in PE (24). Recent observations from our laboratory indicate that activated Hx, in contrast to inactivated Hx, is able to block the AngII receptor-1 (AT-1) of human endothelial cells and monocytes *in vitro*. This may suggest that activated Hx is potentially able to promote the non-responsiveness of the vascular bed to AngII in uncomplicated pregnancy. It is therefore likely that lack of plasma Hx activity in PE might result in a vascular responsiveness upon AngII comparable with the one seen in non-pregnant individuals.

It is generally believed that widespread endothelial dysfunction is a major characteristic of PE (25). Although most authors accept that the endothelial dysfunction occurs systemically, recent evidence suggests that some alterations of vascular endothelium may be restricted to specialized vascular beds, such as in the kidney or in the liver (26). The present biopsies, showing decreased vascular ecto-apyrase activity in PE as well as upregulated ecto-5' nucleotidase activity (Figure 5), do reflect ischemic injury of endothelium of peripheral vessels (11). Although the mechanism of endothelial dysfunction remains unclear, oxidant stress in the placental microvasculature as well as in other capillary beds due to disturbed interaction between vascular endothelial growth factor (VEGF) and endothelium may be implicated; in addition circulating VEGF binding receptor molecules like sFlt-1 may play a role (27, 28).

The present data, together with the preliminary observations mentioned, have lead to the hypothesis that in normal pregnancy activated Hx may promote an expanded vascular bed. As a result of endothelial ischemia in PE, increased placental production of ATP and/or diminished endothelial ecto-apyrase leads to defective hydrolysis of extracellular ATP in these patients, resulting in increased plasma ATP and consequently in inhibited plasma Hx. This inhibited plasma Hx may result in a contracted vascular bed. The possibility that relative high plasma ATP stimulates also inflammatory cells through purinergic (P_{2Y}) receptors to the release of toxic oxygen radicals (29, 30) may fit well in the concept of PE as an systemic inflammatory disorder (30, 31, 32). Future studies in our laboratory are directed towards the relationship between activated Hx, ATP and the AT-1 receptor.

It is clear from the present data that our initial assumption, *i.e.*, plasma Hx activity might be enhanced in proteinuric PE subjects versus non proteinuric pregnant women must be rejected. The possible relationship of extracellular ATP in PE and the lack of Hx activity on the one hand, and anti-angiogenic factors and VEGF on the other hand require further investigation. Preliminary data (to be published separately) showing proteinuria in pregnant versus non-pregnant rats following infusion of extracellular ATP, may point to a potential toxic effect of extracellular ATP in the pregnant condition.

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