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Downregulation of the Angiotensin Receptor 1 by Hemopexin In Vitro

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Abstract—During normal pregnancy, in contrast to preeclampsia, plasma hemopexin activity is increased together with a decreased vascular angiotensin II receptor (AT_1) expression. We now tested the hypothesis that hemopexin can downregulate the AT_1 receptor in vitro. Analysis of human monocytes or endothelial cells by flow cytometry showed decreased membrane density of AT_1 exclusively after incubation with active hemopexin, whereas in supernatants of these cell cultures, AT_1 molecules could be detected (dot blotting). Also, diminished AT_1 was observed in endothelial cell lysates after contact with hemopexin (Western blotting). Hemopexin also induced extracellular signal–regulated kinase 1/2 pathway inhibition in cells after stimulation with angiotensin II in vitro, indicating downregulation of AT_1 by hemopexin. In addition, functional loss of AT_1 occurred after incubation of rat aortic rings with active hemopexin, as reflected by decreased contraction of the aortic rings on stimulation with angiotensin II. It was further demonstrated that plasma from normal pregnant women decreased the AT_1 receptor expression on monocytes as compared with plasma from nonpregnant women or preeclamptic women. Finally, it was shown that plasma hemopexin activity increases during normal gestation from week 10 onward. We concluded that active hemopexin is able to downregulate the AT_1 receptor in human monocytes, endothelial cells, and rat aortic rings. We propose that the physiological role of enhanced hemopexin activity during healthy pregnancy is to downregulate the vascular AT_1 receptor, promoting an expanded vascular bed. Inhibition of hemopexin activity during preeclampsia may result in an enhanced AT_1 receptor expression and a contracted vascular bed. (*Hypertension*. 2009;53:959-964.)

Key Words: pregnancy ■ preeclampsia ■ hemopexin activity ■ angiotensin receptor 1
■ peripheral vascular resistance

Preeclampsia (PE) is a major complication of pregnancy affecting ≈6% of pregnant women in the Western world.[1] The syndrome is clinically characterized by hypertension, proteinuria, and often edema in the second half of pregnancy. Both severe (early onset) and mild forms of this disorder are recognized, as well as severe variants with liver involvement (hemolysis, elevated liver enzymes, and low platelets syndrome).[2] The etiology of PE is unknown, whereas the pathophysiology is poorly understood. The general consensus at this moment is that a systemic inflammatory response associated with endothelial cell dysfunction plays a central role in the pathophysiology of PE. Current thinking focuses on activation of both inflammatory cells and circulating factors, causing endothelial cell dysfunction. For instance, a soluble fms-like kinase 1, an antagonist of vascular endothelial growth factor, may lead to systemic endothelial alterations, including glomerular endotheliosis.[3]

An important physiological adaptation of normal pregnancy is the increase in cardiac output and blood volume, together with a decrease in peripheral vascular resistance.[4,5] These physiological changes are already apparent very early in pregnancy and appear to be maximal between weeks 15 and 25 and remain relatively stable until the end of pregnancy.[4,5] The hemodynamic changes during pregnancy are probably caused (at least in part) by changes in the renin-angiotensin-aldosterone system. One of those changes includes decreased vascular responsiveness to angiotensin II (Ang II), starting approximately at week 10.[6,7] This drop in responsiveness to Ang II may be attributed to decreased vascular expression of the Ang II receptor (AT_1) receptor during normal pregnancy.[8–11] In PE, however, maternal hemodynamics are characterized by relative hypovolemia, enhanced total peripheral resistance, and impaired uteroplacental perfusion.[6,7] The contracted vascular bed in these patients is

associated with the persisting vascular responsiveness on Ang II during the entire gestational period.[6,7] Accordingly, an upregulation of vascular AT_1 receptors, as compared with normal pregnancy, was shown in preeclamptic women.[8–11]

Plasma hemopexin (Hx), a heme binding glycoprotein, has been shown recently to also exert protease activity.[12–14] Thus, Hx is able to affect extracellular matrix (ECM) molecules of renal tissue and endothelial cells in vitro, which can be inhibited by protease inhibitors or nucleotides like ATP.[15] Interestingly, enhanced plasma Hx activity occurs in normal pregnant women as compared with nonpregnant or preeclamptic women.[16] Because this enhanced plasma activity occurs together with downregulation of the vascular AT_1 in healthy pregnancy,[8–11] we proposed that enhanced Hx activity in healthy pregnant women may be responsible for this downregulation. Because of inhibition of Hx activity by enhanced plasma ATP levels observed in PE,[16] it seems likely that AT_1 downregulation does not occur, resulting in a contracted vascular bed in these patients.

In the present article, it is shown that the AT_1 expression can be downregulated by the active isoform of Hx in vitro in various cell types expressing AT_1, including human endothelial cells. Moreover, functional nonresponsiveness on Ang II in blood vessel fragments can be induced by incubation of thoracic artery rings of the rat with active Hx; these data support the notion that the active isoform of Hx may (during pregnancy) downregulate AT_1, resulting in a decrease of peripheral vascular resistance. The lack of vascular expansion in PE in vivo may be related to the absence of active Hx in these patients.

Materials and Methods

Incubation of Peripheral Blood Mononuclear Cells or Human Endothelial Cells With Hx

Peripheral blood mononuclear cells (PBMCs), isolated from EDTA blood samples obtained from healthy volunteers using standard methods, were washed in Hank's balanced salt solution and resuspended in tissue culture medium (RPMI 1640; 1×10^6 cells per mL) and incubated with either active Hx (200 μg/mL) or the same amount of heat-inactivated Hx (Hxi) or washed in saline under standard conditions for 2 hours (37°C; 5% CO_2; Hxi was prepared by heating at 80°C for 60 minutes). After the incubation, cells were centrifuged (2000 rpm) for 10 minutes; supernatants were collected and frozen (−20°C) until measurement of the AT_1 level using Western blot analysis. The cells were resuspended in PBS and analyzed for AT_1 receptor expression using flow cytometry (please see the online data supplement at http://hyper.ahajournals.org). In another set of experiments, cells were incubated with plasma diluted in PBS (1:10) obtained from patients with PE (n=4), healthy pregnant women matched for gestational age (n=5), or age-matched nonpregnant control women (n=5; please see the online data supplement).

Human umbilical venous endothelial cells (HUVECs), passage 3, were obtained from the University Medical Centre Groningen Division of Medical Biology and cultured according to standard methods using 6-well tissue culture plates. Confluent cultures were incubated with either 200 μg/mL of active Hx or the same amount of Hxi or saline as described for PBMCs, harvested using a rubber cell scraper (Corning Inc) after 2 hours, and subsequently washed and prepared for flow cytometry for measurement of AT_1 receptor expression (please see the online data supplement).

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 of the patients. PE patients and healthy pregnant controls were recruited from the antenatal ward of the University Medical Center Groningen. Blood samples were taken from PE patients and healthy pregnant control women, as well as from nonpregnant women. The nonpregnant women were recruited from hospital staff and students (please see the online data supplement).

Dot Blot Assays for the AT_1 Receptor in Supernatants From PBMC Cultures

For dot blot analysis of the AT_1 receptor in culture supernatants in PBMCs, BioBlot nitrocellulose membranes (Costar Cambridge Canada) and the BioDot apparatus (BioRad Laboratories) were used. The AT_1 receptor was measured in supernatants of cultures from PBMCs stimulated with active Hx, Hxi, or saline according to standard methods (please see the online data supplement).

Western Blotting for the AT_1 Receptor in Endothelial Cell Extracts

Confluent human endothelial cell cultures (cell line ECV340) were cultured in medium 199 containing 10% fetal calf serum under standard conditions. After washing with Hanks' balanced salt solution, cultures were incubated for 120 minutes with Hx (200 μg/mL) under serum-free conditions and subsequently washed. Control incubations were done with Hxi. After washing with PBS, cell lysates were prepared and subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membranes (BioRad Laboratories) according to standard methods (please see the online data supplement).

AT_1 Receptor Downregulation by Hx in Smooth Muscle Cell Lysates as Shown by Inhibition of the Phosphorylation of Mitogen-Activated Protein Kinase Extracellular Signal–Regulated Kinase 1/2

This test system is based on phosphorylation of the mitogen-activated protein kinase extracellular signal–regulated kinase (ERK) 1/2 in cells expressing the AT_1 receptor. This phosphorylation occurs on binding of Ang II to the AT_1 receptor. The protocol used was carried out with vascular smooth muscle cells (VSMCs) of Wistar rats, which were kindly provided by L.E. Deelman (Department of Clinical Pharmacology, University Medical Centre Groningen), according to standard methods (please see the online data supplement).

Evaluation of Hx Activity in Plasma From Pregnant Women

For measurement of Hx activity during pregnancy, after signing informed consent, blood samples (EDTA, Vacutainer) were taken of 6 pregnant women throughout the entire period of pregnancy. Sampling started at week 5, and samples were taken every 5 weeks until the end of pregnancy. These women were under standard control with midwives, and their pregnancy remained normal throughout the entire gestational period. Exclusion criteria were the presence of known diseases. Samples were measured for Hx activity using the standard ECM stripping assay.[12–15] This assay is based on the protease activity of Hx evaluated after incubation of kidney tissue with plasma samples with or without anti-Hx IgG. Decrease of expression of glomerular ectoapyrase, reflected by a loss of reaction product, indicates Hx activity of the sample tested and is calculated and expressed as arbitrary units of Hx activity.[14]

Aortic-Ring Contraction Studies

The effect of Hx on stimulation of aorta tissue with Ang II was studied using standard isotonic contraction experiments with thoracic aorta rings of the rat pretreated with N^G-monomethyl-L-arginine (L-NNMA) (10^{-4} mol/L for 15 minutes) to inhibit NO synthase according to standard methods.[17] Because the vascular response to Ang II is mediated through a variety of receptors, including AT_1, Ang II type 2 (AT_2), and possibly additional receptors for cleavage products, without L-NNMA the vascular
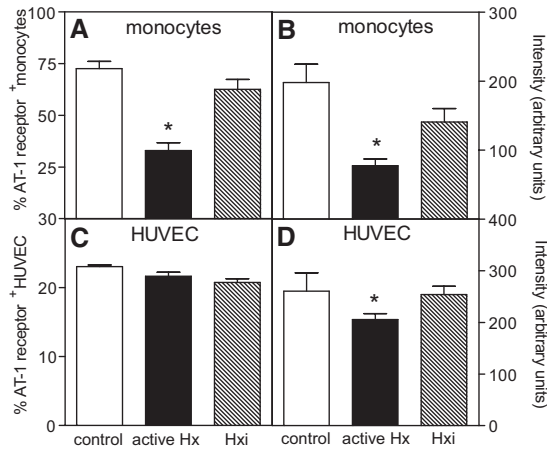
Figure 1. Flow cytometry data showing the mean (±SD) percentage of AT$_1$ receptor–positive human monocytes (n=23; A) or HUVECs (n=5; C) or the mean channel brightness of the AT$_1$ receptor on monocytes (B) or HUVECs (D). Cells have been incubated with either control medium (□), active Hx (■), or Hxi (▨). It can be seen that exclusively after incubation with active Hx, the percentage of cells expressing the AT$_1$ receptor is downregulated in monocytes (A) but not in HUVECs (C). However, mean channel brightness of the AT$_1$ receptor expression is significantly decreased in both monocytes and HUVECs after contact with active Hx (B and D). *P<0.05, Wilcoxon (active Hx vs Hxi).

response for Ang II is highly variable because of differences in receptor types in individual animals. To obtain reproducible and specific responses, L-NNMA is used in this standard assay.[17] Aortic rings from normal Wistar rats were kept in Krebs solution (at 37°C, 5% CO_2, 95% O_2), and the cumulative concentration-response curves for Ang II (10^{-10} to 10^{-6} mol/L) were evaluated after incubation of the tissue with either active Hx (10^{-5} mol/L; n=5) or control medium (n=5). Vasoconstriction responses were expressed as a percentage of maximal contraction after stimulation with phenylephrine (10^{-5} mol/L).

Statistics

For evaluation of the experiments in which PBMCs or HUVECs were incubated with Hx or plasma, statistical analysis was performed using the Wilcoxon's signed-rank test, and differences were considered significant if P<0.05. For the experiments in which aortic rings were incubated with Hx, differences between concentration-response curves were evaluated by 1-way ANOVA for repeated measurements followed by the Bonferroni posthoc test (SPSS for Windows Standard version 8.0, SPSS Inc). Differences were considered significant at a level of P<0.05.

Results

Data obtained after flow cytometry for the detection of the expression of AT$_1$ receptor on monocytes or HUVECs are shown in Figure 1. After incubation of PBMCs with active Hx, the mean percentage of AT$_1$ receptor–positive monocytes shows a significant decrease as compared with monocytes incubated with Hxi (Figure 1A and 1B; Because incubation with saline showed the same results as compared with Hxi throughout all of the experiments, data with saline incubation are not shown). In HUVECs, no significant drop in the percentage of positive cells could be observed (Figure 1C). However, when the mean channel brightness of the AT$_1$ receptor–positive cells was measured, reflecting mean AT$_1$ receptor density, a significant reduction of AT$_1$ receptor
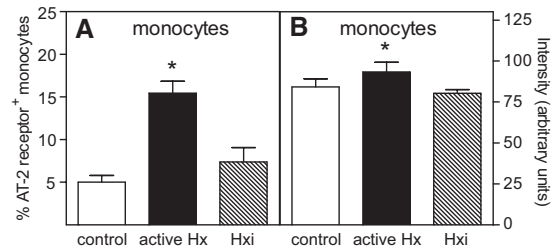

Figure 2. Flow cytometry data showing mean (±SD) percentages of AT$_2$ receptor–positive human monocytes (n=5; A) or the mean channel brightness of the AT$_2$ receptor on monocytes (B). Cells have been incubated with control medium (□), active Hx (■), or Hxi (▨). It can be seen that, exclusively after incubation with active Hx, the percentage of cells expressing the AT$_2$ receptor and the mean channel brightness is upregulated in these cells (P<0.01, active Hx vs Hxi, A; P<0.05, active Hx vs Hxi, B, Wilcoxon).

expression was seen after incubation with active Hx versus Hxi or saline (Figure 1D). In Figure 2, it is shown that the expression of the AT$_2$ receptor has increased after stimulation of cells with active Hx versus Hxi or control medium. With respect to the effect of active Hx on Ang II–mediated contraction of aorta rings in vitro, Hx significantly reduced aortic contraction to cumulative doses of Ang II compared with incubation medium (Figure 3).

Dot blot analysis of supernatants of PBMC cultures after incubation with active Hx shows the presence of increased amounts of AT$_1$ receptor in contrast to control supernatants (ie, after incubation with Hxi or saline; Figure 4A and 4B). In addition, Western blotting of cell lysates prepared from endothelial cell cultures after incubation with Hx showed a decreased amount of the AT$_1$ receptor as compared with incubation with heat-inactivated Hx (Figure 5).

Immunostaining for phosphorylated ERK1/2 using Western blots represented activation of this mitogen-activated protein kinase through stimulation of the AT$_1$ receptor by Ang II. Also this test system showed downregulation of the AT$_1$ receptor by Hx, because it was shown that previous
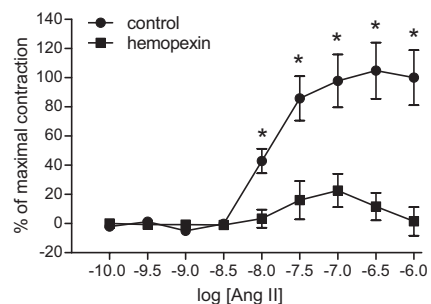

Figure 3. The inhibitory effect of preincubation with active Hx on cumulative Ang II concentration-response curves in thoracic aorta rings of the rat. Thoracic rings were preincubated with vehicle (control; rhombus; n=5) or active Hx (10^{-5} mol/L, squares; n=5) for 15 minutes before administration of Ang II. Experiments were conducted in Krebs solution (37°C, 5% CO_2, 95% O_2) in the presence of L-NNMA (10^{-4} mol/L). Maximum contraction for phenylephrine amounted to 285.1±27.7- and 302.1±60.0-μm transducer displacement in the presence vs the absence of Hx, respectively; this difference is statistically not significant. *Significant difference between curves (ANOVA for repeated measurements, P<0.01).
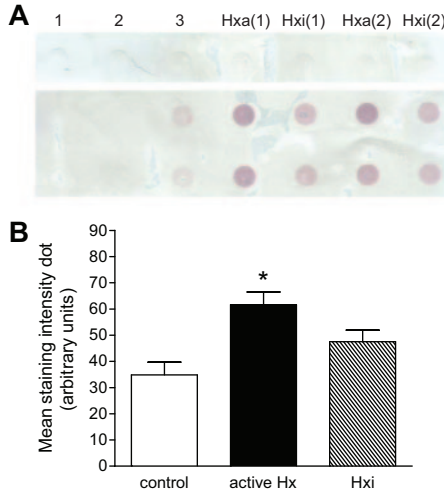
Figure 4. A, Immunostaining of dots of supernatants from PBMC cultures before and after stimulation with 2 different batches of active Hx (Hxa1 and Hxa2) or Hxi, as indicated. Top, Control dots without the first antibody (anti–AT$_1$ receptor); some faint background staining can be seen. Bottom, In duplo: negative dots (lanes 1 and 2) or slight background staining (lane 3) and positive staining of spots containing supernatants from cultures incubated with either active Hx or Hxi. Lanes 1, 2, and 3 contain Tris-buffered saline, tissue culture medium, and supernatant from unstimulated cell cultures, respectively. It can be seen that the most prominent stained dots are derived from supernatants from cultures incubated with active Hx (Hxa1 and Hxa2) vs control culture supernatants derived from cultures stimulated with Hxi (Hxi1 and Hxi2). B, Mean staining intensity (±SD) of dots after staining with anti–AT$_1$ IgG after culture with supernatant from unstimulated cells (▢), active Hx (■), or Hxi (▨). It is shown that contact with active Hx results in increased amounts of AT$_1$ receptor (or receptor fragments) in the medium of the cells vs control cells and cells incubated with Hxi. *$P<0.01$, Hxa vs control and Hxi (Wilcoxon).

incubation of VSMCs with Hx, in contrast to Hxi, leads to decreased staining of the phosphorylated ERK1/2, as reflected in Figure 6.

The studies with plasma samples from either subjects with PE or healthy pregnant women show decreased AT$_1$ receptor expression in monocytes after incubation with plasma from pregnant individuals as compared with cells incubated with plasma from nonpregnant women (Figure 7). The expression of the AT$_1$ receptor in monocytes after contact with PE plasma also showed a loss of AT$_1$ receptor expression, although to a lesser extent as compared with plasma from normal pregnant women. Figure 8 shows mean activities of plasma Hx in samples from healthy pregnant women during
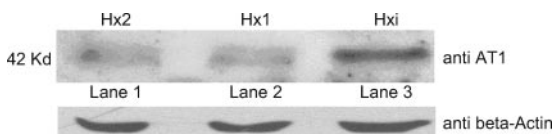

Figure 5. Western blots of endothelial cell lysates after immunostaining for AT$_1$ receptors. Lane 3 (control) shows a band of ≈42 kDa reflecting AT$_1$ receptor molecules. Cell lysates from cell cultures preincubated with active hemopexin (2 different batches, Hx1 and Hx2) show decreased staining of AT$_1$ receptors (lanes 1 and 2) in contrast to lysates from cells incubated with Hxi (lane 3). Bottom, Loading controls of the same cell lysate samples from cells incubated with either Hx or Hxi, stained for β-actine.
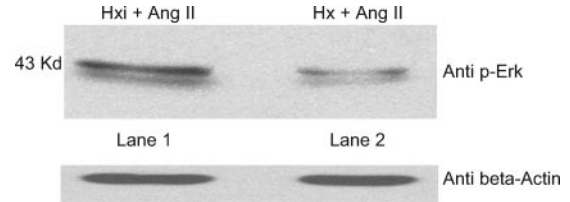

Figure 6. Western blots of VSMC lysates after immunostaining for phosphorylated ERK1/2. It can be seen that lysates from VSMCs derived from Ang II–stimulated cell cultures with previous contact with Hxi show 2 adjacent bands of ≈43 kDa, reflecting phosphorylated ERK1 and ERK2 (lane 1). Decreased staining of phosphorylated ERK1/2 can be seen after preincubation of the stimulated VSMCs with active Hx (lane 2). Stimulation of the cell cultures was done with 1.0 nmol/L angiotensin II. Bottom, Loading controls of the same cell lysate samples from cells incubated with either Hx or Hxi, stained for β-actine.

the entire period of pregnancy. It appeared that Hx activity gradually increased during pregnancy, starting around week 10.

Discussion

The aim of the present study was to test our hypothesis that Hx may be able to downregulate the AT$_1$ receptor in vitro from cells expressing this receptor. Because both PBMCs and endothelial cells showed a loss of expression of the AT$_1$ receptor after contact with active Hx (Figure 1), it is likely that active Hx was indeed able to downregulate this receptor. The data in Figure 4A and 4B clearly show that shedding of the AT$_1$ receptor seems the most likely mechanism. It is clear from Figure 1C and 1D that the outcome of the Hx effect depended on the method of expression of the data. Thus, whereas the mean decrease of the percentage of AT$_1$–positive monocytes reflected the loss of all of the AT$_1$ receptors per cell leading to a decrease of a number of positive cells, the mean decrease of intensity (mean channel brightness) reflected the loss of only a part of the receptors per cell after contact with active Hx. It can be deduced from these data that the downregulation of the AT$_1$ receptors in HUVECs by

Figure 7. Flow cytometry data showing mean percentage (± SD) of monocytes expressing the AT$_1$ receptor after incubation of the cells with plasma from healthy nonpregnant control women (▢; n=5), normal pregnant women (■; n=5), and PE patients (▨; n=4). It can be seen that cells after contact with plasma from pregnant women (containing active Hx) show decreased expression of the AT$_1$ receptor. Although considerable downregulation of the AT$_1$ receptor is also seen after contact of the cells with plasma from PE patients (containing Hxi), this occurs to a significantly lesser extent vs plasma from normal pregnant women. *$P<0.01$, pregnant plasma vs nonpregnant plasma (Wilcoxon). **$P<0.01$, PE plasma vs pregnant plasma (Wilcoxon). #$P<0.01$, PE plasma vs nonpregnant plasma (Wilcoxon).

Figure 8. Mean Hx activity ($\pm$SD) of plasma from nonpregnant (t=0) and normal pregnant women assayed at various time points of pregnancy (n=8). Hx activity, expressed as arbi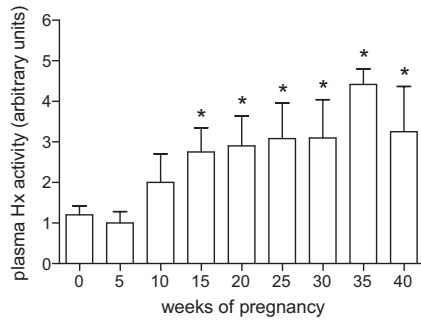trary units, was measured using the standard ECM stripping assay. It can be seen that Hx activity gradually increases during pregnancy, starting approximately on week 10 and plateauing after week 15. Mean Hx activity in plasma samples from weeks 15 to 40 is significantly increased vs mean Hx activity in samples from nonpregnant women (timepoint 0). *$P<0.01$, Wilcoxon.

active Hx was less prominent as compared with this effect in monocytes. The reason for this remains to be investigated. It is conceivable that the nature of AT$_1$ receptor expression in both cell types is not identical, which may explain this discrepancy.

Be this as it may, it is clear that also in endothelial cells a significant reduction of AT$_1$ expression occurred after contact with active Hx. As concomitant with downregulation of AT$_1$, upregulation of the AT$_2$ receptor occurred after stimulation with Hx (Figure 2), and it is clear that exclusively shedding of the AT$_1$ receptor was induced by active Hx. Both Ang II receptor subtypes AT$_1$ and AT$_2$ were able to bind Ang II. However, downregulation of the AT$_1$ receptor often leads to upregulation of the AT$_2$ receptor.[18] The mechanism of AT$_1$/AT$_2$ interaction after binding of Ang II is poorly understood.[19] Because Ang II receptor trafficking between cell membrane and cytosol occurs,[20] it is conceivable that nongenomic mechanisms are involved in upregulation of AT$_2$ receptors concomitantly with enzymatic removal of AT$_1$ receptors after contact with active Hx. Be this as it may, the present data show an antagonistic effect regarding AT$_1$ versus AT$_2$ expression, which is in line with data from other authors.[21]

Downregulation of the AT$_1$ receptor seems also reflected by inhibition of the contraction of aorta rings by active Hx after stimulation with Ang II (Figure 3). However, although a significant decrease of aorta ring contraction during contact with active Hx is seen in these experiments, it cannot be concluded from these data whether this is because of either enzymatic shedding or blocking of this receptor by active Hx. In view of the present data with isolated cells, however (Figures 1 and 4), it is suggested that enzymatic cleavage of the AT$_1$ receptors by active Hx may be considered as a mechanism by which this nonresponsiveness on Ang II stimulation is induced. Because the contraction induced by the $\tilde{\alpha}$-adrenergic receptor agonist phenylephrine was not affected by Hx, it seems unlikely that the effect of Hx was caused by a general influence on signal transduction pathways or calcium handling. Therefore, we feel that downregulation of this vascular response on Ang II by active Hx may be specific.

If active Hx is indeed able to promote shedding of the AT$_1$ receptor from cell surfaces, it should be expected that this molecule is detectable in supernatants of cell cultures treated with active Hx. This indeed appears to be the case. The observed increase of the AT$_1$ receptor in supernatants of PBMC cultures after incubation with active Hx, as shown in Figure 4, therefore supports the assumption that active Hx removed the AT$_1$ receptor from the cells studied. This notion is enhanced by the Western blot data of Figure 5, showing reduced AT$_1$ staining in endothelial cell lysates after incubation of endothelial cells with active Hx. Taking advantage of the immediate phosphorylation of the mitogen-activated protein kinase ERK1/2 after stimulation of the AT$_1$ receptor by Ang II, also using this test system, it is clear that downregulation of the AT$_1$ receptor occurred after contact of the cells with active Hx. Because active Hx contains protease activity, able to enzymatically affect various cell membrane constituents and ECM,[12–14] it is highly likely that this was mediated by the protease activity of active Hx. However, the precise mechanism of this putative shedding of AT$_1$ receptors from cell surfaces remains to be established. Because the blotting technique is based on immunologic detection of AT$_1$ receptor antigens using a polyclonal antibody, it is possible that both the AT$_1$ receptor and fragments of this molecule are recognized. If active Hx also induces shedding of AT$_1$ in vivo, the question as to the function of soluble AT$_1$ emerges. Up to now, a potential function for shedded AT$_1$ receptors or receptor fragments in vivo is unknown. We considered the possibility that active Hx does not enzymatically hydrolyze AT$_1$ directly but acts through stimulation of other membrane-bound enzymes, eg, adamalysine 17. These so-called sheddases are able to promote shedding of various membrane-associated molecules, eg tumor necrosis factor α.[22] Preliminary data, however, showed that inhibition of adamalysine 17, with its inhibitor tumor necrosis factor α protease inhibitor 1, did not affect the downregulation of the AT$_1$ receptor after incubation of the cells with active Hx, suggesting that Hx is able to enzymatically remove the AT$_1$ receptor directly from cell membranes. To exclude the involvement of other sheddases, studies are in progress to test more sheddase inhibitors, including metalloproteinase inhibitors. With respect to the AT$_2$ receptor expression, it is clear that AT$_2$ is significantly upregulated by active Hx, suggesting that the balance between AT$_1$ and AT$_2$ expression may be under the control of active Hx (Figure 2). This is in line with the observation that the AT$_2$ receptor may also be involved in vascular responses in normal and complicated pregnancies.[18] However, as stated above, the molecular mechanism underlying AT$_1$/ AT$_2$ interactions is unclear.

Similar downregulation of the AT$_1$ receptor occurs when PBMCs are incubated with plasma from healthy pregnant individuals (Figure 7). Because these plasma samples contain active Hx,[16] we ascribe this effect to the presence of Hx in these samples. Incubation of cells with plasma samples from subjects with PE unexpectedly also showed significant downregulation of AT$_1$ receptor as compared with plasma from nonpregnant and pregnant control individuals. This may reflect the presence of Hx activity in these plasma samples. However, using the ECM stripping assay, we demonstrated previously that PE plasma does not show Hx activity. This inhibition is because of the relatively high titers of ATP,

which inhibit Hx activity.[16] The apparent discrepancy obtained in the current experiments, however, can be explained by assuming that contact of PE plasma with living cells expressing ectophosphatases on their surface promotes hydrolysis of ATP, leading to reactivation of Hx in the PE plasma samples tested. Indeed, experimental evidence is accumulating that contact of PE plasma with living PBMCs may partially activate Hxi in this plasma (results not shown). In other words, it is likely that this reactivation of Hxi to active Hx has occurred during the incubation steps necessary for the flow cytometry assays. In spite of this disturbing reactivation of Hxi in PE plasma samples, it is clear that plasma from pregnant women contains more Hx activity as compared with that of subjects with PE.

Perspectives

Previously we described enhanced Hx activity in plasma from healthy pregnant women, in contrast to women with PE, occurring in the third trimester of pregnancy.[16] From Figure 8, it can be seen that the rise of Hx activity starts approximately on week 10. Also around the gestational age of week 10 in healthy pregnant individuals, the nonresponsiveness on Ang II is observed.[5,6] This persists throughout the entire period of pregnancy, leading to a relatively expanded vascular bed, beneficial for proper placental perfusion, as compared with age-matched nonpregnant women. Because the nonresponsiveness of vessels on Ang II may be attributable to vascular loss of AT_1,[7] it is tempting to speculate that the function of enhanced Hx activity in normal pregnancy is to promote shedding of the vascular AT_1 receptor, leading to vascular expansion. Interestingly, in healthy pregnant rats, enhanced plasma Hx activity is also associated with decreased AT_1 receptor expression (MM Faas et al, unpublished data, 2009). The hypothesis is in line with the situation in PE lacking Hx activity because of enhanced titers of plasma ATP.[16] In PE, the AT_1 receptor expression is relatively increased[8–11] and vascular responsiveness on Ang II is persisting, leading to a contracted vascular bed and resulting in detrimental consequences for placental perfusion. We feel that potential treatment strategies for PE should focus on reactivation of Hx activity, ie, by treatment of PE patients with alkaline phosphatase, or other ATP hydrolyzing principles. This suggestion is corroborated in vivo, in which smoking, per se, is associated with increased plasma alkaline phosphatase activity and decreased incidence of PE.[23]

Disclosures

None.

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Vascular contraction and preeclampsia. Down regulation of the angiotensin receptor-1 by hemopexin *in vitro*.
Online supplement

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Short title: AT-1 receptor down regulation by hemopexin.

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Expanded 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. Blood samples were taken from PE patients and healthy pregnant control women as well as from 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 women who had 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 mmHg or more on two or more consecutive occasions more than 4 hrs apart and proteinuria of more than 300 mg/24 hours. Maternal blood samples of both pregnant and preeclamptic women were collected during routine blood sampling during pregnancy/preeclampsia. Blood samples were drawn from the antecubital vein into 10-mL tubes containing EDTA (Venoject, Terumo Europe NV, Leuven, Belgium). Samples were immediately stored at 4 °C and centrifuged within 1 hour. They were centrifuged at 130 g for 10 minutes at 4 °C; subsequently the plasma was centrifuged at 700 g for 10 minutes. The platelet poor plasma samples were frozen at -80 °C.

Flow Cytometry of PBMC and HUVEC:
To evaluate the expression of AT-1 receptor upon blood cells or HUVEC, PBMC from healthy female donors were washed (x2) in PBS supplemented with 1% bovine serum albumin (PBS/BSA) and subsequently incubated with monoclonal anti AT-1 IgG (clone TONI, Abcam) or anti AT-2 IgG (clone 364805 R&D ;UK), or non immune mouse IgG, at 0 ºC. After 30 min. cells were washed (x2) in PBS/BSA buffer and incubated with goat-anti-mouse IgG FITC (DAKO) for 30 min at 0º C. For control staining, cells were incubated with the second antibody only (i.e. goat-anti mouse-IgG FITC). After another wash (x2) with BSA/PBS cells were fixed with paraformaldehyde (2%). The fluorescence was measured by flow cytometry using a FACS device (Calibur Beckton Dickinson, USA)). PBMC suspensions containing 1×10^5 cells were assayed. The data were processed using a standard software program (Winlist 6.0). For PBMC analysis, monocytes were gated in the forward-sidescatterplot and this gate was copied to a histogram. For HUVEC a gate was set on the HUVEC population and this gate was also copied to a histogram. In the histogram, control incubations (second antibody only) were gated in such way that 99% of the cells were negative for FITC. This gate was then copied to the samples which were incubated with AT-1 receptor antibody. Percentage positive cells was used as a standard for AT-1 expression; mean channel brightness of the positive cells, reflecting AT-1 receptor density, was also calculated.

Dot blot assays for AT-1 receptor in supernatants from PBMC cultures.
For dot blot analysis of the AT-1 receptor in culture-supernatants in PBMC, BioBlot-nitrocellulose membranes (Costar Cambridge Canada) and BioDot apparatus (BioRad Labs) were used. AT-1 receptor was measured in supernatants of cultures from PBMC stimulated with active Hx, Hxi or saline according to standard methods. Supernatants were applied on the membrane (100 µl) and incubated for 60 minutes at

room temperature. The sheets were washed (x4) with Tris buffered saline (TBS). The sheets were removed from the dot blot device and further incubated for 60 min with 5% skim milk (ELK;Campina, The Netherlands) and subsequently washed with TBS supplemented with Tween-20 (0,05%) (TBS-Tween), followed by incubation overnight with the rabbit-anti-human AT-1 receptor IgG (N10; Santa Cruz, USA) and washed with TBS containing 2.0% ELK. After washing (x2) in TBS-Tween, sheets were incubated with goat-anti-rabbit IgG conjugated with alkaline phosphatase (1:2000) for 60 minutes. Stain development was done with nitro blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate disodium salt (Sigma-Aldrich, Germany). After drying of the sheets, blots were scanned and the staining intensity was quantified using "Quality One" software program (Biorad).

Western blotting for AT-1 receptor in endothelial cell extracts.
Confluent human endothelial cell cultures (cell line ECV340) were cultured in Medium 199 containing 10% fetal calf serum under standard conditions. After washing with Hanks' balanced salt solution (HBSS), cultures were incubated for 120 minutes with hemopexin (200 μg/ml) under serum free conditions and subsequently washed. Control incubations were done Hxi. After washing with PBS, cell lysates were prepared and subjected to 7.5% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membranes (Biorad) according to standard methods. Cell lysates were prepared by using 150 μl lysis buffer per 6 x 10^5 cells (10mM Tris, pH 7.5; 1 mM EDTA, 4% SDS, 20% glycerol, 10% β-mercapto-ethanol, 25mM NaF, containing freshly prepared 100μM Na_3VO_4, 1mM phenylmethane sulfonylfluoride ,4 μg/ml aprotinin and bromophenyl blue). The lysates were heated in a boiling water bath for 5 minutes and subsequently sonicated for 5 seconds followed by centrifugation for 10 minutes at 10.000 x g. Samples were subjected to 7.5% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membranes (Biorad) according to standard methods. The membranes were immunostained with mouse monoclonal antibody to the AT-1 receptor (Abcam, ab9391), followed by anti-mouse IgG conjugated with horseradish peroxidase(HRP) according to standard methods. The immunostaining was visualised by enhanced chemiluminescense (SuperSignal West Pico Chemiluminescent Substrate from Thermo Scientific).
Loading controls were done using rabbit polyclonal anti β-actine IgG (Abcam (ab8227)). Thus, befor restaining, the blots were incubated with stripping buffer (0.1M glycine containing 1%SDS, pH 2.0) for 30 minutes. The blots were subsequently washed (x3) with Tris buffered saline (pH7.4) and incubated with anti β-actine IgG (1:10.000) for 60 minutes. The staining was visualized as described for anti AT-1 IgG.

AT-1 receptor downregulation by hemopexin in smooth muscle cell lysates as shown by inhibition of the phosphorylation of MAP kinase ERK1/2.
This test system is based upon phosphorylation of the MAP kinase ERK1/2 in cells expressing the AT-1 receptor. This phosphorylation occurs upon binding of angiotensin II (Ang II) to the AT-1 receptor. The protocol used was carried out with vascular smooth muscle cells of Wistar rats (VSMC), which were kindly provided by Dr L.E. Deelman (Department of Clinical Pharmacology, UMCG, Groningen), according to standard methods. Confluent cells (passage 3-6) were cultured in

Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum under standard conditions. Before use, cells were washed with HBSS and subsequently incubated with 200 μg/ml Hx or Hxi for 120 minutes under serum free conditions. After incubation cells were washed and incubated with Ang II (1.0 nM/ml)) for 5 minutes followed by the preparation of cell lysates as described above. The lysates were subjected to 10% SDS PAGE followed by blotting to nitrocellulose membranes according to standard methods. Immunostaining was done with a mouse monoclonal antibody against phosphorylated ERK1/2 (Santa Cruz; SC 7383) and by rabbit anti mouse IgG conjugated to HRP (DAKO,PO260) as a second step. The staining was visualized by enhanced chemiluminescence as described above.