

# Hemopexin activity is associated with angiotensin II responsiveness in humans

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**Background:** Hemopexin, an acute phase protein, can downregulate the angiotensin (ang) II type 1 receptor (AT1-R) *in vitro*. Whether hemopexin is involved in the responsiveness to ang II *in vivo* is unknown. Therefore, we tested whether variations in endogenous hemopexin activity are associated with the responsiveness of blood pressure to ang II in healthy volunteers.

**Method:** Healthy men ( $n=33$ , age  $26 \pm 9$ ) were studied in balance on low sodium (50 mmol Na<sup>+</sup> per 24 h) and high sodium (200 mmol Na<sup>+</sup> per 24 h) diet, respectively. After baseline measurements of blood pressure, ang II was infused at 0.3, 1 and 3 ng/kg per min for 1 h per dose. Hemopexin activity was measured at baseline in EDTA-plasma samples by an amidolytic assay with a chromogenic substrate suitable for hemopexin activity evaluation.

**Results:** During high sodium the hemopexin activity was lower;  $1.6 \times 10^5$  ( $0.6 \times 10^5 - 4.7 \times 10^5$ ) versus  $2.8 \times 10^5$  ( $1.1 \times 10^5 - 5.1 \times 10^5$ ) arbitrary units ( $P < 0.01$ ) and the pressor response to 3 ng ang II/kg per minute larger than during low sodium ( $17.6 \pm 6.5$  versus  $14.6 \pm 6.9$  mmHg,  $P < 0.01$ ). Hemopexin activity negatively correlated with the pressor response to ang II during either type of sodium intake (high sodium:  $r = 0.42$ ,  $P < 0.05$ ; low sodium:  $r = 0.35$ ,  $P < 0.05$ ).

**Conclusion:** These in-vivo data obtained in healthy individuals support recent in-vitro data showing that active hemopexin downregulates the availability of the AT1-R. Therefore, activated hemopexin might be considered as a factor mediating ang II effects upon blood pressure by modulating AT1-R availability.

**Keywords:** angiotensin II, angiotensin II type 1-receptor, blood pressure, hemopexin, salt intake

**Abbreviations:** ang II, angiotensin II; APRC, active plasma renin concentration; AT1-R, angiotensin II type I receptor; MAP, mean arterial pressure; S2302, H-D-Pro-Phe-Arg-pNA.2HCl

## INTRODUCTION

Various lines of evidence indicate a major role for angiotensin (ang) II in the pathogenesis of renal and cardiovascular damage, mainly by its effects on the vascular ang II type I receptor (AT1-R) [1]. It has been shown recently that active hemopexin, an acute phase protein with

protease-like activity, is able to downregulate the AT1-R on endothelial cells and human monocytes *in vitro* [2,3]. This suggests that hemopexin activity can modulate the physiological effects of ang II. Whether this is the case *in vivo* is unknown. *In vivo*, the primary vascular effect of ang II leads to systemic vasoconstriction. Observations in individuals with pre-eclampsia suggest that hemopexin controls the availability of AT1-R and consequently the vascular response upon ang II [2,3]. In normal pregnancy, ang II levels are relatively high [4] but do not lead to hypertension, as the vascular responsiveness upon ang II is decreased [5]. This decrease, starting about week 10 of pregnancy, is associated with a significant increase of plasma hemopexin activity, also starting at week 10 of pregnancy [2]. However, in pre-eclamptic women, the physiological decrease of vascular sensitivity for ang II is blunted [5,6], which may initiate the development of hypertension. In addition, individuals with pre-eclampsia show persisting vascular sensitivity for ang II, whereas no rise of hemopexin activity occurs. In other words, their normal pregnancy-induced rise in hemopexin activity is lacking [2].

Ang II responsiveness differs widely between healthy humans and is modulated by dietary sodium intake [7,8]. In this study, we investigated whether variations of endogenous hemopexin activity are associated with the pressor responses to exogenous ang II in young healthy normotensive men during two different sodium intakes, that is 50 mmol Na<sup>+</sup> per 24 h (low sodium) and 200 mmol Na<sup>+</sup> per 24 h (high sodium).

## METHODS

### Participants

The study population consisted of 33 healthy normotensive white men with a median (interquartile range) age of 24

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(21–27) and a mean BMI over both periods of  $23.1 \pm 2.3$  kg/m<sup>2</sup>. Normal blood pressure was confirmed by repeated noninvasive automatic blood pressure assessment (Dinamap) and defined as SBP less than 140 mmHg and DBP less than 90 mmHg. The study was approved by the local medical ethics committee, in accordance with the declaration of Helsinki, and all participants gave written informed consent. All medical histories were without significant disease, and routine physical examination did not reveal any abnormalities.

### Study protocol

Participants were studied at the end of two 7-day periods, during which they used a low sodium diet (50 mmol Na<sup>+</sup> per 24 h) and a high sodium diet (200 mmol Na<sup>+</sup> per 24 h), respectively. Potassium intake was standardized at 80 mmol per 24 h. Otherwise, the participants continued their usual food habits. For assessment of dietary compliance and sodium balance, 24-h urine was collected at day 4 and day 6 during each period. During both periods, the individuals were ambulant and continued their normal activities.

At day 7 of both study periods, the individuals reported at the research unit at 0800 h after having abstained from food and alcohol overnight. Height and body weight were measured at the start of this day and BMI was calculated as the ratio of body weight (kg) and the square of height (m).

At 1100 h, blood was withdrawn for determination of active plasma renin concentration (APRC) and aldosterone. Sodium intake during the day was adjusted according to the actual diet in the concerning diet period. Baseline blood pressure was assessed with an automatic device (Dinamap; GE Medical Systems, Milwaukee, Wisconsin, USA) at 15 min intervals. Mean arterial pressure (MAP) was calculated as diastolic pressure plus one third of the pulse pressure.

### Angiotensin II infusion

Baseline values of blood pressure were obtained between 1000 and 1200 h. Between 1200 and 1500 h, ang II (Clinalfa, Merck Biosciences AG, Läufelfingen, Switzerland) was administered in the left antecubital vein. Between 1200 and 1300 h, ang II was infused at a constant rate of 0.3 ng/kg per min. Thereupon, ang II was infused at a constant rate of 1 ng/kg per min and 3 ng/kg per min, each during 1 h. During the ang II infusions, blood pressure was determined at 5-min intervals.

### Blood and urine sampling and analysis

Blood samples for measurement of plasma hemopexin activity were drawn at 0800 h. Samples for measurement of APRC and aldosterone were drawn at 1100 h in a semi-supine position and stored at  $-20^{\circ}\text{C}$  until analysis.

Aldosterone was measured with a commercially available radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, California, USA). Plasma renin activity was measured as described previously with a radioimmunoassay that detects the amount of ang I produced per hour in the presence of excess angiotensinogen (nanograms of ang I produced per milliliter of plasma per hour) [9].

Urinary and serum concentrations of sodium and potassium were measured by standard autoanalyzer technique (MEGA; Merck, Darmstadt, Germany).

Blood samples for determination of ang II were drawn at 1100 h in prechilled 3 ml vacuum tubes containing 5.4  $\mu\text{g}$  K3EDTA and an additional 0.2 ml angiotensin converting enzyme inhibitor cocktail, containing 1.704  $\mu\text{g}$  phenantroline, 0.16 mg enalaprilat, 1 ml ethanol and 4 mg neomycin. After centrifugation at  $4^{\circ}\text{C}$ , the plasma for determination of ang II was snap-frozen and stored at  $-80^{\circ}\text{C}$  until analysis. Plasma concentration of ang II was measured by a specific radioimmunoassay after SepPak extraction of plasma samples and high-pressure liquid chromatography separation [10].

Hemopexin activity was measured in EDTA-plasma with a standard amidolytic assay with the artificial substrate S2302 (H-D-Pro-Phe-Arg-pNA.2HCl) (Chromogenix, Milano, Italy) as described previously [11]. This assay has been shown to match with the extracellular matrix stripping assay, whereby loss of glomerular apyrase expression after incubation of kidney sections with hemopexin, with or without antihemopexin immunoglobulin G, was quantified as a standard for hemopexin activity [11]. As previous pilot studies indicated that the protease activity of the samples could hardly be inhibited by  $\alpha$ -2-macroglobulin, or antithrombin III, it is unlikely that plasma kallikrein activity occurs in the samples tested.

### Data analysis

Mean values and SDs were calculated for parametric variables. Medians and quartiles were computed for nonparametric data. Paired sample *T*-test (parametric data) and Wilcoxon signed rank test (nonparametric data) were used to compare values between the high-sodium and low-sodium periods, each of the participants being their own control. Data on hemopexin activity were first logarithmically transformed to normality. Pearson correlation was used for analysis of the influence of hemopexin activity on ang II responsiveness.

## RESULTS

In Table 1 parameters on dietary compliance, body weight and circulating rennin–angiotensin–aldosterone system parameters on both diets are displayed. It shows, first, that the change in sodium intake resulted in the expected differences in sodium excretion. Furthermore, the urinary excretion of potassium was close to the target intake of 80 mmol per 24 h. The high sodium intake induced a slight but significant rise in body weight, consistent with a positive volume balance. Serum concentration of potassium was not influenced by the shift in sodium intake, whereas the serum concentration of sodium was slightly but significantly higher during high sodium. As expected, APRC, ang II and aldosterone were suppressed during high sodium.

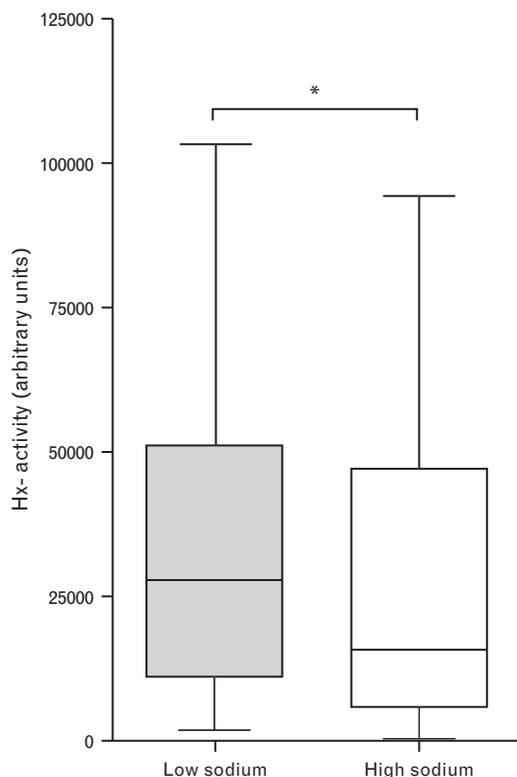
In Fig. 1, the median and interquartile range of hemopexin activity, on both diets, are displayed. It shows that hemopexin activity was slightly but significantly lower during high sodium. There was a strong correlation between individual values of hemopexin activity on low sodium and high sodium ( $r^2 = 0.80$ ,  $P < 0.00001$ ). Table 2 displays hemopexin activity on both diets, MAP before and during incremental doses of ang II and heart rate at baseline

**TABLE 1. Twenty-four-hour excretion of sodium and potassium, serum potassium, body weight, active plasma renin concentration, aldosterone and plasma angiotensin II during low and high sodium intake**

	Sodium intake		P
	50 mmol/24 h	200 mmol/24 h	
U <sub>Na24</sub> (mmol/24-h)	36 ± 22	220 ± 57	<0.01
U <sub>K24</sub> (mmol/24-h)	86 ± 35	84 ± 27	n.s.
Serum K (mmol/l)	3.9 ± 0.2	3.9 ± 0.3	n.s.
Serum Na (mmol/l)	137 ± 3	138 ± 3	<0.05
Body weight (kg)	80 ± 9	81 ± 10	<0.01
APRC (ng ang-1/ml per h)	6.5 (4.8–8.2)	3.0 (1.5–3.6)	<0.01
Aldosterone (ng/l)	130 (82–186)	43 (24–58)	<0.01
Ang II (pmol/l)	10.6 (4.3–13.7)	3.3 (1.2–10.5)	<0.05

Data are expressed as mean ± SD and median (range). Ang II, angiotensin II; APRC, active plasma renin concentration; U<sub>K24</sub>, 24 h urine excretion of K<sup>+</sup>; U<sub>Na24</sub>, 24 h urine excretion of Na<sup>+</sup>.

and during ang II infusion. Baseline MAP was slightly but significantly higher during high sodium. Ang II induced a dose-dependent rise in MAP during both sodium intakes. As anticipated, the pressor responses to ang II were larger during high sodium as compared to low sodium; this difference reached statistical significance at the infusion rates of 1 and 3 ng/kg per min. Also, the responses of DBP and SBP to the incremental doses of ang II were higher during high sodium. However, this did only reach statistical significance during the infusion rate of 3 ng ang II/kg per min ( $P < 0.01$  and  $P < 0.05$ , respectively). There was no significant correlation between the actual SBP and hemopexin activity ( $r = 0.16$ ,  $P < 0.37$ )



**FIGURE 1** Median, interquartile range and 95% confidence intervals of hemopexin (Hx) activity during low sodium and high sodium intake. \* $P < 0.001$  versus low sodium. Wilcoxon's signed rank test. It is shown that low sodium intake, in contrast to high sodium, is associated with enhanced plasma Hx activity.

For individual participants, the correlation between hemopexin activity and the pressor response (MAP) to ang II on high sodium (left panel) and low sodium (right panel) is shown in Fig. 2 (data for 3 ng ang II/kg per min dose). It shows that a higher hemopexin activity is associated with a significantly lower pressor response to ang II infusion, irrespective of the salt intake.

## DISCUSSION

The present study suggests a role for plasma hemopexin activity in mediating vascular responsiveness to ang II *in vivo* in healthy humans. We demonstrated that, on a group level, the enhanced blood pressure response to ang II induced by high sodium intake is associated with a decreased hemopexin activity. Moreover, individual differences in pressor responsiveness to ang II at a given sodium intake correlate negatively with hemopexin activity. In other words, higher responsiveness occurred in individuals with lower endogenous hemopexin activity, whereas low responsiveness correlates with relatively high endogenous hemopexin activity. The present results are in line with previous data, demonstrating that active hemopexin induces downregulation of AT-1R expression on human endothelial cells *in vitro*. Using a functional test system *in vitro*, it was also shown that the ang II-induced vascular contraction in isolated rat aortic rings was blunted after contact with active hemopexin [3]. Therefore, it is conceivable that plasma hemopexin activity in healthy individuals is able to control vascular responsiveness to ang II.

The blood pressure responses to ang II are less pronounced in healthy individuals on low sodium as compared to high sodium, as observed previously [12,13]. To explain this reduced sensitivity, various mechanisms have been proposed. For instance, due to the association of low salt with increased ang II in the circulation, downregulation of the AT-1R and/or increased receptor occupancy by ang II may promote reduced vascular sensitivity for ang II [12,14,15]. Also, ang II-induced AT-1R downregulation by inducing internalization of receptor ligand complexes, whereas, alternatively, transcriptional and posttranscriptional assembling of the receptor have been proposed [16–19]. More recent evidence focuses on AT-1R dimerization and ang II-independent pathways mediating AT-1R availability. It is clear that these putative mechanisms need

**TABLE 2. Baseline hemopexin activity and mean arterial pressure before and during incremental doses of angiotensin II, during low and high sodium intake**

	Sodium intake		P
	50 mmol/24 h	200 mmol/24 h	
Hx activity (arbitrary units)	2.8·10 <sup>5</sup> (1.1·10 <sup>5</sup> –5.1·10 <sup>5</sup> )	1.6·10 <sup>5</sup> (0.6·10 <sup>5</sup> –4.7·10 <sup>5</sup> )	<0.001
SBP (mmHg) baseline	117 ± 10	121 ± 11	<0.01
DBP (mmHg) baseline	69 ± 8	71 ± 7	n.s.
MAP (mmHg) baseline	85 ± 7	87 ± 8	<0.05
ΔMAP (mmHg) 0.3 ng/kg per min	0.1 ± 7.1	1.5 ± 3.8	n.s.
ΔMAP (mmHg) 1 ng/kg per min	7.1 ± 5.6	9.1 ± 6.1	<0.05
ΔMAP (mmHg) 3 ng/kg per min	14.6 ± 6.9	17.6 ± 6.5	<0.01

Data are expressed as mean ± SD, hemopexin activity is in median (range). Hx, hemopexin; MAP, mean arterial pressure; Δ, response to exogenous angiotensin II.

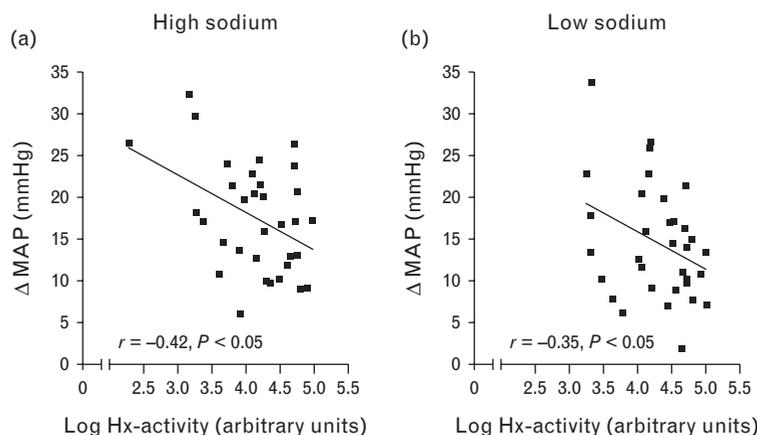
confirmation. However, from the present data from healthy individuals *in vivo*, and previous findings in pregnant individuals [2,3] together with recent *in vitro* data, we propose a role for active hemopexin, which is able to shed the AT-1R from cell surfaces directly [3]. The protease-like activity of hemopexin contributing to the regulation of vascular AT-1R expression may occur in normal human plasma [20].

Active hemopexin can be inhibited by protease inhibitors or by nucleotides, like extracellular ATP [21]. It has been shown that inactive hemopexin, which is complexed by ATP *in vitro* can be reactivated by endothelial ectoapyrase (CD39) [22]. This indicates that intact endothelium may control local hemopexin activity. Different lines of evidence suggest that high sodium intake is associated with endothelial dysfunction [23,24], possibly including affection of endothelial CD39. Recent experiments *in vitro* showed that minor increments of sodium in the culture medium (1%), significantly affected endothelial CD39 (SL Della Penna *et al.* 2012, unpublished observation).

In view of the AT-1R shedding capacity of active hemopexin, we feel that diminished local hemopexin activation *in vivo* due to loss of endothelial CD39 induced by salt may result in increased availability of AT-1R along the endothelial cell membranes. The change of hemopexin activity induced by the switch in sodium intake did not correlate

with the change of ang II responsiveness in individual participants. However, hemopexin activity can be ascribed to only a small percentage of the variability of ang II responsiveness during high and low sodium intake (R-squares of 0.18 and 0.12, respectively). This reflects the fact that the pressor response to ang II depends on many factors resulting in a low sensitivity for the detection of such a quantitative relationship. Nevertheless, the enhanced ang II responsiveness observed in the present study in association with low hemopexin activity during high sodium is remarkably consistent with the findings observed between the participants studied. This within-participant correlation is more pronounced in individuals with lower circulating active hemopexin. As this consistency was confirmed in two sets of salt intake experiments, we feel that this finding supports a role of hemopexin in the regulation of ang II responsiveness in healthy individuals.

Differences between individuals regarding hemopexin activity were relatively large in both high and low sodium intake. The data show further that high sodium induces a decrease in hemopexin activity, and from Fig. 2 it can be seen that individuals with relative low hemopexin activity on one diet have a relatively low hemopexin activity on the other diet as well. This pattern of a high within-participant correlation at different conditions suggest that in addition to environmental factors, individual factors, such as for



**FIGURE 2** Relationship between hemopexin (Hx) reactivity (a) and increase in mean arterial pressure (ΔMAP) (b) in response to angiotensin II (3 ng/kg per min), during high sodium (left panel) and low sodium (right panel).  $r$  = Pearson correlation coefficient. It can be seen that in both, high salt and low salt intake, higher hemopexin activity is associated with lower pressor response induced by angiotensin II infusion.

instance genetic variations in the hemopexin gene expression, are involved in determining the level of hemopexin activity [25].

A limitation of our study is that we applied two 7-day periods of low and high dietary sodium intake, consecutively. It would have been a more ideal design if we had applied the two periods in random order. It cannot be excluded in the present setting that the low sodium diet still had an effect during the high sodium period. However, we found hemopexin activity to be significantly inversely correlated with the pressor response to ang II during both levels of sodium intake, so it is unlikely that carry-over of effects of the low-sodium period to the high-sodium period played an important role in the conclusions drawn from this study. With respect to Na measurement in 24-h urine samples for compliance testing, we are aware that sampling errors may play a role, whereas extrarenal salt loss is not taken into account. Nevertheless, for logistical reasons we selected this protocol concomitantly with proper instructions for the participating volunteers. Although the present study was confined to a posthoc setting for practical reasons, further studies are warranted to link AT-1R expression in monocytes during low versus high salt intake with the present data.

It is clear that the present data support previous in-vitro findings with respect to regulation of the AT1-R by active plasma hemopexin. Although the present study deals with healthy young men, the data fit well within our findings concerning hemopexin activity in disease states such as human pre-eclampsia, in which inhibition of plasma hemopexin activity leads to enhanced vascular responsiveness to ang II and hypertension [2–6].

In conclusion, endogenous hemopexin activity may be considered as a potential determinant of ang II responsiveness *in vivo* in healthy humans, suggesting that hemopexin activity influences ang II-mediated hypertension by decreasing AT1-R availability.

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## Conflicts of interest

There are no conflicts of interest.

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## Reviewer's Summary Evaluations

### Referee 1

Hemopexin is an acute phase protein which can inactivate the angiotensin II type 1 receptor in vitro. In this study, the investigators examined whether variation in hemopexin activity was associated with the responsiveness of blood pressure to an IV infusion of angiotensin II in healthy

humans. The volunteers were studied on both low salt (50 mmol/24 h), and high salt (200 mmol/24 h) diets. Regardless of diet, hemopexin activity negatively correlated with the pressor response to angiotensin II. These in-vivo data support prior in-vitro data, and suggest that hemopexin modification of the angiotensin II type 1 receptor might be an important new factor which may modulate the effects of angiotensin II on blood pressure and possibly also vascular injury.