Early, but not late therapy with a vasopressin V$_{1a}$ antagonist ameliorates the development of renal damage after 5/6 nephrectomy

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**ABSTRACT**  

**INTRODUCTION** Vasopressin, mainly through the V₁a-receptor, is thought to be a major player in the maintenance of hyperfiltration. Its inhibition could therefore lead to a decrease in progression of chronic renal failure. To this end, the effect of the vasopressin V₁a-receptor-selective antagonist, YM218, was studied on proteinuria and focal glomerulosclerosis in early and late intervention after 5/6 nephrectomy in rats, and compared with an angiotensin converting enzyme inhibitor (ACEi).

**METHODS** After 5/6 nephrectomy, early intervention was performed between week 2 and 10 thereafter with the V₁a-receptor-selective antagonist (VRA, 10 mg/kg/day, n=10), enalapril (ACEi, 10 mg/kg/day, n=9), or vehicle (n=8). Late intervention was performed in another group between week 6 and 12 with VRA (10 mg/kg/day, n=7), lisinopril (ACEi, 5 mg/kg/day, n=7), or vehicle (n=7).

**RESULTS** In early intervention, proteinuria and focal glomerulosclerosis were significantly decreased by VRA compared to vehicle (44 ± 7% and 59 ± 8% respectively). ACEi significantly decreased proteinuria (67 ± 7%) and a trend towards a decrease in focal glomerulosclerosis was observed (30 ± 18%). In late intervention, VRA did not decrease proteinuria and focal glomerulosclerosis compared to vehicle (21 ± 20% and 0%, respectively), ACEi significantly lowered proteinuria (92 ± 2%) and a focal glomerulosclerosis (69 ± 1%) lowering trend was observed.

**CONCLUSION** These results indicate that VRA may protect against early progression of renal injury after 5/6 nephrectomy, whereas its effectiveness seems limited in established renal damage.
**INTRODUCTION**

Vasopressin plays an important role in the pathogenesis of several cardiovascular diseases such as heart failure, hypertension, and chronic renal failure. Formerly denoted as antidiuretic hormone (ADH), vasopressin plays an essential role in regulating water balance and cardiovascular homeostasis. It is secreted from the posterior pituitary when plasma osmolarity increases. Its renal effects are mediated through the V1a-receptor, localized in mesangial cells, efferent arteriole, vasa recta, and medullary interstitial cells, which induces an increase in glomerular filtration rate; and the V2 receptor, localized in the collecting ducts, which prevents water and sodium loss.

Variation in vasopressin levels could potentially lead to changes in renal function. Indeed, previous experiments showed that vasopressin contributes to the progression of chronic renal failure. By acting through V1a-receptors, vasopressin causes contraction, proliferation and hypertrophy of the mesangial cells, leading to a decrease in filtration rate and ultrafiltration coefficient. The effect of vasopressin on renal vasculature is not completely clarified, although the effects result in a decrease in glomerular filtration rate. These harmful effects of vasopressin could be inhibited by a vasopressin receptor antagonist. Okada et al. studied the effects of selective V1a- and V2- antagonism in different rat models of renal failure. They found VRA to prevent development of proteinuria and hypertension in the 5/6 nephrectomy model and proteinuria in the adriamycin nephrosis model. However, the late effects of a VRA when renal damage is already established are unknown. This is of special interest, because treatment of chronic renal failure is often initiated in patients once renal function loss is already present.

The aim of the present study was to compare early and late intervention with the V1a-receptor antagonist YM218 in the 5/6 nephrectomy model for renal damage. In order to validate therapeutic responsiveness of the applied model, we compared the effects of the V1a-receptor antagonist with those of an angiotensin converting enzyme inhibitor with proven efficacy in this model. To investigate whether the effects of the V1a-receptor antagonist are mediated through direct effects on renal hemodynamics, we investigated the acute effects of the V1a-receptor antagonist on glomerular filtration rate in awake, unrestrained animals.

**MATERIALS AND METHODS**

**Animals**

Male Wistar rats (Charles River Japan, Inc., Kanagawa, Japan and Harlan, The Netherlands) were housed in a temperature controlled room with a 12-hour light/dark cycle. Animals were fed with a synthetic rat diet containing normal (0.3%) sodium. Tap water was given ad libitum. All protocols were approved by the local Animal Ethics Committee.

**Protocols**

*Early intervention*

Experimental protocol

The protocol is shown in figure 1A. At time point -1 and 0 weeks, 5/6 of the kidneys was removed in two tempi. From week 2 after surgery onwards, either V1a-receptor antagonist, angiotensin converting enzyme inhibitor (ACE inhibitor), or vehicle was given. Proteinuria and systolic blood pressure were measured every other week. The rats were sacrificed after 8 weeks of treatment.
Surgery/induction of disease
Under sodium pentobarbital anesthesia (60 mg/kg, i.p.), rats were subjected to 5/6 nephrectomy in two sessions: surgical excision of approximately 2/3 of the left kidney followed by removal of the contra lateral (right) kidney one week later.

Groups/randomization
Two weeks after 5/6 nephrectomy, rats were randomly assigned to one of the following three treatment groups: vehicle (n=8); 10 mg/kg/day YM218 (V₁a-receptor antagonist, n=10); 10 mg/kg/day enalapril (ACE inhibitor, n=9); or sham-operation, in which animals underwent laparotomy and both kidneys were manipulated, but left intact (n=10). V₁a-receptor antagonist, ACE inhibitor, or vehicle (0.5% methylcellulose) was administered daily by gavage at a volume of 5 ml/kg. After 8 weeks of treatment, rats were sacrificed. The remnant kidney was removed and conserved for histopathological examination.
Late intervention

Experimental protocol

The protocol is shown in figure 1B. At time point 0, 5/6 nephrectomy was performed. From week 5 onwards, proteinuria and systolic blood pressure were measured weekly. From week 6 onwards, either \( V_{1a} \)-receptor antagonist, ACE inhibitor or vehicle was given. After 6 weeks of treatment, rats were sacrificed.

Surgery/induction of disease

Under 1.5\% isoflurane in \( N_2O/O_2 \) (2:1) anesthesia, rats were subjected to 5/6 nephrectomy in one session. The right kidney was removed after ligation of the renal artery, vein and ureter. Likewise, the proximal branch of the renal artery (often responsible for 2/3 of the blood supply to the kidney) was ligated upon visual inspection of interruption of 2/3 of the blood supply to this kidney. If not, additional (smaller) branches of the renal artery were either ligated or coagulated.

Groups/randomization

Six weeks after 5/6 nephrectomy, rats were randomly assigned to one of the following 4 groups: vehicle (n=7), 10 mg/kg/day YM218 (\( V_{1a} \)-receptor antagonist, n=7), 5 mg/kg/day lisinopril (ACE inhibitor, n=7) and a sham-opareted group (n=15). \( V_{1a} \)-receptor antagonist, ACE inhibitor, or the vehicle (0.5\% methylcellulose) were administered daily by gavage at a volume of 5 ml/kg. After 6 weeks of treatment, rats were sacrificed. Remaining renal tissue was removed and conserved for histopathological examination.

Biochemical and hemodynamic parameters

Proteinuria, body weight, water intake and urine output were measured by putting the animals in metabolic cages for 24-h urine collection. After centrifugation, urinary protein concentration was determined using a protein assay kit (Bio-Rad Laboratories; Tokyo, Japan) or by nephelometry (Dade Behring BNII, The Netherlands).

Systolic blood pressure was measured by the tail cuff method (PS-200A; Riken-Kaihatsu; Tokyo, Japan and IITC Life Sciences, Woodland Hills, CA, USA). Before the experiments, animals were allowed to adapt to the equipment and the procedure in a 4-week training period to minimize stress-induced artifacts. For each animal, the systolic blood pressure recorded for any given time represented the mean of three to five pressure recordings obtained at a single session. In the early intervention group, creatinine was enzymatically measured by an automatic analyzer (type 7250, Hitachi, Tokyo, Japan).

Histopathological Examination

At the end of the treatment periods, the left kidney was excised and immersion fixed in 10\% phosphate-buffered formalin, and embedded in paraffin for examination by light microscopy. Sections of 3 \( \mu m \) were stained with periodic acid Schiff (PAS). The degree of focal glomerulosclerosis was assessed in 50 glomeruli by scoring semi-quantitatively on a scale of 0 to 4. focal glomerulosclerosis was scored positive when mesangial matrix expansion and adhesion to Bowman's capsule was present in the same quadrant. When 25\% of the glomerulus was affected, a score of 1+ was adjudged, 50\% was scored as 2+, 75\% as 3+ and 100\% as 4+. Overall focal glomerulosclerosis score is expressed in percentage of the maximum score of 200. An examiner blinded for the groups evaluated all sections.
Acute effects of $V_{1a}$-receptor antagonist on renal hemodynamics in the late intervention model

**Groups**
A separate group of rats were instrumented 5 weeks after 5/6 nephrectomy (ligation model) and stratified for proteinuria. Renal function was evaluated one week later (*i.e.* 6 weeks after initial surgery). Groups were as follows: vehicle (n=11), YM218 ($V_{1a}$-receptor antagonist, n=11), and control (n=5). After equilibration, two clearance periods were carried out under infusion of vehicle. Then, $V_{1a}$-receptor antagonist (10 mg/kg) or vehicle was infused and its effect evaluated during three subsequent clearance periods.

**Cannulation**
In these rats, the right jugular vein was catheterized with silicone tubing and the left carotid artery using polyethylene tubing fused to a silicone tube. The proximal end of the catheters were tunneled subcutaneously, exteriorized and fixed to the skull using small screws and dental cement. Catheters were filled with a 55% polyvinylpyrrolidone solution in saline containing heparin (500 IU/ml, Leo Pharmaceuticals, The Netherlands) to prevent blood (cloths) from entering and obstructing the cannula.

**Measurement of renal function – clearance protocol**
Renal function was evaluated as described before. In short, cannulated rats were put unrestrained and awake in a metabolic cage and connected to an infusion pump through a swivel. To ensure a stable diuresis during the experiment, an infusion with 5% dextrose solution (2 ml/h) via the jugular vein was started a few hours before start of the experiment. A loading dose of 0.08 MBq $^{125}$I-iothalamate and 0.16 MBq $^{131}$I-hippuran in 300 μl 5% dextrose was administered followed by continuous infusion (2 ml/h) of 0.02 MBq $^{125}$I-iothalamate and 0.04 MBq $^{131}$I-hippuran per ml 5% dextrose. After an equilibration period of at least 120 minutes to establish steady state hippuran and iothalamate plasma levels, collection of spontaneously voided urine portions was started. Following every spontaneous voiding, a blood sample of 300 μl was collected via the jugular catheter and replaced with an equal volume of 5% dextrose. Renal function was evaluated until five clearance periods were completed. Activities of $^{125}$I-iothalamate and $^{131}$I-hippuran were determined in 150 μl of urine, in 150 μl of plasma and in 20 μl of standard infusion solution using a two-channel scintillation counter (Packard Instruments, USA).

**Compounds**
Enalapril (enalapril maleate) and lisinopril were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The $V_{1a}$-receptor-selective antagonist YM218 was obtained from Astellas Pharma Inc. (Ibaraki, Japan). The dose of 10 mg/kg chosen for YM218 proved to be orally effective in rats. A dose finding study in rats resulted in an effective plasma concentration of 30 ng/ml. To be sure that the lack of effect in the late intervention protocol was not due to plasma concentrations below this level, actual plasma levels of the $V_{1a}$-receptor antagonist were measured, showing mean levels of $50 \pm 10$ ng/ml.

**Data Analysis**
Data are expressed as mean ± standard error of the mean (SEM). Differences between treatment groups at one time point were compared by one-way ANOVA with a Dunnet post hoc test to identify the
groups that were different from the vehicle treated animals. Paired samples t-test was used to compare differences before and after acute infusion with vehicle and $V_{1a}$-receptor antagonist. Differences were considered significant at P-values less than 0.05.

**RESULTS**

**Early intervention protocol**

*Body weight and urine output*

Although all rats gained weight during the experiment, rats that underwent 5/6 nephrectomy gained less weight than sham-operated animals (table 1). Body weight in the $V_{1a}$-receptor antagonist and the ACE inhibitor groups were significantly higher compared with vehicle after 8 weeks of treatment. At start of treatment, urine output was significantly increased in the groups that underwent 5/6 nephrectomy compared to SHAM (47 ± 3 compared to 27 ± 6 ml/24h respectively). The urinary output was unaffected by $V_{1a}$-receptor antagonist and ACE inhibitor treatment (table 1).

*Systolic blood pressure*

At start of treatment, SBP was increased to 143 ± 5 mmHg in the vehicle treated group compared to 122 ± 1 mmHg in the SHAM group. In the $V_{1a}$-receptor antagonist and ACE inhibitor treated group, SBP remained 133 ± 5 and 136 ± 3 mmHg respectively. At the end of the follow-up, systolic blood pressure was markedly elevated in rats that underwent 5/6 nephrectomy as compared to SHAM (179 ± 7 versus 123 ± 3 mmHg). In 5/6 nephrectomy, $V_{1a}$-receptor antagonist and the ACE inhibitor did not significantly lower systolic blood pressure (174 ± 7 versus 157 ± 10 mmHg, figure 2), while at the end of the treatment period the systolic blood pressure in the ACE inhibitor treated group was lower than in the vehicle treated group (versus 179 ± 7 mmHg, P= 0.07).

![Figure 2. Effect treatment on systolic blood pressure, given as delta blood pressure before and after treatment. VRA, $V_{1a}$-receptor antagonist, ACE-I: angiotensin converting enzyme inhibitor. * : P<0.05 versus start treatment; #: P<0.05 versus VRA.](image)
Table 1. Rat characteristics at the end of the study

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Data shown as mean ± SEM, SHAM, sham-operation; VEH, vehicle; VRA, V₁₅-receptor-selective antagonist; ACEi, angiotensin converting enzyme inhibitor. *: P< 0.05 versus VEH.

Renal characteristics

Two weeks after 5/6 nephrectomy, proteinuria increased in all groups to a level of around 100 mg/24h, while the proteinuria in the SHAM-group only amounted 21 mg/24h during the whole experiment. Rats were treated between week 2 and 8. In this period, proteinuria in the vehicle group steadily increased to a level of 715 ± 82 mg/24h, which was significantly different from SHAM. Proteinuria was significantly prevented in the V₁₅-receptor antagonist treated group (44 ± 7 % reduction) and the ACE inhibitor treated group (67 ± 7 % reduction) compared to vehicle (figure 3A). There was no significant difference between both treatments.

![Figure 3](image-url)  
* : P< 0.05 versus vehicle.

At the end of the experiment, focal glomerulosclerosis was significant higher in the vehicle group compared to SHAM (figure 4A). V₁₅-receptor antagonist treatment prevented focal glomerulosclerosis by 59 ± 8% (P= 0.06), whereas there was a numerical, although non-significant prevention in the ACE inhibitor treated group (30 ± 18%, P= 0.6).
Creatinine clearance was measured as a parameter for renal function. After 5/6Nx creatinine was significant lower compared to SHAM operated animals (0.50 ± 0.11 versus 3.4 ± 0.18 ml/min). The V₁a-receptor antagonist and ACE inhibitor treatment showed no significant effect on creatinine clearance (0.95 ± 0.10 and 0.91 ± 0.11 ml/min respectively).

Late intervention protocol

Body weight and urine output

Although all rats gained weight during the experiment, rats that underwent 5/6 nephrectomy gained less weight than sham-operated animals (table 1). At start of treatment there were no differences in urine output between the treatment groups, vehicle treated animals and the SHAM group: urine output was 21 ± 2 ml/24h. In accordance with the reported diuretic effect of ACEi15-17, urine output was significantly increased in the ACE inhibitor treated group compared with the vehicle group (table 1).

Systolic Blood Pressure

In the vehicle and ACE inhibitor group, systolic blood pressure increased to about 168 mmHg at randomization (week 6), while in the sham group blood pressure remained at a normal level (136 ± 3 mmHg). By change, blood pressure in the V₁a-receptor antagonist group was not increased at randomization (142 ± 4 mmHg). At the end of the follow-up, no differences were observed in rats that underwent 5/6 nephrectomy as compared to SHAM (132 ± 8 versus 131 ± 4 mmHg). In 5/6 nephrectomy, V₁a-receptor antagonist did not lower systolic blood pressure, while in the ACE inhibitor group a significant decrease by 25 ± 2% in systolic blood pressure was observed (figure 2).

Proteinuria

Five weeks after 5/6 nephrectomy, proteinuria in all groups increased to a level of around 40 mg/24h, while the proteinuria in the SHAM-group only amounted 12 mg/24h during the whole experiment. During the treatment period, proteinuria in the vehicle group steadily increased to a level of 132 ± 26 mg/24h, which was significantly different from SHAM. No effect on proteinuria was observed in the
V$_{1a}$-receptor antagonist treated group, although a significant reduction by 92 ± 2% was seen in the ACE inhibitor treated group (figure 3B).

At the end of the experiment, focal glomerulosclerosis was significant higher in the vehicle group compared to SHAM (figure 4). No effect was observed on focal glomerulosclerosis in the V$_{1a}$-receptor antagonist treated group, while a trend towards prevention was observed in the ACE inhibitor treated group (P= 0.29).

**Figure 5.** Effects of acute infusion with V$_{1a}$-receptor antagonist on glomerular filtration rate (GFR), filtration fraction (FF), effective renal plasma flow (ERPF), and urine flow. ■: before infusion, ■: after infusion with vehicle or V$_{1a}$-receptor antagonist. *: P < 0.05 versus control, *: P< 0.05 versus vehicle.

**Renal hemodynamics**

In the 5/6 nephrectomy-groups, baseline glomerular filtration rate, filtration fraction, and effective renal plasma flow were significant lower compared to control (figure 5). Urine flow in 5/6 nephrectomy was similar compared to control. Acute treatment with V$_{1a}$-receptor antagonist in 5/6 nephrectomy caused a significant increase in glomerular filtration rate by 0.04 ml/min, while no effect on effective renal plasma flow, filtration fraction and urine flow were measured. In the control group and the 5/6 nephrectomised rats, the filtration fraction was significant increased in the second period of two consecutive periods of infusion with vehicle. This increase in filtration fraction was not observed in 5/6 nephrectomised rats after infusion with the V$_{1a}$-receptor antagonist.
DISCUSSION

This is the first study reporting the effects of a V$_{1a}$-receptor antagonist in a late intervention setting, and showed its inadequacy to reduce proteinuria and focal glomerulosclerosis. In contrast, in early intervention, V$_{1a}$-receptor antagonist did show renoprotection. These data suggest that the therapeutic efficacy of a V$_{1a}$-receptor antagonist is dependent on the pre-existing level of renal damage.

The effects of vasopressin receptor antagonism on blood pressure and proteinuria reported in the literature are controversial. Okada et al. monitored the effects of early intervention with a V$_{1a}$-receptor antagonist in different models. In partially surgical 5/6 nephrectomized and salt-loaded spontaneous hypertensive rats, a V$_{1a}$-receptor antagonist significantly prevented the development of proteinuria and prevented a rise in blood pressure. This effect was explained by antagonism of the increased vasopressin levels caused by the salt load and partial renal ablation.

In adriamycin nephrosis, an antiproteinuric effect and reduction of focal glomerulosclerosis of a V$_{1a}$-receptor antagonist were found as well, but no effect was seen on blood pressure in this normotensive model. Consequently, the beneficial effects of the V$_{1a}$-receptor antagonist upon early intervention in our study are in agreement with the studies of Okada et al. These effects were in contrast with results of Brooks et al., who studied the effects of a V$_{1a}$-receptor antagonist on partially nephrectomized Brattleboro (vasopressin deficient) and Long Evans control rats. No evidence was found for vasopressin to be involved in the pathogenesis of hypertension, proteinuria, and renal damage in this ligation model. It should be noted that in all studies mentioned above, administration of the V$_{1a}$-receptor antagonist was started directly after induction of the disease, i.e. prevention.

While the V$_{1a}$-receptor antagonist was effective in early intervention in 5/6 nephrectomy, it proved substantially less effective in late intervention. Taking into account these settings, the question is why a V$_{1a}$-receptor antagonist is effective in reducing proteinuria and focal glomerulosclerosis in early, but not in late intervention. First, renal damage can be prevented by blood pressure reduction, and therefore a difference in antihypertensive effect could account for the difference in reduction of renal damage. Intervention with the V$_{1a}$-receptor antagonist had no effect on systolic blood pressure in both early and late intervention. During treatment with the V$_{1a}$-receptor antagonist, blood pressure is increased, because damage is still developing in the early intervention protocol. In the late intervention protocol were damage is already rather established at start of treatment, V$_{1a}$-receptor antagonist is ineffective. Therefore, the beneficial pharmacological effects of the V$_{1a}$-receptor antagonist in early intervention could not be explained by a difference in blood pressure reduction, while part of the beneficial effect of the ACE inhibitor could be.

Secondly, the decline in the development of proteinuria in early intervention can be the consequence of inhibition of the vasoconstrictive effect of increased vasopressin levels and a decrease in contraction, proliferation and hypertrophy of the mesangial cells. Tahara et al. showed YM218 to be a potent inhibitor of the vasopressin-induced response of human mesangial cells. In the late intervention protocol, renal damage is characterized by irreversible hyperfiltration-induced processes caused by a rise in intraglomerular pressure. No decrease in proteinuria is seen in the V$_{1a}$-receptor antagonist treated group, only a flattening of the proteinuria curve. This means that chronic treatment with a V$_{1a}$-receptor antagonist has limited applicability in the deterioration of progression of chronic renal failure and does not redress features of chronic renal failure, as in the case of ACE inhibition. This is partly confirmed by our results on acute infusion of V$_{1a}$-receptor antagonist 6 weeks following 5/6 nephrectomy where
the $V_{1a}$-receptor antagonist did not reduce, but slightly increase glomerular filtration rate. In contrast, a decrease in glomerular filtration rate was previously reported after acute infusion of a $V_{1a}$-receptor antagonist in normal rats\(^7\), which might indicate differences in efficacy of this type of antagonism in early versus late intervention. It has to be mentioned however, that efficacy of chronic antiproteinuric medication is more often not reflected in changes in renal function upon acute infusion.

Thirdly, an increase in plasma vasopressin levels could have blunted the effects of the $V_{1a}$-receptor antagonist in the late intervention model. From literature is known that the endogenous vasopressin concentration increases with the degree of renal insufficiency\(^22\). Moreover, the antagonism of the $V_{1a}$-receptor by YM218 might have caused an increase in endogenous vasopressin release. It is known that $V_2$-receptor stimulation by the vasopressin like agonist dDAVP caused an increase in urine albumin excretion, independent of creatinine excretion\(^23\).

Some study limitations have to be mentioned about the animal models used. The differences in the operation techniques used in the early and late intervention protocol are not likely to explain the variation in response to the $V_{1a}$-receptor antagonist. The distinction in 5/6 nephrectomy between surgical excision and ligation is the difference in blood pressure. After ligation of the renal arteries, hypertension is usually observed, probably caused by activation of the renin angiotensin aldosterone system, which is not always present after surgical excision of the renal parenchyma\(^24,25\). In the present experiment, hypertension was observed after both techniques, indicating comparable pathophysiology through hyperfiltration and activation of the renin angiotensin system and as a result a comparable amount of focal glomerulosclerosis. Because comparable levels of focal glomerulosclerosis were observed, the discrepancy in level of proteinuria must be caused by the different methods for proteinuria measurement.

**CONCLUSION**

We conclude that the $V_{1a}$-receptor antagonist, YM218, protects against the early progression of renal injury caused by a reduction in nephron number, whereas its effectiveness seems limited in established renal damage caused by deteriorating nephron function of previously healthy nephrons. These protective effects appear not to be mediated through short-term effects on renal hemodynamics. $V_{1a}$-receptor-selective antagonism might be useful in renal protection only in the prevention of renal damage in renal failure caused by an acute reduction in nephron number.

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Vasopressin antagonism in renal disease
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