Chronic Administration of Oral Vasopressin Type 2 Receptor Antagonist, Tolvaptan Exerts Both Myocardial and Renal Protective Effects in Rats with Hypertensive Heart Failure

Morooka et al: Vasopressin Type 2 Receptor Antagonist in Heart Failure

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Abstract

Background—Although recent clinical trials have demonstrated the efficacy of the oral vasopressin (AVP) type 2 receptor (V2R) antagonist, tolvaptan, its long-term effects on the myocardium and kidney in heart failure (HF) are not clear. We examined the chronic effects of tolvaptan administration on both the myocardium and kidney in a rat hypertensive HF model.

Methods and Results—Not only circulating AVP level, but also myocardial AVP and V1a receptor (V1aR) expressions, renal V1aR, and V2R expressions were significantly up-regulated during the transition to HF. The animals were chronically treated with low-dose or high-dose (HD) tolvaptan or vehicle from the LV hypertrophic stage. Chronic tolvaptan treatment persistently increased urine volume, but did not affect blood pressure. In the HD group, the animal survival significantly improved (log-rank test, p<0.01). At the HF stage, the progression of LV dysfunction was prevented and lung congestion was suppressed. Activation of ANP, endothelin-1 (ET-1), AVP and V1aR mRNA levels were significantly suppressed in the LV myocardium. Meanwhile, renal histopathological damage was ameliorated and renal function was improved in the HD group at the HF stage. Concomitantly, not only activation of aquaporin-2, but also those of V2R, V1aR, renin and ET-1 in the kidney were significantly suppressed (all p <0.05).

Conclusions—These results indicate chronic tolvaptan treatment has beneficial effects by preventing not only the progression of LV dysfunction, but also that of renal injury in hypertensive rats with HF. The underlying mechanism may be related to the suppression of myocardial and renal neurohumoral activation.

Key Words: heart failure; vasopressin type 2 receptor antagonist; kidney; myocardium
Neurohumoral abnormalities contribute to the process of subsequent cardiac remodeling and transition to heart failure (HF), and successful approaches to improving the prognosis of patients with HF are based largely on therapeutic interruption of activated neurohumoral systems such as renin-angiotensin-aldosterone (RAA) and the sympathetic nervous system (SNS) (1,2). In contrast, excess vasopressin levels have long been recognized in patients with HF, particularly those with severe clinical manifestations (3,4). They have the potential to exert deleterious effects on various physiologic processes in HF (5). However, the usefulness of long-term blockade of the vasopressin system has not been fully addressed in cardiac remodeling or HF, especially in the clinical setting.

Among the three distinct receptor subtypes: V1a, V1b, and V2 receptors, V2 receptor (V2R) is abundantly expressed on the renal collecting duct (CD) cells and is linked to the adenylate cyclase pathway (6). Activation of this receptor leads to increased aquaporin 2 (AQP2) water channels, thus increasing the water permeability of the CD. Recently, V2R antagonists have shown promise for use in patients with HF, by increasing free-water excretion and serum sodium levels (7). The oral V2R antagonist tolvaptan caused an early and sustained reduction in body weight and improvement in serum sodium in the EVEREST trial (8,9). However, it lacked outcome benefits such as reduced mortality and reduced recurrent HF hospitalization. It was suggested that the population may not have been appropriately selected for optimal long-term benefit from V2R blockade (10). This means that further exploration into the pathophysiologica

This means that further exploration into the pathophysiological mechanism in HF related with V2R activation or blockade is required. Also, although it is well documented that AVP plays an essential role through V2R in the development of
water retention and subsequent hyponatremia in advanced HF, its relative contribution to the cardiovascular and renal dysfunction in HF is less-well understood, particularly in terms of the long-term effects (11). We thus tested chronic tolvaptan administration in a rat hypertensive HF model and examined the functional and pathological effects on both the myocardium and kidney. We further explored the molecular mechanism of long-term V2R blockade in hypertensive cardiac and renal dysfunction.

Methods

Animals and experimental design (Supplemental Figure 1)

Male inbred Dahl salt-sensitive (DS) rats were fed an 8% high-salt diet after the age of 6 weeks. They were chronically treated with tolvaptan (Tolv group: n=41) or vehicle (Cont group: n=22) from the left ventricular (LV) hypertrophic stage (LVH stage: 11 weeks of age) until 27 weeks (12). The tolvaptan group was further divided into two groups; low dose (LD Tolv, 0.01% tolvaptan in diet) and high dose (HD Tolv, 0.05% tolvaptan in diet). In a separate low salt (LS) group, rats (n=6) were given a 0.3% low-salt diet throughout the course, and did not show hypertension or cardiac hypertrophy throughout the course. In the first series of experiment, animals (n=31) were monitored and death was recorded every day. In the second experiment, animals (n=32) were sacrificed to collect serum samples and to harvest cardiac and renal tissues for measurement of mRNA expression and histological examination at 18-19 weeks.

The animals were treated in accordance with the “Position of the American Heart Association on Research Animal Use”, adopted by the Association in November 1984, and with the institutional guidelines of Kinki University Faculty of Medicine.
Hemodynamic and echocardiographic studies

*In vivo* LV geometry and contractile measurements were serially assessed by transthoracic echocardiography at 11, 15 and 18 weeks as previously reported (12). The short axis view of the LV was recorded to measure the LV end-diastolic dimension (LVDd) and LV end-systolic dimension (LVDs). LV fractional shortening (FS) was calculated. In the same period, heart rate (HR) and systolic blood pressure (SBP) were measured by the tail-cuff method.

Urine and serum measurements

The rats were placed individually in metabolic cages and urine samples were collected for 24 hours after treatment at 11 and 18 weeks. The collected urine was used for measurement of urine parameters (urine osmolality, sodium, potassium, nitrogen, creatinine, and total protein). At 18 weeks the rats were decapitated and the collected serum from trunk blood was used for measurement of serum parameters (sodium, potassium, urea nitrogen, creatinine, osmolality, BNP, AVP, aldosterone, plasma renin activity) by commercial available kits.

Histological analysis

The excised hearts were separated into RV and LV, and fixed in formalin. After being embedded in paraffin, LV tissue was sliced into 5μm sections. To determine the level of myocardial interstitial fibrosis, sections were stained with Masson’s trichrome or Picrosirius Red and quantified by three independent observers using Image J software. The excised kidneys were separated into upper and lower poles, and fixed. The renal tissue was also sliced, and sections were stained with either hematoxylin-eosin, Masson’s trichrome or Picrosirius Red. Semi-quantitative analysis was performed as previously described (13). In addition, immunohistochemical staining for aquaporin-2
(AQP2) was performed using the UltraTek HRP Anti-Polyvalent (DAB) Staining System (Scytek Laboratories) according to the manufacturer’s instruction. The primary antibody used was rabbit polyclonal anti-AQP2 antibody (Abcam, 1:10000). The sections were faintly counterstained with haematoxylin. The specificity and results obtained with the primary antibody were checked by omission of the primary antibodies and use of a non-immune rabbit IgG antibody (DAKO) as a negative control.

**Quantitative real-time PCR**

The total RNA was prepared using Trizol® and treated with DNase I (Invitrogen). Single-strand cDNAs were generated by the reverse transcription of RNA samples using the SuperScript first-strand synthesis system kit (Invitrogen) and then subjected to quantitative real-time PCR with SYBR Green PCR Master Mix (ABI) using an ABI PRISM 7900 HT Sequence Detection System. mRNA levels (Supplemental Table) were measured and normalized with an endogenous control, GAPDH mRNA.

**Calculations and Statistical analysis**

To clarify the effect of tolvaptan, electrolyte-free water clearance (E-CH₂O) and electrolyte clearance (E-Cosm) were calculated (14). The formulas used were as follows: E-CH₂O = UV - E-Cosm , E-Cosm = (Uₙa+Uₖ)UV/Pₙa , where UV is the urine volume, Uₙa is the urine sodium concentration, Uₖ is the urine potassium concentration, and Pₙa is the serum sodium concentration.

All data are expressed as the means ± S.D. Statistical significance of differences between mean values was analyzed by one-way analysis of variance (ANOVA) with post hoc comparisons by Fisher’s protected least significant difference test. The two-way repeated measures ANOVA was used for the analysis in the echocardiographical data. Survival was analyzed by the standard Kaplan-Meier analysis.
with log-rank test, where two separate tests in HD Tolv vs. Cont and LD Tolv vs. Cont were performed. In all tests, a value of $P<0.05$ was considered statistically significant.

Results

Vasopressin system during the transition from LVH to HF (Supplemental Figure 2)

Serum BNP levels showed a modest increase at the LVH stage and a further increase in accordance with the transition from LVH to HF (around 18 weeks) in the Dahl rat HF model. Although serum sodium was not changed, serum osmolality and serum AVP levels increased significantly during the transition from LVH to HF. At the LVH stage, renal V1aR and V2R mRNA levels and myocardial AVP, V1aR, V1bR mRNA levels did not differ from those in the age-matched LS rats. However, at the HF stage, they were all increased compared with those at the LVH stage.

Kaplan-Meier survival analysis (Figure 1A)

All rats of the Cont group died of pulmonary congestion with severe LV dysfunction after 14 weeks. By Kaplan-Meier analysis, the survival in the HD Tolv group showed significant improvement (mean survival rate: $22.9 \pm 3.5$ weeks, $p<0.01$) compared with the Cont group, although that in the LD Tolv group showed only a trend toward improvement ($19.2 \pm 3.9$ weeks, $p=0.184$).

Hemodynamic and echocardiographic measurements

There were no significant differences in SBP and HR among the 3 experimental groups (Cont, LD Tolv, and HD Tolv) during the course (Table1). By echocardiography, the Cont group showed an increase of LVDd and a decrease of FS during the transition from LVH to HF. Although the LD Tolv group showed no significant changes, LVDd tended to be smaller in the HD Tolv group, while FS was significantly preserved compared
with that in Cont group (41.7 ± 16.7 % vs. 27.5 ± 6.5 %; p<0.05) (Figure 1B).

**Histopathological parameters at 18 weeks**

There were no significant differences in BW and LV/tibial length (TL) among the experimental groups at 18 weeks. Both the LD and HD Tolv groups showed significantly-decreased Kidney/TL ratios. However, only the HD Tolv group showed a significantly-decreased Lung/TL ratio (P<0.05), indicating that lung congestion was suppressed. Although the HD group tended to exhibit less myocardial fibrosis than the Cont or LD Tolv groups, the effect did not reach statistical significance (Table 1 and Supplemental Figure 3).

**Biochemical and hormonal data of serum and urine (Table2)**

At 11 weeks (3 days after the initiation of treatment), the tolvaptan treatment increased urine volume (Cont:214.2 ± 46.2, LD Tolv:250.4 ± 88.7, HD Tolv:357.4 ± 62.1 ml/kg/day) and fluid intake (Cont:272.9 ± 58.4, LD Tolv:295.4 ± 93.9, HD Tolv:395.4 ± 71.2 ml/kg/day), and decreased osmolality in a dose-dependent manner (Cont:853.7 ± 154.5, LD Tolv:764.3 ± 199.3, HD Tolv:531.3 ± 95.6 mOsm/kgH2O). No significant changes were observed in other parameters (data not shown). At 18 weeks, there were no significant differences in serum sodium, potassium, urea nitrogen, creatinine, AVP, aldosterone, and plasma renin activity among the three experimental groups. Serum BNP was significantly suppressed in HD group as compared with Cont or LD Tolv group. LD Tolv demonstrated only trends, but HD Tolv treatment significantly increased the urine volume (94% increase) and decreased urine osmolality (43% decrease) at 18 weeks. There were no significant differences in urine nitrogen, creatinine, and total protein among the groups. In the Cont group, electrolyte-free water clearance (E-CH2O) and electrolyte clearance (E-Cosm) were decreased as compared with those in the LS
group and only E-CH\textsubscript{2}O was improved in the HD Tolv group. In addition, creatinine clearance and sodium excretion were significantly improved in the HD Tolv group (p<0.05). The rats in three experimental groups showed the increased fluid intake compared with those in LS group (p<0.01). Among the experimental groups, although there was a trend toward increasing fluid intake, it was not significant. The difference between fluid intake and urine volume was significantly decreased in HD Tolv group as compared with Cont or LD tolvaptan group, indicating negative or improved fluid balance existed in HD group at 18 weeks (HF phase).

**mRNA expressions in the LV (Figure 2)**

Activation of myocardial mRNAs such as AVP, V1aR, and V1bR was significantly suppressed in the HD Tolv group. Also, atrial natriuretic peptide (ANP), angiotensin-converting enzyme (ACE), and endothelin-1 (ET-1) expressions in the LV were significantly decreased in the HD Tolv group. In contrast, those of collagen type Ia (Colla1) and matrix metalloproteinase-2 (MMP2) were not significantly different among the groups.

**Histological changes in the kidney**

Figure 3A shows that the glomeruli, arterioles, and interstitium of LS group kidneys were mostly intact. In contrast, the focal segmental or global glomerulosclerosis observed in the Cont group was less frequently observed in Tolv-treated groups. Also, the tubulointerstitium of Tolv-treated groups showed fibrosis around the sclerosing glomeruli and arterioles to a lesser extent than in the Cont group. Semi-quantitative analysis showed that increases in the degree of glomerulosclerosis (GS score), crescent glomeruli (CG score), tubulointerstitial fibrosis (TF), tubular dilatation (TD) were seen in the Cont group compared with the LS group, and a significant suppression of GS
score or TF was observed in the HD Tolv group (Figure 3B). The images of immunohistochemical staining in inner medulla collecting ducts revealed increased labeling of AQP2 in the apical plasma domains of principal cells. Tolvaptan treatment decreased and redistributed the AQP2 protein evenly to intracellular domains (Figure 4A). Concomitantly, activations of AQP2 and AQP3 mRNA expression were significantly suppressed in the Tolv groups (p<0.01, Figure 4B).

mRNA expression in the kidney (Figure 5)

In addition to those of AQP-2 and -3, mRNA levels of V2R, V1aR, epithelial Na\(^+\) channel (SCNN1:ENaC \(\alpha\)-subunit) and renin were significantly suppressed in the HD Tolv group compared with the Cont group. Also, those of ET-1, Colla1, and fibronectin (FN), which are associated with renal fibrosis, were significantly suppressed in the HD Tolv group.

Discussion

The role of vasopressin in heart failure

The pathogenesis of HF involves activation of the neurohumoral axis including stimulation of the SNS, the RAAS, and AVP systems (2,15). In Dahl salt-sensitive hypertensive rats, we previously observed the systemic and local activation of the SNS, RAAS, and endothelin system, and that their chronic blockade had beneficial effects on LV remodeling and HF (12,16). In the present study, we found that the AVP system was activated not only at the systemic level, but also in the LV myocardium. In the LV myocardium, AVP mRNA was 3.5-fold increased, followed by increases of V1aR and V1bR mRNA expressions, which was concordant with a previous report by Hupf et al. (17). They showed that the stressed heart expressed AVP in concentrations sufficient to
cause local and potentially systemic effects in the rat. In the human heart, AVP production and excretion were observed in patients with volume overload (18). Although the mechanism for activating local AVP system has not been elucidated, hemodynamic stress or other neurohumoral factors might regulate it. Recently, Li et al. utilized transgenic mice with myocyte-specific overexpression of V1aR and showed that chronic V1aR activation in myocytes causes cardiac hypertrophy and the development of HF (19). Taken together, in addition to the increased AVP level in circulation, activation of the myocardial AVP system might play a pivotal role during the transition to HF.

The systemic and cardiac effects of V2R blockade in HF

Tolvaptan is a selective nonpeptide V2 receptor antagonist and inhibits the binding of AVP to the V2R on the CDs of the kidneys resulting in aquaresis, the electrolyte sparing excretion of water. The published trials (SALT-1, -2 and ACTIV in CHF) demonstrated the benefit of tolvaptan in terms of raising or normalizing sodium levels in hyponatremic HF patients, most performed even without fluid restriction (7,20). However, the efficacy is unknown in HF patients with normal or even hypernatremia. In addition, the EVEREST trial has not shown what types of HF patients are optimal for long-term benefits from tolvaptan treatment (8,9). In the present study, Dahl rats showed progressive myocardial and renal dysfunction/damage by volume and pressure overload, but no hyponatremia due to high-salt diet. However, through the treatment course, HD tolvaptan caused persistent aquarectic effects without adversely affecting the hemodynamics, urinary potassium excretion, serum electrolytes, or neurohumoral activation, and improved the animal survival. Since we did not find V2R expression in the LV myocardium (data not shown), the beneficial effects on the LV myocardium
may be achieved indirectly through hemodynamic or neurohumoral alterations. In the present study, we observed no significant change in SBP during the experimental course in the Dahl salt-sensitive rat. This is a similar observation to the studies in HF patients after acute and chronic tolvaptan administration (20,21). It suggests absence of significant peripheral vasodilative or vasoconstrictive responses after tolvaptan treatment and may be because tolvaptan is a selective V2R antagonist without blocking V1aR, or no significant or only a little increase of serum AVP is observed after tolvaptan treatment. Chronic effects of V2R antagonists have been examined in other experimental HF models induced by immunization with porcine cardiac myosin (22) and coronary artery ligation (23). However, they neither showed significant beneficial effects on cardiac geometry or function, nor ascertained significant improvement in the survival rate. The differences in HF pathogenesis such as the extent of volume-overload or renal damage might underlie the conflicting results. Recently, Costello et al. reported that, in an animal pacing model of HF, co-administration of tolvaptan and BNP resulted in a beneficial profile of renal, neurohumoral, and hemodynamic actions (24). Addition of BNP attenuated some of the adverse hemodynamic and neurohumoral effects seen with tolvaptan alone. In the present study, the plasma BNP levels were already (about two-fold) increased at the LVH stage and further increased (about five-fold) during the transition to HF. This may be associated with the beneficial effects of tolvaptan seen in the present study.

The renal effect of tolvaptan in HF

A major therapeutic aim in patients with HF is to induce renal excretion of water and sodium to reduce congestion without impairing renal function. Diuretics such as loop diuretics and thiazides act primarily as natriuretics by blocking sodium channels in the
luminal membrane of tubular cells. The disadvantages are that increased sodium in the tubule can reduce GFR through tubuloglomerular feedback, promote hypertrophy of tubular cells in more distant nephron segments, and increase the potential for electrolyte imbalance such as hyponatremia (25). In this regard, tolvaptan for HF patients is one of the strategies to enhance water excretion without inducing renal dysfunction and electrolyte imbalance as shown in this study. In human stable HF, acute V2R antagonism with tolvaptan was shown to enhance aquaresis without adversely affecting the renal hemodynamics and urinary sodium or potassium excretion, or neurohumoral systems (21). However, little is known about the long-term effects on renal function and morphology in HF. This study demonstrates that HF is associated with dysregulation of AQP2 in the renal collecting duct. Immunochemical study showed an increase in abundance of AQP2 in the apical plasma membrane of CD principal cells and redistribution of AQP2 from apical to intracellular domains by treatment with tolvaptan, in good agreement with the previous findings in other HF models (26) or acute V2R blockade study (27). We demonstrated for the first time that chronic tolvaptan treatment improved not only the AQP-V2R dysregulation but also the increased expressions of V1aR, SCNN1 (ENaC) and renin mRNA. Furthermore, it ameliorated renal fibrosis as demonstrated by histology and reduced the expressions of ET-1, Col1a1, and FN mRNA. However, it is unclear why chronic V2R antagonism ameliorated renal damages and mRNA expressions other than AQPs and V2R.

Chronic blockade of RAAS is known to improve renal damages including fibrosis in various experimental models of HF. Onozato et al. reported that NADPH oxidase and renal TGF-β were increased in the kidney of Dahl salt sensitive rats with HF and related to the changes in FN and Na-K-ATPase in the renal tubules with the development of
glomerulosclerosis and renal fibrosis (28). They also showed that ACE inhibitor or aldosterone blocker had beneficial effects on these changes, resulting in improvement of renal damage. In the present study, although there were no significant differences in mRNA expression of TGF-β (data not shown), mRNA expressions of Col1a1 and FN were significantly suppressed, and renal fibrosis was ameliorated in the Tolv groups. An ACE inhibitor has been also reported to reduce the expressions of V2R and AQP-2 mRNA in the kidney of the cardiomyopathic hamster (29). Recently, Lütken et al. demonstrated that increased expression of AQP2 as well as ENaC and NHE3 in post-infarcted rats with HF were, at least in part, reversed by AT1 receptor antagonism (30). AVP could potentially stimulate renin secretion directly via activation of V2R or indirectly through reduction of the sodium concentration at the macula densa (31). When considering that renin mRNA was markedly suppressed in HD tolv group, chronic V2R antagonism in the kidney might be associated with attenuation of the intra-renal RAAS system in the present study.

V2R antagonism has been attempted for the treatment of chronic kidney disease. For example, it was reported to limit the AVP-mediated exacerbation of renal damage in rats with streptozocin-induced diabetes mellitus (32) or with puromycin aminonucleoside nephropathy (33). Urinary albumin excretion or podocyte damage was attenuated, where an indirect mechanism is supposed such as reduced glomerular hypertension/hyperfiltration, and reduced the renal hypertrophy. Also, a V2R-mediated increase in intracellular cAMP levels and the attendant increase in cell calcium favor cysts growth in experimental models of polycystic kidney disease (ADPKD) (34) and V2R antagonists have been tried for the treatment of patients with ADPKD (35). Interestingly, a recent study has shown that continuous AVP infusion for at least 3 days
in normal kidneys induces a proliferative response in cells expressing V2R (thick ascending limb of loop of Henle and CD) that is blocked by V2R antagonist, suggesting that prolonged stimulation of V2R can convert these cells to a cAMP-dependent proliferative phenotype (36). Further studies are needed to elucidate whether such a mechanism may be associated with the renal protective effects of tolvaptan observed in the present study.

**Study limitations**

This study has several limitations. First, any invasive hemodynamic or telemetric analysis was not performed in the present study. Chronic effects of tolvaptan on hemodynamics may be underestimated. Instead of invasive hemodynamic study using a pressure catheter, we have performed serial echocardiography under light anesthesia, since previous studies have shown the changes of echocardiographic measurements represented the functional changes of LV myocardium well in this Dahl salt-sensitive HF model (12,37). Second, this study has been performed in a very specific disease model with inbred Dahl salt-sensitive rats. Hence, it may not be applicable to other HF models such as post-myocardial infarction, myocarditis, and post-aortic banding (22,23). Lastly, we have not compared tolvaptan treatment with that of standard diuretics (e.g. thiazide or furosemide) directly. However, previous studies clearly demonstrated that tolvaptan treatment may have beneficial effects on renal hemodynamics, electrolyte disturbance and neurohumoral activation in HF compared with other diuretics (21,22).

**Conclusion**

These results indicate that chronic tolvaptan treatment had beneficial effects by preventing the progressions of both LV dysfunction and renal injury in hypertensive rats with HF. The underlying mechanism may be related to the amelioration of local
(myocardial and renal) neurohumoral activation. Chronic V2R antagonism may have the therapeutic potential, especially in HF patients with progressive renal impairment, the so-called cardio-renal syndrome.

Acknowledgements

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Disclosures

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Table 1. Hemodynamic and histopathological parameters in Dahl salt sensitive rats treated with vehicle or tolvaptan at 18 weeks

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<tr>
<td>BW (g)</td>
<td>305±39</td>
<td>325±27</td>
<td>326±37</td>
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<td>SBP (mmHg)</td>
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<td>220±15</td>
<td>216±16</td>
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<td>HR (beat/min)</td>
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<td>452±60</td>
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<td>475±47</td>
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<td>LV/TL (g/mm)</td>
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<td>Lung/TL (g/mm)</td>
<td>1.01±0.44</td>
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<td>Kidney/TL (g/mm)</td>
<td>3.99±0.76</td>
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<td>3.40±0.36</td>
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<td>Area of fibrosis (%)</td>
<td>23.1±8.1</td>
<td>23.0±3.7</td>
<td>20.4±5.7</td>
<td>10.1±1.7‡</td>
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Values are means ± SD. BW, body weight; SBP, systolic blood pressure; HR, heart rate; LV, left ventricular weight; TL, tibial length. Cont, rats given 8% high salt diet; LD Tolv, rats treated with 0.01% tolvaptan; HD Tolv, rats treated with 0.05% tolvaptan; LS, rats given 0.3% low salt diet. *p<0.05, †p<0.01 versus Cont, ‡p<0.05 versus Cont, LD Tolv, or HD Tolv.
Table 2. Biochemical, hormonal, and urine parameters in Dahl salt sensitive rats treated with vehicle or tolvaptan at 18 weeks

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<td><strong>Serum parameters</strong></td>
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<td>serum sodium (mEq/l)</td>
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<td>serum potassium (mEq/l)</td>
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<td>serum urea nitrogen (mg/dl)</td>
<td>27.3±5.78</td>
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<td>20.1±1.51†</td>
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<td>serum creatinine (mg/dl)</td>
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<td>serum osmolality (mOsm/kgH₂O)</td>
<td>315.5±11.5</td>
<td>321.0±7.72</td>
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<td>serum BNP (pg/ml)</td>
<td>280.0±44.7</td>
<td>211.4±83.6</td>
<td>161.7±64.9†</td>
<td>74.4±8.8#</td>
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<td>serum AVP (pg/ml)</td>
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<td>3.43±1.96</td>
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<tr>
<td>serum aldosterone (pg/ml)</td>
<td>78.7±67.4</td>
<td>127.6±79.8</td>
<td>96.0±58.7</td>
<td>152.7±83.7‡</td>
</tr>
<tr>
<td>plasma renin activity (pg/ml/h)</td>
<td>4.77±1.56</td>
<td>3.87±2.00</td>
<td>3.80±0.92</td>
<td>2.08±0.69‡</td>
</tr>
<tr>
<td><strong>Urine parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV (ml/kg/day)</td>
<td>224.6±93.6</td>
<td>270.3±109.3</td>
<td>329.1±146.7*</td>
<td>40.7±11.5‡</td>
</tr>
<tr>
<td>Uosm (mOsm/kgH₂O)</td>
<td>707.3±277.6</td>
<td>603.4±273.8</td>
<td>489.2±163.7*</td>
<td>1187.5±428.5‡</td>
</tr>
<tr>
<td>urine sodium (mEq/kg/food)</td>
<td>2.55±1.34</td>
<td>3.35±0.78</td>
<td>3.59±0.91*</td>
<td>0.19±0.19‡</td>
</tr>
<tr>
<td>urine potassium (mEq/kg/food)</td>
<td>0.71±0.83</td>
<td>0.83±0.74</td>
<td>0.78±0.59</td>
<td>1.01±0.81‡</td>
</tr>
<tr>
<td>urine nitrogen (mg/kg/day)</td>
<td>861.2±17.3</td>
<td>815.6±151.2</td>
<td>818.8±149.3</td>
<td>751.5±192.1‡</td>
</tr>
<tr>
<td>urine creatinine (mg/kg/day)</td>
<td>34.3±6.06</td>
<td>32.8±7.02</td>
<td>35.2±6.97</td>
<td>32.8±2.31</td>
</tr>
<tr>
<td>urine total protein (mg/kg/day)</td>
<td>942.7±463.6</td>
<td>917.2±432.0</td>
<td>798.5±406.7</td>
<td>73.7±13.2‡</td>
</tr>
<tr>
<td>E-CH₂O (ml/kg/day)</td>
<td>-134.6±82.5</td>
<td>-88.7±56.4</td>
<td>-27.3±93.2†</td>
<td>-9.75±19.4‡</td>
</tr>
<tr>
<td>E-Cosm (ml/kg/day)</td>
<td>0.39±0.11</td>
<td>0.36±0.10</td>
<td>0.32±0.14</td>
<td>0.05±0.02‡</td>
</tr>
<tr>
<td>creatinine clearance (mg/min/100gBW)</td>
<td>0.534±0.12</td>
<td>0.593±0.096</td>
<td>0.749±0.31*</td>
<td>0.868±0.087‡</td>
</tr>
<tr>
<td>FENa (%)</td>
<td>3.91±1.96</td>
<td>3.46±1.20</td>
<td>3.24±1.83</td>
<td>0.05±0.01‡</td>
</tr>
<tr>
<td><strong>Fluid intake (ml/kg/day)</strong></td>
<td>276.5±117.2</td>
<td>299.2±116.4</td>
<td>338.3±131.5</td>
<td>52.7±19.3‡</td>
</tr>
<tr>
<td><strong>Difference between fluid intake and urinary volume (ml/kg/day)</strong></td>
<td>54.1±37.0</td>
<td>34.0±51.1</td>
<td>9.1±39.7**</td>
<td>11.9±17.7#</td>
</tr>
</tbody>
</table>

Values are means ± SD. AVP, vasopressin; E-CH₂O, electrolyte-free water clearance; E-Cosm, electrolyte clearance; FENa, fractional excretion of sodium.; Uosm, urine osmolarity, UV; urine volume. Cont, rats given 8% high salt diet; LD Tolv, rats treated with 0.01% tolvaptan; HD Tolv, rats treated with 0.05% tolvaptan; LS, rats given 0.3% low salt diet,*p<0.05, †p<0.01 versus Cont, ‡p<0.05 versus Cont, LD Tolv, or HD Tolv, #p<0.05 versus Cont or LD Tolv.
Figure Legends

**Figure 1.** The chronic effects of tolvaptan treatment on animal survival (A) and echocardiographic parameters (B). LVDd, Left ventricular dimension in diastole; FS, fractional shortening. The values represent means ± SD. *p<0.05.

**Figure 2.** Quantitative RT-PCR analysis in the LV myocardium. AVP, arginine vasopressin; V1aR, V1a receptor; V1bR, V1b receptor; ANP, atrial natriuretic peptide; ET-1, endothelin-1; ACE, angiotensin converting enzyme; Col1a1, collagen type Ia; FN, fibronectin; MMP2, matrix metalloproteinase-2. The mRNA values represent arbitrary units (the values for the LS rats were set at 1.0 and the remaining values were adjusted accordingly) and means ± SD. *p<0.05; †p<0.01.

**Figure 3.** Histological analysis in the kidney. Representative images of glomerular sections with hematoxylin-eosin staining (A-1) and of tubulointerstitial sections with Picrosirius Red staining (A-2), and semiquantitative analysis of the renal injuries (B). GS, glomerulosclerosis; CG, crescent glomeruli; TF, tubulointerstitial fibrosis; TD, tubular dilation. The values represent means ± SD. *p<0.05; †p<0.01.
Figure 4. Expression of the aquaporins in the kidney. Immunohistochemical staining of AQP2 in inner medullary collecting duct (A), and quantitative RT-PCR analysis of AQPs (B). AQP2, aquaporin-2; AQP3, aquaporin-3. The mRNA values represent arbitrary units (the values for the LS rats were set at 1.0 and the remaining values were adjusted accordingly) and means ± SD. †p<0.01.

Figure 5. Quantitative RT-PCR analysis in the kidney. V2R, V2 receptor; V1aR, V1a receptor; SCNN1, epithelial Na+ channel; NHE3, Na+/H+ exchanger; SGK1, serum/glucocorticoid regulated kinase 1; ET-1, endothelin-1; Col1a1, collagen type Ia; FN, fibronectin; The mRNA values represent arbitrary units (the values for the 11WLS rats were set at 1.0 and the remaining values were adjusted accordingly) and means ± SD. *p<0.05; †p<0.01.
Figure 1.

(A) Survival rate over time for Cont, LD Tolv, and HD Tolv groups.

(Cont versus HD Tolv: log-rank test: P<0.01)

![Graph showing survival rates](image)

(B) Changes in LVDd and FS over time for Cont, LD Tolv, and HD Tolv groups.

![Graph showing LVDd and FS changes](image)
Figure 2.

- **Cont**
- **LD Tolv**
- **HD Tolv**
- **LS**

Bar charts showing various mRNA levels:
- ANP mRNA
- VinR mRNA
- V1bR mRNA
- ET-1 mRNA
- ACE mRNA
- Col1a1 mRNA
- FN mRNA
- MMP2 mRNA

Significance levels indicated by * and †.
Figure 3.
Figure 4.
Figure 5.
Chronic Administration of Oral Vasopressin Type 2 Receptor Antagonist, Tolvaptan Exerts Both Myocardial and Renal Protective Effects in Rats with Hypertensive Heart Failure
Hanako Morooka, Yoshitaka Iwanaga, Yodo Tamaki, Toru Takase, Yasumitsu Akahoshi, Yoshimasa Nakano, Hiroyuki Fujiki and Shunichi Miyazaki

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SUPPLEMENTAL MATERIAL
<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>ACE</td>
<td>AAAGCTGCAGAGATCGA</td>
<td>TTGCCGTTGGAGATAGGA</td>
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<tr>
<td>ANP</td>
<td>GATCTGCCTCTGAAAGCA</td>
<td>TGGCTGTATCTTCGTTACCG</td>
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<tr>
<td>AQP2</td>
<td>TTGCCATGTCTCTCTTCTTCC</td>
<td>GGCGTTGGTGGAGAGCAT</td>
</tr>
<tr>
<td>AQP3</td>
<td>CCATCTACACACTGGCCACAGA</td>
<td>GACCATAACAGTGTTCCAGA</td>
</tr>
<tr>
<td>AVP</td>
<td>CTCGCCCATGATGCTCAACACT</td>
<td>TGTCTCAGCTCCATGTCGGAT</td>
</tr>
<tr>
<td>Col1a1</td>
<td>ACGCATGGCCAGAAGACATC</td>
<td>TTTCGATAGCACGCCATCG</td>
</tr>
<tr>
<td>ET-1</td>
<td>CTTCGCCACCTGGACATCA</td>
<td>TCCCTGGTTGCTGTGGCTTTG</td>
</tr>
<tr>
<td>FN</td>
<td>GAAGAAGCAGAGATGAGGTG</td>
<td>GGGAGTCCAGACCTGTCTTTC</td>
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<tr>
<td>GAPDH</td>
<td>AGGTCCGGTGTGAACGGATTG</td>
<td>TGACTGTGCCACCTGGCAGTCAG</td>
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<tr>
<td>MMP2</td>
<td>CTTCGCCCATCATCAAGTT</td>
<td>CCTTCAACACAAAGAGGTTC</td>
</tr>
<tr>
<td>NHE3</td>
<td>ATGGAAGCTGCTCTATGGAGA</td>
<td>TCAATTCTGCACCAGAGTCA</td>
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<tr>
<td>Renin</td>
<td>GGAGTCACAGGAGACAGACGA</td>
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<tr>
<td>SCNN1</td>
<td>ATTACGGGCACGTACTGAGA</td>
<td>GTCACAGAACTCAACTCTCTTT</td>
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<tr>
<td>SGK1</td>
<td>CGGAGAGCTGTGCTACATCT</td>
<td>GACGATGTGTCCGTTGAGT</td>
</tr>
<tr>
<td>V1aR</td>
<td>GCCGCCTTCTCTCTAGTCCA</td>
<td>GTTGCAGAGCTGTTCAAGGA</td>
</tr>
<tr>
<td>V1bR</td>
<td>GCCACCTCTTCTCCAGTGCCA</td>
<td>TGAAGCCATGTACATCCAGG</td>
</tr>
<tr>
<td>V2R</td>
<td>ATGCTCTCGGTGCTACACATT</td>
<td>AGCAGTGCCAGTTCAGAAGGG</td>
</tr>
</tbody>
</table>

ACE, angiotensin converting enzyme; ANP, atrial natriuretic peptide; AQP2, aquaporin-2; AQP3, aquaporin-3; AVP, arginine vasopressin; Col1a1, collagen type Ia; ET-1, endothelin-1; FN, fibronectin; MMP2, matrix metalloproteinase-2; NHE3, Na⁺/H⁺ exchanger; SCNN1, epithelial Na⁺ channel; SGK1, serum/glucocorticoid regulated kinase 1; V1aR, V1a receptor; V1bR, V1b receptor; V2R, V2 receptor.
Supplemental Figure 1.

BP&UCG
Metabolic cage
Sacrifice

LVH stage
HF stage

0  5  6  11  15  18  19  22 weeks

8% High salt diet (LS)  Cont (n=22)
Tolvaptan 0.01%  LD Tolv (n=20)
Tolvaptan 0.05%  HD Tolv (n=21)
0.3% Low salt diet (LS)  LS (n=6)
Supplemental Figure 2.
Supplemental Figure 3.
Supplemental Figure Legends

Supplemental Figure 1. Diagram of the study design. LVH stage, LV hypertrophic stage; HF stage, heart failure stage.

Supplemental Figure 2. Vasopressin system in Dahl salt-sensitive rat heart failure model during transition from LVH to HF. Serum levels of BNP (A), sodium (B), Osmolality (C), AVP (D) and mRNA levels of V1aR (D), V2R in the kidney and AVP (G), V1aR (H), V1bR (I) mRNA in the LV myocardium at the LVH and HF stages. AVP, arginine vasopressin; V1aR, V1a receptor; V1bR, V1b receptor; V2R, V2 receptor. The mRNA values represent arbitrary units (the values for the 11WLS rats were set at 1.0 and the remaining values were adjusted accordingly) and means ± SD. *p<0.05.

Supplemental Figure 3. Histological analysis in the LV myocardium. Representative images of (A) whole LV and (B) high-power LV stained with Masson’s trichrome, (C) hematoxylin-eosin and (D) Picrosirius Red in the 3 experimental and LS groups.