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

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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 upregulated during the transition to HF. The animals were chronically treated with low-dose or high-dose (HD) tolvaptan or vehicle from the left ventricular (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 atrial natriuretic peptide, endothelin-1, AVP, and V1aR mRNA levels were significantly suppressed in the LV myocardium. Meanwhile, renal histopathologic 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 endothelin-1 in the kidney were significantly suppressed (all \( P<0.05 \)).

Conclusions—These results indicate that 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. (Circ Heart Fail. 2012;5:484-492.)

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 the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system (SNS). In contrast, excess vasopressin levels have long been recognized in patients with HF, particularly those with severe clinical manifestations, and have the potential to exert deleterious effects on various physiological processes in HF. However, the usefulness of long-term blockade of the vasopressin system has not been fully addressed in cardiac remodeling or HF, especially in the long-term blockade of the vasopressin system has not been fully addressed in cardiac remodeling or HF, especially in the long-term blockade of the vasopressin system has not been.

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Among the 3 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. Activation of this receptor leads to increased aquaporin 2 (AQ2P2) 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. The oral V2R antagonist tolvaptan caused an early and sustained reduction in body weight and improvement in serum sodium in the EVEREST trial. 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. 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 hypotension 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. 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
Male inbred Dahl salt-sensitive 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 28 weeks. The tolvaptan group was further divided into 2 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 euthanized to collect serum samples and to harvest cardiac and renal tissues for measurement of mRNA expression and histological examination at 18 to 19 weeks (online-only Data Supplement Figure I).

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. The short-axis view of the LV was recorded to measure the LV end-diastolic dimension (LVDD) and LV end-systolic dimension (LVSD). LV fractional shortening (FS) was calculated. 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 euthanized to collect serum samples and to harvest cardiac and renal tissues for measurement of mRNA expression and histological examination at 18 to 19 weeks (online-only Data Supplement Figure I).

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, brain natriuretic peptide [BNP], AVP, aldosterone, plasma renin activity) by commercial available kits.

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, brain natriuretic peptide [BNP], AVP, aldosterone, plasma renin activity) by commercial available kits.

Histological Analysis
The excised hearts were separated into right ventricle (RV) and LV and fixed in formalin. After being embedded in paraffin, LV tissue was sliced into 5-mm sections. To determine the level of myocardial interstitial fibrosis, sections were stained with Masson trichrome or Picrosirius red and quantified by 3 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 and eosin, Masson trichrome, or Picrosirius red.

Semiquantitative analysis was performed as previously described. 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 hematoxylin. The specificity and results obtained with the primary antibody were checked by omission of the primary antibodies and use of a nonimmune 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 (online-only Data Supplement 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. The formulas used were as follows: E-CH2O=UV−E-Cosm, E-Cosm=(UNa(UK+PNa)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 means±SD. Statistical significance of differences between mean values was analyzed by 1-way analysis of variance (ANOVA) with post hoc comparisons by Fisher protected least-significant difference test. The 2-way repeated-measures ANOVA was used for the analysis in the echocardiographic data. Survival was analyzed by the standard Kaplan-Meier analysis with log-rank test, where 2 separate tests in HD Tolv versus Cont and LD Tolv versus 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
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 V1αR and V2R mRNA levels and myocardial AVP, V1αR, V1βR 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 (online-only Data Supplement Figure II).

Kaplan-Meier Survival Analysis
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±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±3.9 weeks, P=0.184) (Figure 1A).

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 (Table 1). 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, whereas FS was significantly preserved compared with that in the Cont group (41.7±16.7% versus 27.5±6.5%; P<0.05) (Figure 1B).

**Histopathologic Parameters at 18 Weeks**

There were no significant differences in body weight 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 online-only Data Supplement Figure III).

**Biochemical and Hormonal Data of Serum and Urine**

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 per day) and fluid intake (Cont: 272.9±58.4, LD Tolv: 295.4±93.9, HD Tolv: 395.4±71.2 mL/kg per 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/kg H2O). 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 3 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-CH2O 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 3 experimental groups showed the increased fluid intake compared with those in LS group (P<0.01). Among the experimental

<table>
<thead>
<tr>
<th>Table 1. Hemodynamic and Histopathological Parameters in Dahl Salt-Sensitive Rats Treated With Vehicle or Tolvaptan at 18 Weeks</th>
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<tbody>
<tr>
<td><strong>Hemodynamic data</strong></td>
</tr>
<tr>
<td><strong>Body wt, g</strong></td>
</tr>
<tr>
<td>305±39</td>
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<tr>
<td><strong>SBP, mmHg</strong></td>
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<tr>
<td><strong>HR, beats/min</strong></td>
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<tr>
<td><strong>Histopathology</strong></td>
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<tr>
<td><strong>LV/TL, g/mm</strong></td>
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<tr>
<td><strong>Lung/TL, g/mm</strong></td>
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<tr>
<td><strong>Kidney/TL, g/mm</strong></td>
</tr>
<tr>
<td><strong>Area of fibrosis, %</strong></td>
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</table>

Cont indicates 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; SBP, systolic blood pressure; HR, heart rate; LV, left ventricular weight; and TL, tibial length.

Values are mean±SD.

*P<0.05.

†P<0.05 versus Cont or LD Tolv.

‡P<0.05 versus Cont, LD Tolv, or HD Tolv.
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 groups, indicating negative or improved fluid balance existed in HD group at 18 weeks (HF phase) (Table 2).

mRNA Expressions in the LV
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 (Figure 2).

Table 2. Biochemical, Hormonal, and Urine Parameters in Dahl Salt-Sensitive Rats Treated With Vehicle or Tolvaptan at 18 Weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cont</th>
<th>LD Tolv</th>
<th>HD Tolv</th>
<th>LS</th>
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</thead>
<tbody>
<tr>
<td>Serum sodium, mEq/L</td>
<td>157.6±4.45</td>
<td>158.8±7.49</td>
<td>158.3±4.41</td>
<td>151.8±5.19</td>
</tr>
<tr>
<td>Serum potassium, mEq/L</td>
<td>6.48±0.34</td>
<td>6.28±0.36</td>
<td>6.03±0.38</td>
<td>7.12±0.39</td>
</tr>
<tr>
<td>Serum urea nitrogen, mg/dL</td>
<td>27.3±5.78</td>
<td>23.2±5.97</td>
<td>23.2±1.93</td>
<td>20.1±1.51‡</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>0.42±0.09</td>
<td>0.42±0.07</td>
<td>0.39±0.09</td>
<td>0.26±0.01‡</td>
</tr>
<tr>
<td>Serum osmolality, mosm/kg H₂O</td>
<td>315.5±11.5</td>
<td>321.0±7.72</td>
<td>321.6±14.1</td>
<td>295.0±6.57‡</td>
</tr>
<tr>
<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>
</tr>
<tr>
<td>Serum AVP, pg/mL</td>
<td>4.03±2.42</td>
<td>3.43±1.96</td>
<td>4.65±2.94</td>
<td>1.50±1.47‡</td>
</tr>
<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 per 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>Urine parameters</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>UV, mL/kg per d</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/kg H₂O</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 per 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 per 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 per d</td>
<td>861.2±217.3</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 per d</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 per d</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-CH₃O, mL/kg per d</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 per d</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 per 100 g body wt</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>Fluid intake, mL/kg per d</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>Difference between fluid intake and urinary volume, mL/kg per d</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>

Cont indicates 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; BNP, brain natriuretic peptide; AVP, vasopressin; UV, urine volume; Uosm, urine osmolality; E-CH₃O, electrolyte-free water clearance; E-Cosm, electrolyte clearance; and FENa, fractional excretion of sodium.

Values are mean±SD.
*P<0.05 versus Cont.
†P<0.01 versus Cont.
‡P<0.05 versus Cont, LD Tolv, or HD Tolv.
§P<0.05 versus Cont or LD Tolv.

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 tubulointerstitial fibrosis of Tolv-treated groups showed fibrosis around the sclerosing glomeruli and arterioles to a lesser extent than in the Cont group. Semiquantitative analysis showed that increases in the degree of glomerulosclerosis (GS score), crescent glomeruli (CG score), tubulointerstitial fibrosis (TF), and 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

In addition to those of AQP-2 and AQP-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, Col1a1, and fibronectin (FN), which are associated with renal fibrosis, were significantly suppressed in the HD Tolv group (Figure 5).

Discussion

Role of Vasopressin in HF

The pathogenesis of HF involves activation of the neurohumoral axis including stimulation of the SNS, the RAAS, and AVP system.$^{2,15}$ 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.$^{19}$ used transgenic mice with myocyte-specific overexpression of V1aR and showed that

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**Figure 2.** Quantitative RT-PCR analysis in the left ventricular (LV) myocardium. AVP indicates arginine vasopressin; V1aR, V1a receptor; V1bR, V1b receptor; ANP, atrial natriuretic peptide; ET-1, endothelin-1; ACE, angiotensin-converting enzyme; Colla1, collagen type Ia; FN, fibronectin; MMP2, matrix metalloproteinase-2; Cont, rats given 8% high-salt diet; LD Tolv, rats treated with 0.01% tolvaptan; HD Tolv, rats treated with 0.05% tolvaptan; and LS, rats given 0.3% low-salt diet. 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 and eosin staining (A-1) and of tubulointerstitial sections with Picrosirius red staining (A-2) and semiquantitative analysis of the renal injuries (B). GS indicates glomerulosclerosis; CG, crescent glomeruli; TF, tubulointerstitial fibrosis; TD, tubular dilation; Cont, rats given 8% high-salt diet; LD Tolv, rats treated with 0.01% tolvaptan; HD Tolv, rats treated with 0.05% tolvaptan; and LS, rats given 0.3% low-salt diet. Values represent mean±SD. *$P<0.05$; †$P<0.01$. 

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chronic V1αR activation in myocytes causes cardiac hypertrophy and the development of HF. 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.

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 aquaretics, the electrolytes 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 aquaretic 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 and coronary artery ligation. 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, coadministration of tolvaptan and BNP resulted in a beneficial profile of renal, neurohumoral, and hemodynamic actions. 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 2-fold) increased at the LVH stage and further increased (about 5-fold) during the transition to HF. This may be associated with the beneficial effects of tolvaptan seen in the present study.

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 glomerular filtration rate through tubuloglomerular feedback, promote hypertrophy of tubular cells in more distant nephron segments, and increase the potential for electrolyte imbalance such as hyponatremia. 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. 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 or acute V2R blockade study. 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 AQP2 and V2R.

Chronic blockade of the RAAS is known to improve renal damage, 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. They also showed that ACE inhibitors or aldosterone blockers 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 expression of Col1a1 and FN was 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. Recently, Lütken et al demonstrated that increased expression of AQP2 as well as ENaC and NHE3 in postinfarcted rats with HF were, at least in part, reversed by AT1 receptor antagonism. AVP could potentially stimulate renin secretion directly via activation of V2R or indirectly through reduction of the sodium concentration at the macula densa. 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 intrarenal 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 or with puromycin aminonucleoside nephropathy. 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 cyst growth in experimental models of polycystic kidney disease (ADPKD), and V2R antagonists have been tried for the treatment of patients with ADPKD. 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. 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 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. 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. Last, we have not compared tolvaptan treatment with that of standard diuretics (eg, 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.

Conclusions
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 cardiorenal syndrome.

Acknowledgments
We thank for Atsuko Murata for technical assistance.

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References


**CLINICAL PERSPECTIVE**

Diuretic therapy in heart failure (HF) may eventuate in activation of the neurohormonal cascade, electrolyte depletion, and renal injury. Blockade of the vasopressin type 2 receptors (V2R) increases free-water excretion in the renal collecting duct. Although recent clinical trials of the oral V2R antagonist tolvaptan have shown signals of short-term efficacy and neutral long-term effects on outcomes in HF patients, the long-term effects on the myocardium and kidney are not clear. Therefore, we tested chronic tolvaptan administration in a rat hypertensive HF model and explored the chronic effects both on myocardium and kidney. We have demonstrated that chronic high-dose tolvaptan treatment was associated with persistent aquaretic effects without disturbing electrolyte balances through the course and improved survival. It was associated with amelioration of left ventricular and renal dysfunction/damage. In the myocardium, activation of the local vasopressin system, atrial natriuretic peptide, endothelin-1, and angiotensin-converting enzyme were significantly suppressed. In the kidney, histopathologic damage was ameliorated and activation of the local vasopressin system and profibrotic factors was suppressed. These results indicate that chronic tolvaptan treatment had beneficial effects by preventing the progression of both left ventricular dysfunction and renal injury in hypertensive rats with HF. Chronic V2R antagonism may offer the therapeutic potential in such a setting.
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, Yoshimasu Nakano, Hiroyuki Fujiki and Shunichi Miyazaki

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SUPPLEMENTAL MATERIAL
**Supplemental Table. Primer sequences used by real-time quantitative RT-PCR**

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<th>Genes</th>
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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 11 15 18 19 22 weeks

HF stage

0 5 6

Cont (n=22)
LD Tolv (n=20)
HD Tolv (n=21)

[Diagram showing intervention points]
- LS 8% High salt diet
- Tolvaptan 0.01%
- Tolvaptan 0.05%
- 0.3% Low salt diet (LS)
Supplemental Figure 2.
Supplemental Figure 3.

(A)  

(B)  

(C)  

(D)  

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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) Picosirius Red in the 3 experimental and LS groups.