Tolvaptan Improves Left Ventricular Dysfunction after Myocardial Infarction in Rats

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Background—Arginine vasopressin, which promotes the reabsorption of renal water is increased in chronic heart failure. Here, we compared the effects of tolvaptan, a newly developed nonpeptide V$_{1a}$ receptor antagonist, with those of furosemide, a loop diuretic, and a combination of these 2 agents in rats with left ventricular dysfunction after myocardial infarction (MI).

Methods and Results—After 10 weeks of MI induction, the rats were separated them into the following 6 groups adjusted to the infarct size: a vehicle group, a group treated with 15 mg·kg$^{-1}$·day$^{-1}$ of furosemide, 2 groups treated with 3 or 10 mg·kg$^{-1}$·day$^{-1}$ of tolvaptan; and 2 groups treated with 15 mg·kg$^{-1}$·day$^{-1}$ of furosemide plus 3 or 10 mg·kg$^{-1}$·day$^{-1}$ of tolvaptan. Each treatment agent was administered for 4 weeks, and all groups had similar blood pressure levels and infarct size. The tolvaptan-treated groups were found to have lower levels of left ventricular end-diastolic and systolic cardiac volumes than the vehicle group did. Furthermore, the improvement in the ejection fraction in the tolvaptan-treated groups was significantly greater than those in the vehicle group. ED-1 immunostaining and Sirius red staining revealed that tolvaptan significantly repressed MI-induced macrophage infiltration and interstitial fibrosis in the left ventricle, respectively. Tolvaptan attenuated the MI-induced mRNA expressions of atrial and brain natriuretic peptides, monocyte chemotactic protein-1, transforming growth factor-β1, arginine vasopressin V$_{1a}$ receptor, and endothelin-1 in the marginal infarct region.

Conclusions—Tolvaptan may improve cardiac dysfunction after MI, which is partially mediated by the suppression of V$_{1a}$ receptor, neurohumoral activation and inflammation. (Circ Heart Fail. 2012;5:794-802.)

Key Words: arginine vasopressin • diuretic • echocardiography • left ventricular dysfunction • tolvaptan

Chronic heart failure (HF) is a clinical syndrome characterized by decreases in cardiac function, exercise tolerance, and quality of life; it has been associated with high morbidity and mortality. Although angiotensin-converting enzyme inhibitors, angiotensin receptor II blockers, β-blockers, and antialdosterone drugs have improved cardiac remodeling and function, therapeutic agents available for chronic HF, especially refractory HF, are still not optimal. Chronic HF patients may have body fluid retention because of the development of ischemic or valvular heart disease, excessive fluid intake, or stress of infection and can, therefore, present with symptoms of congestive HF. To prevent the occurrence of congestive HF, diuretics are used to regulate the body fluid volume. Several classes of diuretics are available for the treatment of congestive HF; these include loop diuretics, thiazide diuretics, potassium-sparing diuretics (antialdosterone drugs), and carbonic anhydrase inhibitors. However, in some cases of chronic HF, these diuretics may be insufficient to control the body fluid volume. Furthermore, the use of these diuretics poses the risk of electrolyte imbalance and renal dysfunction. Hilleg et al reported that impaired renal function (glomerular filtration rate) is a stronger predictor of mortality than impaired cardiac function is in advanced chronic HF and that the former is associated with increased levels of N-terminal atrial natriuretic peptide. Thus, renal-sparing water diuretics are preferable for the treatment of chronic HF.

Clinical Perspective on p 802

Arginine vasopressin (AVP) is a 9-amino acid peptide secreted from the posterior pituitary, in response to high plasma osmolality and hypotension. AVP is known to play an important role in water metabolism by inducing water reabsorption at the renal collecting duct via the V$_{2}$ receptor (V,R). AVP is also involved in the maintenance of blood pressure.
Tolvaptan, a nonpeptide AVP V₄R antagonist, is a newly developed diuretic drug. This drug exerts an aquaretic effect by blocking the V₄R at the renal collecting ducts and thereby inhibiting water reabsorption. A placebo-controlled study was performed to assess the clinical applicability of tolvaptan in patients with HF and reduced LV systolic function. The results indicated that the tolvaptan-treated patients had significantly lower rates of mortality or hospitalization for worsening HF than those administered the placebo. The tolvaptan group tended to show a better LV ejection fraction (LVEF) than the placebo group, but there was no significant effect of tolvaptan therapy on LV volumes observed during 1 year of therapy. Moreover, the large, multicenter trial, Efficacy of Vasopressin Antagonism in Heart Failure Outcome Study with Tolvaptan (EVEREST), revealed that chronic tolvaptan administration was safe and induced aquaresis, which was evidenced by a reduction in the body weight during the initial hospitalization for acute decompensated HF. In addition, tolvaptan improved the dyspnea, although it neither improved nor worsened the primary outcome variable, which was mortality. Although V₄R antagonism can aid in the management of congestion through aquaretics, it lacks natriuretic action, and its cardiovascular actions remain unclear.

In the present study, we investigated whether tolvaptan is a more effective diuretic than furosemide in LV dysfunction, by using an experimental model of chronic LV dysfunction after myocardial infarction (MI). We obtained evidence that tolvaptan has the beneficial effect of improving cardiac remodeling in chronic LV dysfunction.

Methods

Animals and Experimental Design
Furosemide purchased from Sigma Chemical Co. (St. Louis, MO) and tolvaptan prepared by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan) were used in this study. All procedures were performed in accordance with the Osaka City University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Wistar rats, aged 8 to 9 weeks and weighing 260 to 290 g, were purchased fromCLEA Japan, Inc. (Tokyo, Japan). After administering pentobarbital sodium (50 mg/kg, intraperitoneally) as an anesthetic, MI was induced by the ligation of the left coronary artery, as described previously. Excluding the suturing of the coronary artery, the same surgical procedure was performed on a control group of rats. Ten weeks after MI induction, we measured the BP and heart rate (HR) of the rats by the tail cuff method (BP98A; Softron, Tokyo, Japan) and assessed cardiac function by echocardiography (SONOS 5500; Philips Medical Systems, Best, The Netherlands). Because rats with less than 50% of LVEF were defined as having chronic LV dysfunction, the present study only excluded 2 rats. On the basis of the findings, we separated the MI-affected rats into 6 groups: a nontreated vehicle (V) group, a group treated with 15 mg kg⁻¹•day⁻¹ of furosemide (F), 2 groups treated with 3 or 10 mg kg⁻¹•day⁻¹ of tolvaptan (TL or TH, respectively), and 2 groups treated with a combination of 15 mg kg⁻¹•day⁻¹ of furosemide and 3 or 10 mg kg⁻¹•day⁻¹ of tolvaptan (FTL or FTH, respectively). In the present study, we used 3 or 10 mg kg⁻¹•day⁻¹ of tolvaptan because these doses significantly increased urine volume in rats, but 1 mg kg⁻¹•day⁻¹ of tolvaptan did not change in the previous report. Furthermore, diuretic action of tolvaptan 3 mg kg⁻¹•day⁻¹ seemed to be as same as 15 mg kg⁻¹•day⁻¹ of furosemide. Age-matched Wistar rats subjected to the sham operation were used as the control (C) group. The diet, which was prepared by Oriental Yeast Co. Ltd. (Tokyo, Japan), was mixed with the respective treatment agents and administered to the MI-affected rats. The rats were placed individually in metabolic cages, where they had free access to food and water such that assessment of the fluid and food intake and urine volume was possible.

Four weeks after treatment, the BP and HR of the rats were measured. Cardiac function was monitored by echocardiography under anesthesia, as described below. Immediately after echocardiography, the rat abdomen was cut open and a blood sample was collected from the inferior vena cava. The hearts were then immediately excised, and the ventricle was separated from the atrium and weighed. The infarct size was calculated as the ratio of the scar area to the entire cardiac muscle area, which was measured using a digital image analyzer. The ventricle was separated into the upper and lower portions, and then the upper portion of the left ventricle was divided into the marginal zone and noninfarcted zone; the specimens obtained were then immediately frozen in liquid nitrogen and stored at −80°C until further use. The lower portion was fixed in 10% formaldehyde overnight and embedded in paraffin. Other detailed procedures are described in online-only Data Supplement.

Statistical Analysis
All data are presented as means±standard error of mean (SEM) or median±interquartile range. Differences between group means were compared with the V group with Dunnett test, by using StatView (SAS Institute, Inc., Cary, NC). The differences between the values before and after treatment were analyzed by using Wilcoxon signed-rank tests. The differences were considered statistically significant at a value of P<0.05.

Results

Effects of Tolvaptan and Furosemide on Metabolic Parameters
Nine rats for the C, F, and FTL groups, 10 for the V and TH groups, and 11 for the TL and FTH groups were used in this study.

Mean BP (mBP) and HR at 10 weeks after MI induction ranged from 97 to 107 mm Hg and from 352 to 393 beats per minute, respectively (data not shown). The measurements of the metabolic and hemodynamic parameters at 4 weeks after treatment are shown in Figure 1. The food intake in all the groups was similar, and the planned treatment was successfully administered to all rats. The drinking and urine volume in the FTH group were significantly increased compared with that in the V group.

Effects of Tolvaptan and Furosemide on Hemodynamic and Organ Weights
As shown in Table 1, there was no significant difference in the HR, mBP, and body weight (BW) between the treated groups and the V group. The infarct sizes of the V, F, TL, TH, FTL, and FTH groups were similar (35.8±2.4%, 35.8±3.5%, 35.0±2.6%, 35.0±5.2%, 36.0±2.4%, and 33.3±1.7%, respectively, Figure 1A). The values ofventricular weights/BW in the V group were significantly higher than those in the C group, whereas those in the FTL and FTH groups were significantly lower (2.3±0.1 mg/g and 2.3±0.1 mg/g, respectively) than those in the V group (2.6±0.1 mg/g; Figure 1B). Thus, the MI-induced increase in the value of ventricular weight/BW was inhibited by the combination therapy.
To evaluate the extent of organ congestion, we measured the lung and liver weights. In the present study, neither the lung weight nor the liver weight had a significant difference among the MI groups.

Blood and Urine Chemical Analysis

The measurements of the blood and urine chemical parameters are shown in Table 2. The serum creatinine level did not show any significant difference. Although the urine sodium and potassium levels were lower in the FTH group than in the V group, the serum Na and K levels did not show any significant difference.

The brain natriuretic peptide level, a marker of cardiac load, was significantly higher in the V group than in the C group, but the treatment groups did not differ significantly.

Cardiac function

Figure 2A shows the LV end-diastolic volume, the LV end-systolic volume, and LVEF measurements obtained by echocardiographic studies. Compared with the V group, the tolvaptan-treated groups had significantly lower LV end-diastolic volume and LV end-systolic volume and significantly higher LVEF. Furthermore, after 4 weeks of administration, tolvaptan produced a significant improvement in the LVEF (Figure 2B), whereas furosemide did not result in any change in the LVEF.

Doppler echocardiographic analysis of the LV inflow pattern in each group is shown in Figure 2C and Table 3. MI-induced restrictive inflow pattern tended to exhibit normal pattern by tolvaptan, although it was difficult to evaluate the LV inflow pattern quantitatively because the heart rates were not equalized adequately in the echocardiographic study. Especially, elevated early rapid filling (E) wave velocity by MI was significantly lowered in the TL group.

Estimation of Macrophage Infiltration and Interstitial Fibrosis in LV

The extent of macrophage infiltration in the marginal area of the infarct was estimated by using an antibody against ED-1. Figure 3A and 3B shows macrophage infiltration in the

<table>
<thead>
<tr>
<th>Table 1. Metabolic and Hemodynamic Parameters</th>
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<tr>
<td>MI</td>
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<tr>
<td>---------------------------------------------</td>
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<tr>
<td>Drinking volume, mL/day</td>
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<tr>
<td>Urine volume, mL/day</td>
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<tr>
<td>Food intake, g/day</td>
</tr>
<tr>
<td>Mean blood pressure, mm Hg</td>
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<tr>
<td>Heart rate, bpm</td>
</tr>
<tr>
<td>Body weight, g</td>
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</table>

Value are median±IQR. MI indicates myocardial infarction; C, sham-operated control rats; V, nontreated vehicle rats with MI; F, 15 mg·kg⁻¹·day⁻¹ of furosemide-treated rats with MI; TL or TH, 3 or 10 mg·kg⁻¹·day⁻¹ of tolvaptan-treated rats with MI, respectively; and FTL or FTH, a combination of 15 mg·kg⁻¹·day⁻¹ of furosemide and 3 or 10 mg·kg⁻¹·day⁻¹ of tolvaptan-treated rats with MI, respectively.

*P<0.05 vs V.
Table 2. Blood and Urine Chemical Parameters

<table>
<thead>
<tr>
<th></th>
<th>C (n=9)</th>
<th>V (n=10)</th>
<th>F (n=9)</th>
<th>TL (n=11)</th>
<th>TH (n=10)</th>
<th>FTL (n=9)</th>
<th>FTH (n=11)</th>
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<td>Serum</td>
<td></td>
<td></td>
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<tr>
<td>Blood urea nitrogen</td>
<td>23.3±0.7</td>
<td>23.2±1.0</td>
<td>24.4±0.3</td>
<td>22.8±1.1</td>
<td>22.4±0.8</td>
<td>26.4±0.8</td>
<td>23.8±1.1</td>
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<td>Creatinine, mg/dL</td>
<td>0.32±0.01</td>
<td>0.34±0.02</td>
<td>0.29±0.01</td>
<td>0.32±0.01</td>
<td>0.30±0.01</td>
<td>0.31±0.01</td>
<td>0.32±0.05</td>
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<tr>
<td>Sodium, mEq/L</td>
<td>139.0±0.5</td>
<td>139.1±0.5</td>
<td>137.3±1.4</td>
<td>136.8±1.4</td>
<td>139.1±0.6</td>
<td>137.0±0.8</td>
<td>139.4±0.5</td>
</tr>
<tr>
<td>Potassium, mEq/L</td>
<td>5.5±0.3</td>
<td>5.7±0.1</td>
<td>5.7±0.1</td>
<td>6.5±0.2</td>
<td>6.4±0.3</td>
<td>6.2±0.2</td>
<td>5.6±0.3</td>
</tr>
<tr>
<td>Chloride, mEq/L</td>
<td>102.1±0.8</td>
<td>103.1±0.6</td>
<td>100.5±1.2</td>
<td>101.6±1.1</td>
<td>103.6±0.6</td>
<td>100.1±0.8</td>
<td>101.6±0.4</td>
</tr>
<tr>
<td>Osmolality, mOsm/kgH_2O</td>
<td>315.8±2.3</td>
<td>314.3±4.8</td>
<td>307.2±3.7</td>
<td>307.3±3.7</td>
<td>309.7±1.8</td>
<td>311.9±2.4</td>
<td>308.1±0.7</td>
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<td>Plasma</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain natriuretic peptide, pg/mL</td>
<td>119.9±4.5*</td>
<td>162.2±3.6</td>
<td>152.2±8.5</td>
<td>156.4±7.9</td>
<td>151.0±8.0</td>
<td>144.4±8.2</td>
<td>161.8±4.4</td>
</tr>
<tr>
<td>Plasma renin activity, ng mL⁻¹ hr⁻¹</td>
<td>5.3±0.8</td>
<td>7.8±2.7</td>
<td>6.4±1.8</td>
<td>6.0±1.6</td>
<td>4.9±1.6</td>
<td>7.6±1.6</td>
<td>5.9±1.9</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sodium, mEq/L</td>
<td>97.5±5.0</td>
<td>103.0±7.4</td>
<td>107.2±5.7</td>
<td>93.9±6.0</td>
<td>94.4±4.1</td>
<td>102.5±4.5</td>
<td>81.2±3.5*</td>
</tr>
<tr>
<td>Potassium, mEq/L</td>
<td>252.7±13.9</td>
<td>272.1±16.5</td>
<td>296.6±10.5</td>
<td>263.1±9.9</td>
<td>244.7±11.5</td>
<td>267.4±11.6</td>
<td>223.2±12.8*</td>
</tr>
<tr>
<td>Chloride, mEq/L</td>
<td>116.2±7.5</td>
<td>124.2±7.7</td>
<td>125.9±4.5</td>
<td>119.3±10.2</td>
<td>117.2±5.7</td>
<td>120.0±5.4</td>
<td>103.5±6.5</td>
</tr>
<tr>
<td>Osmolality, mOsm/kgH_2O</td>
<td>2045±137</td>
<td>1966±105</td>
<td>2087±97</td>
<td>1916±78</td>
<td>1717±81</td>
<td>2034±86</td>
<td>1709±84</td>
</tr>
</tbody>
</table>

Value are mean±SEM. MI indicates myocardial infarction; C, sham-operated control rats; V, nontreated vehicle rats with MI; F, 15 mg·kg⁻¹·day⁻¹ of furosemide-treated rats with MI; TL or TH, 3 or 10 mg·kg⁻¹·day⁻¹ of tolvaptan-treated rats with MI, respectively; and FTL or FTH, a combination of 15 mg·kg⁻¹·day⁻¹ of furosemide and 3 or 10 mg·kg⁻¹·day⁻¹ of tolvaptan-treated rats with MI, respectively.

*P<0.05 vs V.

**marginal area of the infarct. The increase of macrophage infiltration by MI was significantly suppressed in the FTH group.

The extent of interstitial fibrosis in the marginal zone of the infarct is shown in Figure 4A and 4B. The extent of interstitial fibrosis in the tolvaptan-treated (TL, TH, FTL, and FTH) groups was significantly lower than the V group, whereas this effect was not shown in the F group.

Effect of Tolvaptan on Cardiac Gene Expressions

As shown in Figure 5, the mRNA expressions in the LV marginal area in each group of rats were measured by quantitative real-time polymerase chain reaction, and differences between group means were compared with the V group with Dunnett test. mRNA expressions of atrial natriuretic peptide and brain natriuretic peptide, which are factors closely associated with cardiac load, were increased by 36.1- and 3.8-fold (*P<0.05), respectively, by MI induction. The MI-induced upregulation of atrial natriuretic peptide or brain natriuretic peptide expressions was significantly decreased in the TL and TH groups (Figure 5A) or TH group (Figure 5B), respectively.

MI-induced an 8.4- and 2.4-fold (*P<0.05) increase in the V₃,R and endothelin-1 (ET-1), which are factors closely associated with vasoconstriction, were significantly increased by MI induction. This upregulation of V₃,R and ET-1 was significantly decreased in the tolvaptan-treated groups (Figure 5G) or TH and FTH groups (Figure 5H), respectively.

Discussion

This study revealed that tolvaptan may improve cardiac remodeling in LV dysfunction because of old MI. This is the first attempt to compare the effects of tolvaptan with those of furosemide and their combination in a rat model of LV dysfunction.

Previous clinical studies, such as EVEREST and the Effect of Tolvaptan on Hemodynamic Parameters in Subjects with Heart Failure (ECLIPSE) trial, have shown that tolvaptan increases urine output in a dose-dependent manner. However, no significant differences were reported for the secondary end points of BP, HR, systemic vascular resistance, or cardiac index. Notably, most of these patients were on concomitant medication. Therefore, from these studies alone, it is impossible to elucidate whether tolvaptan can independently improve LVEF in chronic HF patients.

In the present study using an experimental rat model of LV dysfunction, the influences of secondary factors affecting cardiac remodeling, such as BP, other medications, and infarct size were similar in all MI-induced groups. Thus, we found that the LVEF in the tolvaptan-treated groups was significantly greater than that in the furosemide-treated group. In addition, tolvaptan also partially improved the increase of...
E wave velocity by MI, suggesting that tolvaptan may also improve LV diastolic function because it has been reported that there is a weak correlation between LV end-diastolic pressure and E wave velocity and E deceleration time in MI rats. These findings suggest that the effect of tolvaptan in improving the LV dysfunction may not be entirely attributable to its volume-reducing effect. The ventricular weight in the combination therapy group was significantly lower than that in the

Table 3. Echocardiographic Measurements of Left Ventricular Diastolic Function At 4 Weeks After Each Treatment

<table>
<thead>
<tr>
<th>MI</th>
<th>C (n=9)</th>
<th>V (n=10)</th>
<th>F (n=9)</th>
<th>TL (n=11)</th>
<th>TH (n=10)</th>
<th>FTL (n=9)</th>
<th>FTH (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early rapid filling(E) wave, cm/sec</td>
<td>77.2±3.7*</td>
<td>92.8±4.6</td>
<td>88.3±3.4</td>
<td>77.2±3.9*</td>
<td>87.0±3.3</td>
<td>80.4±3.8</td>
<td>87.5±4.3</td>
</tr>
<tr>
<td>Atrial contraction(A) wave, cm/sec</td>
<td>31.3±3.0</td>
<td>26.9±3.7</td>
<td>25.3±2.1</td>
<td>29.0±2.6</td>
<td>35.7±4.4</td>
<td>26.9±2.3</td>
<td>26.4±3.0</td>
</tr>
<tr>
<td>The ratio of E wave to A wave</td>
<td>2.80±0.45</td>
<td>4.10±0.60</td>
<td>3.65±0.30</td>
<td>2.89±0.47</td>
<td>2.90±0.48</td>
<td>3.16±0.32</td>
<td>3.86±0.52</td>
</tr>
<tr>
<td>E deceleration, cm/sec²</td>
<td>1523±115*</td>
<td>2116±155</td>
<td>1809±108</td>
<td>1377±106*</td>
<td>1695±111*</td>
<td>1698±165</td>
<td>1670±101*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MI indicates myocardial infarction; C, sham-operated control rats; V, nontreated vehicle rats with MI; F, 15 mg·kg⁻¹·day⁻¹ of furosemide-treated rats with MI; TL or TH, 3 or 10 mg·kg⁻¹·day⁻¹ of tolvaptan-treated rats with MI, respectively; FTL or FTH, a combination of 15 mg·kg⁻¹·day⁻¹ of furosemide and 3 or 10 mg·kg⁻¹·day⁻¹ of tolvaptan-treated rats with MI, respectively.

*P<0.05 vs V.
untreated vehicle group and the monotherapy groups. This suggests that the addition of tolvaptan to the classical diuretic therapy (furosemide only) could improve cardiac remodeling after MI. Furthermore, tolvaptan with or without furosemide could prevent cardiac remodeling because tolvaptan alone could improve LVEF but furosemide alone failed.

Previous evidences suggest that the inflammatory response is a key component of the structural deterioration associated with post-MI remodeling of the LV. MCP-1, a C-C chemokine with potent chemotactic and activating effects on monocytes, is known to be a major contributor to the pathogenic role of inflammation in cardiovascular diseases. MCP-1 contributes to the progression of atherosclerosis and vascular remodeling. Recent reports have indicated that the expression of MCP-1 in the myocardium increases during the early phase of MI and that the targeted deletion or pharmacologic inhibition of MCP-1 prevents early post-MI LV remodeling. Furthermore, the second phase of the inflammatory response occurs in the noninfarcted myocardium. It has been reported that MCP-1 expression and macrophage infiltration are increased in the noninfarcted region of the post-MI remodeled myocardium. Recent studies have shown that the marginal myocardial area might be a novel therapeutic target in the treatment of post-MI LV remodeling. Extension of the macrophage-mediated inflammation in the infarcted region might cause the inflammatory response in the marginal region, although the mechanism underlying the inflammatory response in the latter has not been fully elucidated. In addition, geometric changes in hearts with LV aneurysm after MI are known to increase wall stress in the marginal region, and pressure-overload–induced cardiac fibrosis is mediated through MCP-1 induction and macrophage accumulation.

In the present study, ED-1 immunostaining and quantitative real-time polymerase chain reaction revealed that tolvaptan significantly suppressed MI-induced macrophage infiltration and MCP-1 expression in the marginal myocardial area of the infarct.

Macrophages infiltrating the infarcted myocardium are known to produce a variety of inflammatory cytokines and growth factors, which in turn amplify the inflammatory response and promote myocardial fibrosis. In addition to its chemoattractant activity toward monocytes, MCP-1 itself is involved in the occurrence of fibrosis through the stimulation of fibroblast collagen expression via the generation of profibrotic cytokines. These findings suggest that MCP-1 might be a key mediator in the development of LV remodeling.

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**Figure 3.** Estimation of the extent macrophage infiltration. Myocardial infarction-induced macrophage infiltration in rats. A. Representative photomicrographs of cross-sections of macrophage infiltration stained with ED-1 (dark brown cells) on left ventricular marginal area sections (original magnification×400). Bar, 200 μm. B. Quantitative results of macrophage infiltration (/hpf). Macrophage infiltration in the FTH group was lower than that in the V group. C indicates sham-operated control rats; V, nontreated vehicle rats with MI; F, 15 mg·kg⁻¹·day⁻¹ of furosemide-treated rats with MI; TL or TH, 3 or 10 mg·kg⁻¹·day⁻¹ of tolvaptan-treated rats with MI, respectively; and FTL or FTH, a combination of 15 mg·kg⁻¹·day⁻¹ of furosemide and 3 or 10 mg·kg⁻¹·day⁻¹ of tolvaptan-treated rats with MI, respectively. Values are mean±standard error of mean (SEM) (n=9–11). *P<0.05 vs V.

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**Figure 4.** Estimation of the extent of interstitial fibrosis. Myocardial infarction-induced interstitial fibrosis in rats. A. Representative photomicrographs of the cross-sections showing myocardial interstitial fibrosis by staining left ventricular marginal area sections (original magnification×200) with Sirius red (red). Bar, 500 μm. B. Quantitative results of relative area of interstitial fibrosis (%). Tolvaptan-treated groups showed significant suppression of MI-induced interstitial fibrosis compared with the V group. C indicates sham-operated control rats; V, nontreated vehicle rats with MI; F, 15 mg·kg⁻¹·day⁻¹ of furosemide-treated rats with MI; TL or TH, 3 or 10 mg·kg⁻¹·day⁻¹ of tolvaptan-treated rats with MI, respectively; and FTL or FTH, a combination of 15 mg·kg⁻¹·day⁻¹ of furosemide and 3 or 10 mg·kg⁻¹·day⁻¹ of tolvaptan-treated rats with MI, respectively. Values are mean±standard error of mean (SEM) (n=9–11). *P<0.05 vs V.
through macrophage infiltration and fibrosis in the marginal zone. Sirius red staining and quantitative real-time polymerase chain reaction revealed that MI-induced interstitial fibrosis and fibrosis-related genes, such as transforming growth factor-$\beta_1$ and collagen type III, were significantly inhibited by tolvaptan. We have, thus, for the first time, shown that tolvaptan has anti-inflammatory and antifibrotic activity in chronic stage of MI-induced LV dysfunction. However, furosemide does not have such beneficial effects.

Reports have indicated the existence of an interaction between the renin-angiotensin system (RAS) and MCP-1.27,29 Angiotensin II can induce the synthesis of MCP-1 both in vitro and in vivo, thereby suggesting that Angiotensin II participates in the recruitment of inflammatory cells by increasing MCP-1 expression.30 However, inflammatory cells express all the components of the RAS,31 and the activity of the RAS is remarkably high during monocyte/macrophage differentiation.32 Therefore, Ang II-induced MCP-1 expression promotes the activation of inflammatory cells, which in turn can activate the RAS and increase local Angiotensin II generation, thereby contributing to the progression and perpetuation of inflammation. Hirano et al33 reported that tolvaptan induces aquaresis, resulting in increased serum sodium levels, without affecting the RAS. In contrast, furosemide induces natriuresis, which results in decreased serum sodium levels and the activation of the RAS, thereby suggesting that furosemide may accelerate cardiac remodeling.

The direct effect of tolvaptan on the cardiac muscle is difficult to explain because tolvaptan is pharmacologically a V$_2$R antagonist,6 and V$_2$R is unknown to be expressed in cardiac muscles. LV V$_2$R mRNA expression was undetectable in this model. (data not shown). However, our study showed

**Figure 5.** mRNA expression. Gene expressions in the marginal infarct region of the vehicle-treated and medicated rats, and left ventricle of the control rats. The bar graph shows the value of each mRNA, corrected for the Glyceraldehyde 3-phosphate dehydrogenase mRNA value. Mean values in the control rat group are represented as 1. All parameters of mRNA expressions were upregulated by MI induction. A and B. The MI-induced upregulation of ANP and BNP was significantly decreased in the TL and TH groups. C and D. MCP-1 or TNF-$\alpha$ mRNA expression was significantly decreased in the TH and FTH groups or TH group, respectively. E and F. TGF-$\beta_1$ or Col III mRNA expression was significantly decreased in the TH and FTH groups or TH group, respectively. G and H. MI-induced V$_1$R or ET-1 mRNA expression was also significantly decreased in the tolvaptan-treated groups or TH and FTH groups, respectively. ANP indicates atrial natriuretic peptide; BNP, brain natriuretic peptide; MCP-1, monocyte chemoattractant protein-1; TNF-$\alpha$, tumor necrosis factor-$\alpha$; TGF-$\beta_1$, transforming growth factor-$\beta_1$; Col III, collagen type III; V1aR, vasopressin V$_1$ receptor; ET-1, endothelium-1; C, sham-operated control rats; V, nontreated vehicle rats with MI; F, 15 mg·kg$^{-1}$·day$^{-1}$ of furosemide-treated rats with MI; TL or TH, 3 or 10 mg·kg$^{-1}$·day$^{-1}$ of tolvaptan-treated rats with MI, respectively; and FTL or FTH, a combination of 15 mg·kg$^{-1}$·day$^{-1}$ of furosemide and 3 or 10 mg·kg$^{-1}$·day$^{-1}$ of tolvaptan-treated rats with MI, respectively. Values are mean±standard error of mean (SEM) (n=9–11). *P<0.05 vs V.
that the ventricular weight was significantly lower in the group treated with a combination of furosemide and tolvaptan, and echocardiography showed significant improvement in the LVEF in the tolvaptan-treated groups. Further, because the groups did not differ in terms of BW, we can infer that the effect of tolvaptan may not be entirely explained by its volume-reducing effect. Some possibilities are being considered to explain how tolvaptan works. Previous studies have shown that furosemide increases plasma renin activity, whereas the combination of furosemide and tolvaptan does not increase plasma renin activity.\(^{33,34}\) Unfortunately, furosemide did not elevate plasma renin activity levels in the present study of chronic phase after MI. The \(V_1R\), which exists in cardiac or vascular smooth-muscle cells, causes cardiac hypertrophy, blood-vessel contraction, and cardiac remodeling.\(^{35,36}\) In the present study, MI-induced cardiac \(V_1R\) mRNA expression was significantly suppressed by the treatment of tolvaptan. Li et al.\(^{37}\) have shown that chronic \(V_1R\) activation in myocytes causes cardiac hypertrophy and the development of HF by using transgenic mice with myocyte-specific overexpression of \(V_1R\), suggesting that tolvaptan may partially contribute to prevent cardiac dysfunction by downregulation of \(V_1R\) of which activation plays a pivotal role during cardiac remodeling after MI. A very recent study also showed that tolvaptan not only prevented the progression of LV dysfunction but also suppressed \(V_1R\) activation of myocardium in Dahl salt-sensitive rats.\(^{37}\) Furthermore, hemodynamic stress or other neurohumoral factors might regulate local AVP system, though the mechanism has not been elucidated. Therefore, we investigated the effect of tolvaptan on cardiac ET-1 expression, one of neurohumoral factors. Interestingly, tolvaptan decreased MI-induced ET-1 expression. Taken together with a recent study that showed that tolvaptan suppressed ET-1 expression of myocardium in Dahl salt-sensitive rats and prevented the progression of LV dysfunction,\(^{37}\) the underlying beneficial mechanism of tolvaptan may be partially related to the suppression of neurohumoral activation.

### Study Limitations

Any invasive hemodynamic or telemetric analysis was not performed in this study. Cardiac function was estimated by echocardiography. It is unknown from our data whether tolvaptan has a cardioprotective effect on acute MI. The effect of tolvaptan on cardiac RAS is also unclear. Further studies are needed to elucidate the precise mechanism of the effects of tolvaptan and the efficacy of combination therapy with tolvaptan and an RAS inhibitor in chronic HF.

In conclusion, our present study provides the in vivo evidence showing the beneficial effect of tolvaptan in the improvement of cardiac dysfunction. This effect may be attributed to its anti-inflammatory and antifibrotic actions and its volume-reducing effect. We propose tolvaptan as a new therapeutic agent in LV dysfunction.

### Acknowledgments

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### Disclosures

Dr. Fujiyki is an employee of Otsuka Pharmaceuticals Co., Ltd. The other authors have no conflicts to report.

### References


**CLINICAL PERSPECTIVE**

Previous studies have shown that plasma arginine vasopressin levels may be significantly elevated in patients with HF, leading to volume overload and possibly vasoconstriction. We compared the effects of tolvaptan, a nonpeptide V₂ receptor antagonist, with those of furosemide and a combination of these 2 agents in rats with left ventricular dysfunction after myocardial infarction. During 4 weeks of treatment, all groups had similar blood pressure levels and infarct size. The tolvaptan-treated groups were found to have lower levels of left ventricular end-diastolic and systolic cardiac volumes than the vehicle group. Tolvaptan significantly repressed macrophage infiltration and interstitial fibrosis in the left ventricle, attenuated the perivascular inflammatory response, and suppressed angiotensin II-induced target organ damage in hypertensive heart disease.
Tolvaptan Improves Left Ventricular Dysfunction after Myocardial Infarction in Rats
Takanori Yamazaki, Yasukatsu Izumi, Yasuhiro Nakamura, Naoto Yamashita, Hiroyuki Fujiki, Mayuko Osada-Oka, Masayuki Shiota, Akihisa Hanatani, Kenei Shimada, Hiroshi Iwao and Minoru Yoshiyama

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SUPPLEMENTAL MATERIAL
Experimental Procedures

Echocardiographic study

Transthoracic echocardiographic studies were performed on rats according to a previously described method \(^1\), \(^2\). In brief, rats were anesthetized with tiletamine (10 mg·kg\(^{-1}\), ip) and xylazine (10 mg·kg\(^{-1}\), ip). A two-dimensional short-axis view of the left ventricle was obtained at the level of the papillary muscles. The LVEF was calculated by measuring the LV end-diastolic volume (LVEDV) and the LV end-systolic volume (LVESV), by using a modified Simpson’s method. Pulsed wave Doppler spectra (early rapid filling (E) wave and atrial contraction (A) wave) of mitral inflow velocities were recorded from the apical four chamber view, with the sample volume placed near the tips of the mitral leaflets and adjusted to the position at which velocity was maximum and the flow pattern was laminar. The ratio of E wave velocity to A wave velocity (E/A ratio) and E deceleration were calculated.

ED-1 immunostaining and estimation of macrophage infiltration of the left ventricle

The area of interstitial macrophage infiltration in the marginal region of the infarct was measured using a previously described method \(^3\). In short, serial sections were stained with antibodies against ED-1 (Serotec Inc., NC), a marker for tissue-resident macrophages. Eight microscopic fields in each tissue block were examined for the presence of macrophages, and macrophage infiltration was expressed as the number of positive cells per high power field (×200).

Estimation of cardiac fibrosis

The area of interstitial fibrosis in the marginal area of the infarct was measured, as described
previously \(^4,5\). In short, 4-μm-thick sections were cut and stained with Sirius red stain for the measurement of the area with interstitial fibrosis. The area of interstitial fibrosis was calculated as the ratio of the sum of the total area of interstitial fibrosis to the sum of the total connective tissue area plus the area of cardiomyocytes in the marginal area of the LV. Each field was analyzed using image-analyzing software (Micro Analyzer, Japan Poladigital, Tokyo, Japan).

**RNA preparation and analysis**

RNA from the marginal area of the LV was isolated using ISOGEN (Nippon Gene, Toyama, Japan) \(^4,5\). To elucidate the gene expression levels, we subjected the RNA samples to quantitative real-time RT-PCR (qRT-PCR, 7500 Fast; Applied Biosystems, Carlsbad, CA). One-step qRT-PCR reactions were performed using 100 ng of total RNA per reaction. TaqMan primers and probes were designed using Primer 3 (v. 0.4.0); see Supplemental Table. For normalization, the transcript levels were compared to those of GAPDH.
Supplementary References


### Supplemental Table. Sequences of the quantitative real-time RT-PCR probes and primers used in this study

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Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Nppa, atrial natriuretic peptide; Nppb, brain natriuretic peptide; Ccl2, monocyte chemoattractant protein-1; Tnf, tumor necrosis factor-α; Tgfb1, transforming growth factor -β1; Col3, collagen type III; Avpr1a, V1a receptor; Edn1, endothelin-1.