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Effects of nonpeptide vasopressin V_2 antagonist tolvaptan in rats with heart failure

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ARTICLE INFO

Article history:
Received 30 May 2007
Accepted 18 July 2007

Keywords:
Vasopressin
Vasopressin receptor antagonists
Hyponatremia
Water excretion
Chronic heart failure
Aquaporin 2

ABSTRACT

Similar to other neurohormones that are activated in chronic heart failure (CHF), circulating arginine vasopressin (AVP) is elevated in patients with CHF. The precise role of AVP in the pathophysiology of cardiovascular disease is controversial. AVP is a peptide hormone that contributes to water retention and vasoconstriction in CHF through effects on V_2 and V_{1a} receptors, respectively. In the present study, the effect of V_2 receptor (V_2R) blockade using tolvaptan was assessed in a rat model of myosin-induced experimental autoimmune myocarditis. CHF was elicited in Lewis rats by immunization with porcine cardiac myosin, and 28 days after immunization rats were treated for 28 days with oral tolvaptan (3 or 10 mg/(kg day)) or vehicle. CHF was characterized by left ventricular remodeling and impaired systolic and diastolic function. Chronic V_2R blockade increased urine volume and urinary AVP excretion and decreased urine osmolality but had no natriuretic effect, and as a result caused increases in plasma osmolality and sodium. High doses of tolvaptan markedly elevated electrolyte-free water clearance. V_2R blockade did not activate the renin–angiotensin system, not influence cardiac remodeling, cardiac function, or survival. The upregulation of aquaporin 2 protein in the kidney of CHF rats was inhibited by the administration of V_2R antagonist. These results suggest that in a rat model of CHF, AVP plays a major role in water retention through the renal V_2R.

1. Introduction

Congestive heart failure is a disorder often characterized by decreased cardiac output and arterial pressure, left ventricular (LV) dysfunction and activation of sodium and water retaining hormones, including the sympathetic nervous system, the renin–angiotensin–aldosterone system (RAAS), and the antidiuretic hormone arginine vasopressin (AVP) [1]. However, chronic activation of these systems can lead to excessive ventricular preload and afterload, adverse ventricular remodeling, pulmonary and systemic congestion, and electrolyte abnormalities, such as hyponatremia [2]. Current therapy for chronic heart failure (CHF) is still not optimal and the possible benefit of blocking vasopressin actions in patients with CHF remains to be evaluated [3]. Using loop diuretics, the principal therapy for congestion is possible to cause electrolyte abnormalities (loss of sodium and other essential electrolytes, which may exacerbate the hyponatremia) and renal dysfunc-

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doi:10.1016/j.bcp.2007.07.027

tion [4,5]. Furthermore, serum sodium concentration is one of the best predictors of cardiovascular mortality, with hyponatremia patients showing substantially shorter survival than patients with a normal sodium concentration [6]. It is not known whether neurohormonal activation and decreased serum sodium level are the result of more advanced heart failure or whether they contribute directly to the progression of mortality. An alternative approach to relieve symptoms of CHF may be the use of selective water diuretics.

Similar to other neurohormones that are activated in CHF, circulating AVP is elevated in patients with CHF [7]. The precise role of AVP in the pathophysiology of cardiovascular disease is controversial. AVP acts via three receptor types: V_{1a}, V_{1b} (V_3), and V_2. Through activation of V_{1a} and V_2 receptors, AVP regulates various physiological processes including vascular tone regulation, cardiovascular contractility and body fluid regulation, respectively [7]. V_2 receptors are located in the renal collecting duct, where AVP binding to the V_2 receptor (V_2R) leads to a rise in intracellular adenosine 3′,5′-cyclic monophosphate. This promotes renal water reabsorption via translocation of intracellular vesicles containing the water channel aquaporin 2 (AQP2) into the apical plasma membrane and increased transcription of AQP2, thereby increasing the intravascular volume and diluting the sodium concentration [8]. Water reabsorption in the collecting ducts is regulated by short-term and long-term mechanisms, both of which have been shown to depend critically on AQP2. Short-term regulation occurs as a result of exocytic insertion of AQP2 water channels into the apical plasma membrane in response to vasopressin [8–10]. Long-term regulation of collecting duct water permeability is characterized by an increase in AQP2 mRNA and protein content during fluid restriction and AVP infusion in diabetes insipidus rats. This increase in AQP2 is paralleled by a comparable increase in osmotic water permeability, demonstrating a direct role of vasopressin in the long-term regulation of AQP2 [11].

The recent development of nonpeptide orally active AVP-receptor antagonists has allowed reevaluation of the precise role of AVP in experimental animal models of diseases, including hypertension [12,13] and heart failure [14]. Tolvaptan is a modified benzazepine derivative that was selected as a potent human V_2R antagonist through a series of structural conversions of mozavaptan. The potent aquaretic properties of tolvaptan in rats and its pharmacological profile were reported by Yamamura et al. [15]. The first human study using this V_2R antagonist (tolvaptan) in patients with CHF was performed by Gheorghiade et al. [16]. They found that, patients with blockade of V_2 receptors had an increase in urine volume and a decrease in body weight that were maintained throughout the study. Its oral bioavailability and a long half-life time permit the studies that address the physiological significance of V_2R in CHF and also the development of V_2R antagonists as therapeutic agents. Taking the known facts all together, it would appear as though blocking the renal effects of AVP with selective V_2R antagonists would produce diuresis and maintenance of renal function by antagonizing elevated endogenous AVP levels rather than unphysiologically blocking sodium reabsorption.

To date, the effects of V_2R antagonism in a rat model of myosin-induced CHF are unknown. Cardiac myosin-induced experimental autoimmune myocarditis (EAM) is characterized by extensive myocardial necrosis, congestive heart failure and appearance of multinucleated giant cells and reflective to human giant cell myocarditis. EAM was demonstrated to progress into the clinicopathological state similar to dilated cardiomyopathy (DCM) in the chronic phase, and was characterized by the enlargement of the heart, dilatation of both ventricles, diffuse and extensive myocardial fibrosis, and hypertrophic and atrophic changes of myocardial fibers, resembling human cardiomyopathy [17–19]. DCM is a serious disorder and the most common cause of heart failure. Difficulties in finding effective therapies are mainly based on the fact that the pathogenesis of DCM is still poorly understood. The establishment of an animal model that mimics human DCM will provide useful information with regard to the pathogenesis of DCM and the development of effective therapies. Thus the present study was designed to assess the effects of long-term blockade of V_2R on renal and myocardial functions, related hormones and AQP2 protein expression in a rat model of CHF.

2. Materials and methods

2.1. Materials

Tolvaptan (7-chloro-5-hydroxy-1-[2-methyl-4-(2-methylbenzoylamino)benzoyl]-2,3,4,5-tetrahydro-1*H*-1-benzazepine) was a gift from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). AQP2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were purchased from Santa Cruz Biotechnology Inc., CA, USA. All other reagents used, unless otherwise specified, were obtained from Sigma (Tokyo, Japan). Lewis rats (male, 8 weeks old) were purchased from Charles River Japan Inc., Kanagawa, Japan.

2.2. Experimental design

All experiments were carried out using 8-week-old male Lewis rats and were performed in accordance with the guidelines of our institute [19]. CHF in rats was induced by immunization with porcine cardiac myosin into their footpads by subcutaneous injection. Porcine cardiac myosin was prepared from the ventricular muscle according to the procedure described previously [17,19]. The morbidity of EAM was 100% in rats immunized by this procedure [17,19]. Rats immunized with myosin became ill and immobile on day 14, and their activity gradually recovered beginning at the fourth week. Six (25%) of the 24 rats died between days 14 and 28 after immunization. All hearts from the dead rats showed extensive myocardial necrosis and pericardial effusion. Twenty-eight days after immunization, the postmyocarditis DCM develops in the rats. The surviving 18 rats were divided into three groups and received oral administration (p.o.) of tolvaptan (3 mg/(kg day), Tol 3 group, $n = 6$; 10 mg/(kg day), Tol 10 group, $n = 6$), or vehicle (1% hydroxylpropyl methyl cellulose, CHF group, $n = 6$) for 28 days. Eight-week-old Lewis rats without immunization used as normal controls were also divided into three groups which received treatment according to the above pattern as follows; normal rats treated with tolvaptan (3 mg/(kg day),

Ntol 3 group, $n = 5$; or 10 mg/(kg day), Ntol 10 group, $n = 5$), or vehicle-treated control group, $n = 5$. The dose used in the experiments was determined on the basis of the aquaretic properties of tolvaptan described in earlier reports [15,20].

2.3. Treatments and measurements

Tolvaptan was administered orally to the groups as indicated in Table 1. Immediately after administration, the rats were placed individually in metabolic cages and urine samples were collected for 4 h after drug administration on days 1 and 28. The urinary volume and body weight (BW) were noted. The collected urine was used for measurement of urinary parameters (urine osmolality and sodium and AVP). Urinary AVP excretion was determined by radioimmunoassay (RIA) using the method reported previously [21].

2.4. Hemodynamic and echocardiographic studies

Rats were anesthetized with 2% halothane in O_2 and subjected to surgical procedures to measure hemodynamic parameters on day 56. After the instrumentation, the concentration of halothane was reduced to 0.5% to record steady-state hemodynamic data. Hemodynamic parameters such as mean blood pressure (MBP), peak LV pressure (LVP), central venous pressure (CVP), LV end-diastolic pressure (LVEDP) and the rate of intra-ventricular pressure rise and decline ($\pm$dP/dt) were recorded as described previously [19]. Two-dimensional echocardiographic studies were performed under 0.5% halothane using an echocardiographic machine equipped with a 7.5-MHz transducer (SSD-5500; Aloka, Tokyo, Japan). M-mode tracings were recorded from the epicardial surface of the right ventricle, and the short axis view of the left ventricle was recorded to measure the LV posterior wall diameter (LVPWD), LV dimension in diastole (LVDd) and LV dimension in systole (LVDs). LV fractional shortening (FS) was calculated using the following formula: $(LVDd - LVDs)/LVDd \times 100$ (%). The study was performed in a blinded manner.

2.5. Analytical methods

After the measurement of myocardial functional analysis, blood was withdrawn for measurement of plasma osmolality, sodium, potassium and plasma aldosterone and atrial natriuretic peptide (ANP). Urine and plasma electrolytes (sodium and potassium) were measured using an electrolyte autoanalyzer (ATWill EA-06, Yokohama, Japan). Plasma and urine osmolality were determined by freezing point depression with an osmometer (Dai-ichi Kagaku OM-6040; Kyoto, Japan). Plasma aldosterone and ANP were determined by standardized RIA and EIA kit, respectively.

2.6. Histopathological analysis

After the performance of myocardial functional analyses, rats were sacrificed and the heart and kidney were excised. The

Table 1 – Histopathology, hemodynamic and echocardiographic parameters in control and CHF rats after 4 weeks treatment with vehicle or tolvaptan

	Control			CHF		
	Vehicle, $n = 5$	Ntol 3, $n = 5$	Ntol 10, $n = 5$	Vehicle, $n = 5$	Tol 3, $n = 6$	Tol 10, $n = 6$
Histopathology						
BW (g)	359 ± 9.7	354 ± 4.5	368 ± 8.1	327 ± 6.8*	324 ± 4.5*	322 ± 10*
HW (g)	0.954 ± 0.04	0.967 ± 0.04	1.003 ± 0.04	1.234 ± 0.06*	1.330 ± 0.04**	1.260 ± 0.06**
H/B (g/kg)	2.652 ± 0.05	2.733 ± 0.07	2.724 ± 0.08	3.78 ± 0.23**	4.11 ± 0.16**	3.92 ± 0.13**
KW (g)	1.14 ± 0.04	1.13 ± 0.02	1.22 ± 0.02	1.11 ± 0.02	1.12 ± 0.03	1.08 ± 0.03
K/B (g/kg)	3.17 ± 0.04	3.20 ± 0.02	3.30 ± 0.05	3.40 ± 0.08	3.50 ± 0.08	3.40 ± 0.06
Area of fibrosis (%)	3.0 ± 0.47	3.0 ± 0.47	3.0 ± 0.47	29 ± 3.3**	20.8 ± 4.5**	20 ± 2.2**
Hemodynamic data						
CVP (mmHg)	2.56 ± 1.1	4.52 ± 2.1	2.77 ± 1.0	7.5 ± 1.3*	5.8 ± 1.0	4.6 ± 0.6
MBP (mmHg)	102 ± 7.4	111 ± 0.7	110 ± 7.7	65 ± 8.4**	72 ± 3.4**	79 ± 4.5*
LVP (mmHg)	126 ± 6.4	133 ± 3.5	136 ± 7.7	82 ± 10**	85 ± 4.4**	93 ± 3.9**
LVEDP (mmHg)	5.57 ± 1.3	8.2 ± 0.3	6.25 ± 0.3	14.0 ± 1.1**	12.1 ± 0.6**	10.8 ± 0.7**
+dP/dt (mmHg/s)	6037 ± 239	6137 ± 308	6729 ± 332	3901 ± 569	4646 ± 316	6004 ± 1100
−dP/dt (mmHg/s)	4986 ± 419	4247 ± 404	4372 ± 301	2775 ± 369	3544 ± 305	4269 ± 894
HR (beats/min)	407 ± 18	437 ± 32	425 ± 16	353 ± 30	330 ± 13.1	350 ± 13
Echo data						
LVPWD (mm)	2.2 ± 0.2	2.2 ± 0.1	2.3 ± 0.2	1.5 ± 0.06**	1.6 ± 0.08**	1.7 ± 0.1*
LVDd (mm)	6.5 ± 0.3	5.6 ± 0.3	6.1 ± 0.3	8.0 ± 0.8*	8.9 ± 0.4**	8.4 ± 0.3**
LVDs (mm)	3.0 ± 0.3	2.4 ± 0.3	2.9 ± 0.4	6.8 ± 0.6**	7.4 ± 0.4**	6.5 ± 0.3**
FS (%)	55 ± 2.9	53 ± 4.3	54 ± 4.7	11 ± 2.7**	16 ± 3.2**	22 ± 2.1**
EF (%)	89 ± 1.8	88 ± 2.8	88 ± 2.8	27 ± 6.0**	38 ± 7.0**	50 ± 3.5**

Results are means ± S.E.; n, number of rats. BW, body weight; HW, heart weight; H/B, ratio of heart weight to body weight; KW, kidney weight; K/B, ratio of kidney weight to body weight; CVP, central venous pressure; MBP, mean blood pressure; LVP, left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; ±dP/dt, rate of intra-ventricular pressure rise and decline; HR, heart rate; LVPWD, left ventricular posterior wall diameter; LVDd, left ventricular dimension in diastole; LVDs, left ventricular dimension in systole; FS, fractional shortening; EF, ejection fraction. Tol 3, rats treated with tolvaptan 3 mg/(kg day); Tol 10, rats treated with tolvaptan 10 mg/(kg day). *$P < 0.05$, **$P < 0.01$ CHF vs. control.

heart and kidney weight (HW and KW, respectively) were measured immediately, and their ratios to BW were calculated. The excised hearts were cut into about 2-mm transverse slices and fixed in 10% formalin. After being embedded in paraffin, several transverse sections were obtained from the ventricle, and stained with the Azan-Mallory staining. The area of myocardial fibrosis was measured quantitatively using a color image analyzer (CIA-102; Olympus, Tokyo, Japan), making use of the differences in the Azan-Mallory stained color (blue fibrotic area opposed to red myocardium). The results were presented as the ratio of the fibrotic area to the whole area of myocardium [19].

2.7. Western blotting analyses

Kidney homogenates were prepared from rats treated as described above for 28 days and age-matched vehicle-treated control rats. For the determination of the protein level of AQP2, equal amounts of protein extracts (30 μg) were separated by 10% SDS-PAGE (Bio-Rad, CA, USA), and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in TBS-T (20 mM Tris, 137 mM NaCl, and 0.05% Tween, pH 7.6) for 3 h, and incubated with AQP2 antibody (diluted 1:500) overnight at 4 °C. After incubation with primary antibody, the bound antibody was visualized with the respective horseradish peroxidase-conjugated secondary antibody (diluted 1:1500) (Santa Cruz Biotechnology Inc., CA, USA) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, UK). The level of GAPDH was estimated in every sample. Films were scanned, and band densities were quantified with densitometric analysis using of the Scion Image program (Epson GT-X700, Tokyo, Japan). Finally, Western blot data were normalized by those for kidney GAPDH.

2.8. Calculations

To clarify the effects of tolvaptan, electrolyte-free water clearance (E-CH_2O) and electrolyte clearance (E-Cosm) were calculated as described previously [22]. The formula used were

$$\text{E-CH}_2\text{O} = \text{UV} - \text{E-Cosm}$$

$$\text{E-Cosm} = \frac{(U_{Na} + U_K)\text{UV}}{P_{Na}},$$

where UV is the urine volume, U_{Na} the urinary sodium concentration, U_K the urinary potassium concentration, and P_{Na} is the plasma sodium concentration. We calculated E-Cosm except the values for plasma potassium concentration, because plasma potassium concentration is low

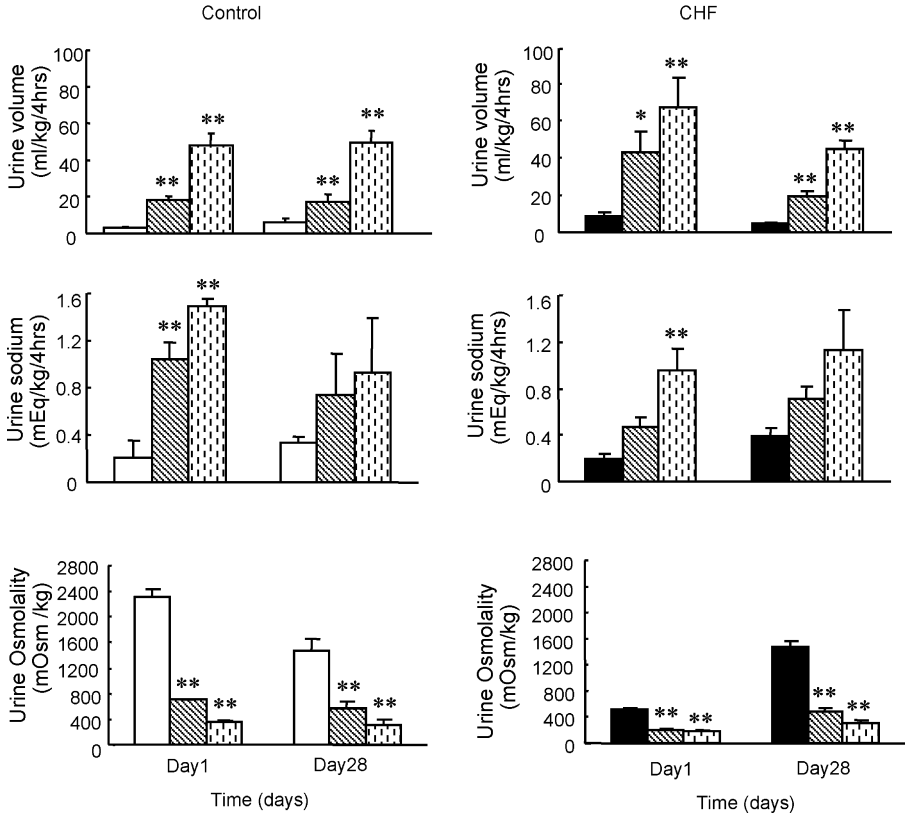

Fig. 1 – Results of metabolic studies to assess V_2R blockade after 1 and 28 days treatment in both control and CHF rats. Rats (n = 15/groups for control; n = 18/groups for CHF) were placed into individual metabolic cages, and urine volume, sodium and osmolality were assessed. Values are expressed as means ± S.E. Open bars, vehicle treated control rats; closed bars, vehicle-treated CHF rats; hatched bars, rats treated with tolvaptan 3 mg/(kg day); dotted bars, rats treated with tolvaptan 10 mg/(kg day). Left panel shows control rats treated with vehicle or tolvaptan, right panel shows CHF rats treated with vehicle or tolvaptan. *P < 0.05; **P < 0.01 vs. vehicle.

enough to be negligible compared with plasma sodium concentration.

2.9. Statistical analysis

All values are expressed as means ± S.E. Statistical analysis of differences between the groups was performed by one-way ANOVA, followed by Tukey's or Bonferroni's method and two-tailed *t*-test when appropriate. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Aquaretic effect of multiple-dosing of tolvaptan in control and CHF rats

Tolvaptan was orally administered once daily at 3 or 10 mg/(kg day) for 28 days. Only one rat died in the vehicle treated CHF group between days 28 and 56 but none of the rats died in the tolvaptan treated and control groups. BW at day 0 (before treatment) did not differ among the groups. However, mean BW at day 1 was significantly reduced ($P < 0.05$) in the CHF rats treated with tolvaptan (10 mg/(kg day)) in comparison with vehicle treated CHF rats. No differences in BW gain were seen between the CHF groups with or without tolvaptan treatment (Table 1) at the end of study period. However, BW was significantly decreased ($P < 0.05$) in rats from CHF groups in comparison to control rats. Fig. 1 shows the metabolic caging parameters such as urine volume, osmolality and sodium excretion in control and CHF rats. V_2R blockade significantly increased urine volume and decreased urine osmolality in control and CHF rats, and these effects were seen during 0–4 h post-dosing on days 1 and 28 (Fig. 1). Chronic V_2R blockade tended to increase the sodium excretion in both control and CHF rats, but the effect did not attain statistical significance. On day 1, the effect on sodium excretion was more in control rats compared to that in CHF rats and the effect was significant in control and CHF rats. Fig. 2 shows a comparison of the effects of tolvaptan on E-CH_2O and E-Cosm between control and CHF rats. On day 28, V_2R blockade markedly elevated E-CH_2O to a positive value in a dose-dependent manner (Fig. 2 right panel). Tolvaptan treatment had no significant effect on E-Cosm in either control or CHF rats (Fig. 2 left panel). Tolvaptan treatment significantly increased urinary AVP excretion in both control and CHF rats, except at a dose of 3 mg/(kg day) on day 28 in control rats (Fig. 3 left and right panel, respectively). It is important to note that, in addition to the constant change in urine osmolality, the constant AVP excretion during the study period further supports the conclusion that repeated administration did not alter the aquaretic effect of tolvaptan (Figs. 1 and 3).

3.2. Histopathology

There were no significant differences in KW and K/B among the groups. The HW ($P < 0.05$) and H/B ($P < 0.01$) were significantly increased in CHF rats compared to control rats. Chronic V_2R blockade had no effect on these parameters in either CHF or control rats (Table 1). The area of fibrosis was significantly larger ($P < 0.01$) in vehicle-treated CHF rats compared to control rats. Although V_2R blockade tended to decrease the fibrotic area compared with that in CHF rats, the effect did not reach statistical significance. There was little or no evidence of fibrosis in the control rats (Table 1).

3.3. Hemodynamic measurements

Although heart rate (HR) and ±dP/dt were not different among the six groups of rats, CVP ($P < 0.05$) and LVEDP ($P < 0.01$) were significantly higher, and MBP and LVP ($P < 0.01$) were significantly lower in vehicle-treated CHF rats in comparison to control rats, indicating systolic and diastolic dysfunction in vehicle-treated CHF rats. In particular, V_2R blockade had no significant adverse or beneficial effect on cardiac function in control or CHF rats (Table 1).

3.4. Echocardiographic assessments

Echocardiographic studies in vehicle-treated CHF rats showed evidence of left ventricular remodeling, with increased LVDd ($P < 0.05$) and LVDs ($P < 0.01$) and reduced FS ($P < 0.01$), indicating impaired systolic function compared with that in control rats. In addition, LV wall of diseased rats is significantly thinner ($P < 0.01$) than that of normal rats. Tolvaptan treatment tended to increase FS and ejection fraction (EF) compared with those in CHF rats, but the effect

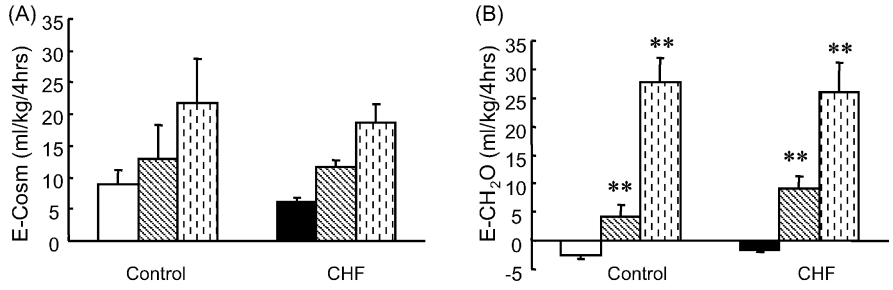

Fig. 2 – Effects of tolvaptan on electrolyte clearance (E-Cosm) (A) and electrolyte-free water clearance (E-CH_2O) (B) in control and CHF rats after 4 weeks treatment. Data are presented as means ± S.E. of 15, and18 animals in control and CHF groups, respectively. Open bars, vehicle treated control rats; closed bars, vehicle-treated CHF rats; hatched bars, rats treated with tolvaptan 3 mg/(kg day); dotted bars, rats treated with tolvaptan 10 mg/(kg day). **$P < 0.01$ vs. vehicle.

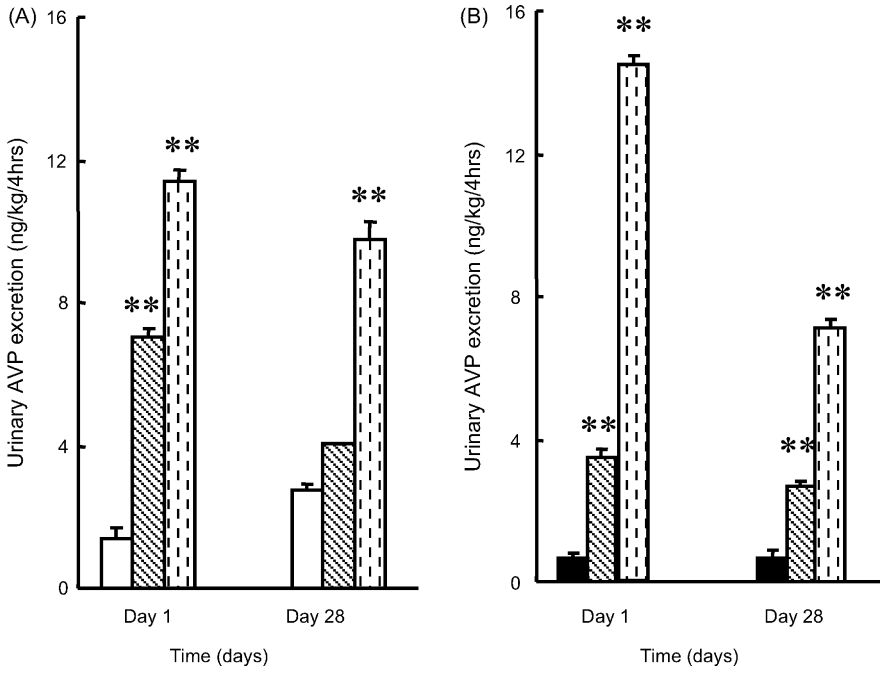

Fig. 3 – Effects of tolvaptan on urinary AVP excretion after 1 and 28 days treatment in control (A) and CHF (B) rats. Values are expressed as means ± S.E. Open bars, vehicle treated control rats; closed bars, vehicle-treated CHF rats; hatched bars, rats treated with tolvaptan 3 mg/(kg day); dotted bars, rats treated with tolvaptan 10 mg/(kg day). **P < 0.01 vs. vehicle.

was not significant. Moreover, treatment with tolvaptan had no significant effect on cardiac geometry or function in either control or CHF rats (Table 1).

3.5. *Biochemical and hormonal data*

Table 2 shows the effects of V_2R blockade on biochemical and hormonal data in control and CHF rats. Plasma aldosterone was significantly elevated ($P < 0.01$) and ANP concentrations tended to be elevated in CHF compared with control rats. These parameters were lowered by V_2R blockade in CHF rats, and the effects attained a statistically significance level only in tolvaptan group (10 mg/(kg day)) with respect to plasma aldosterone ($P < 0.05$). The plasma sodium concentration was significantly decreased ($P < 0.01$) in vehicle treated CHF rats compared to those in control rats. Tolvaptan treatment significantly increased plasma sodium concentration and plasma osmolality in CHF rats as compared to CHF rats treated with vehicle. However, these variables were unchanged by V_2R blockade in control rats.

3.6. *V_2R blockade on AQP2 expression*

Protein extracts of the kidney were immunoblotted and the immunoblots showed a band at 29 kDa, representing AQP2, as well as high molecular weight band between 35 and 45 kDa, which represented the glycosylated protein form of AQP2. The levels of immunoreactivity of the AQP2 protein (29 kDa) and its glycosylated form (35–45 kDa) were significantly increased in CHF rats (6.7- and 4.9-fold, $P < 0.01$, respectively) compared to those in control rats. The increases of APQ2 protein and its glycosylated form in CHF rats were significantly attenuated by the administration of tolvaptan (Fig. 4).

Table 2 – Biochemical and hormonal data in control and CHF rats measured after 4 weeks treatment with vehicle or tolvaptan

	Control			CHF		
	Vehicle, $n = 5$	Ntol 3, $n = 5$	Ntol 10, $n = 5$	Vehicle, $n = 5$	Tol 3, $n = 6$	Tol 10, $n = 6$
Plasma sodium (mequiv./l)	142 ± 2.6	141 ± 5.2	142 ± 2.1	133 ± 1**	139 ± 1.1#	142 ± 1.1##
Plasma potassium (mequiv./l)	6.7 ± 0.5	6.4 ± 0.4	6.8 ± 0.2	6.9 ± 0.4	6.7 ± 0.08	6.2 ± 0.2
Plasma osmolality (mosmol/kg)	315 ± 3	317 ± 2.4	317 ± 1.4	304 ± 7.7	322 ± 3.9#	327 ± 3.9##
Plasma aldosterone (pg/ml)	305 ± 8	ND	ND	464 ± 12**	396 ± 70	362 ± 45#
Plasma ANP (ng/ml)	0.36 ± 0.04	ND	ND	0.51 ± 0.02	0.39 ± 0.08	0.37 ± 0.05

Results are means ± S.E. *n*, number of rats. ANP, atrial natriuretic peptide. ND, not determined. Tol 3, rats treated with tolvaptan 3 mg/(kg day); Tol 10, rats treated with tolvaptan 10 mg/(kg day). **$P < 0.01$ vs. control; #$P < 0.05$; ##$P < 0.01$ vs. CHF.

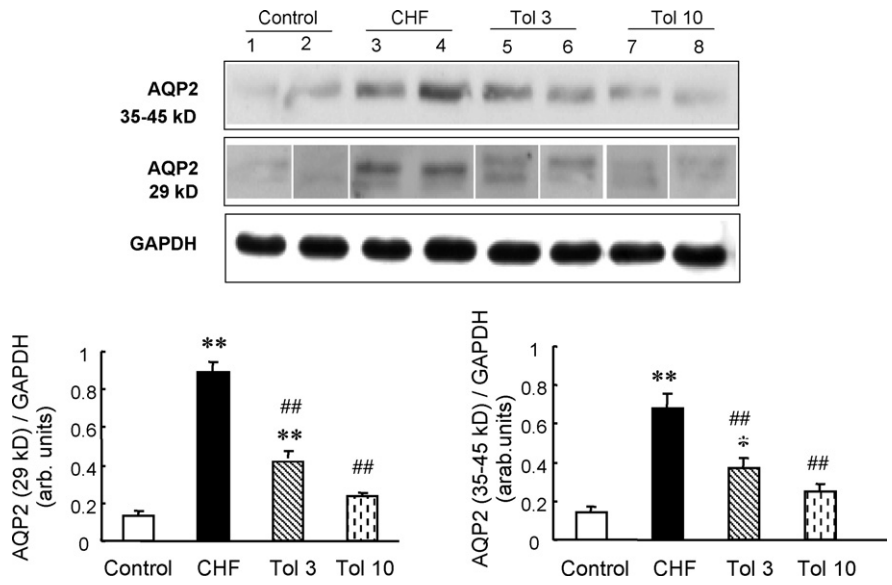

Fig. 4 – Effect of V_2R antagonist, tolvaptan on AQP2 protein expression in CHF rats (top). Western blot analysis for AQP2 protein expressed in rat kidney after 4 weeks of treatment using a polyclonal antibody against AQP2. Thirty micrograms of protein extract was loaded in each lane. (Lanes 1 and 2) control group, rats treated with vehicle; (lanes 3 and 4) CHF group, immunized rats treated with vehicle; (lanes 5 and 6) Tol 3 group, CHF rats treated with tolvaptan 3 mg/(kg day); (lanes 7 and 8) Tol 10 group, CHF rats treated with tolvaptan 10 mg/(kg day). These bands are representative of five separate experiments. Two bands are detectable: a band of 29 and 35–45 kDa corresponding to the predicted molecular mass of AQP2 and its glycosylated form. Densitometric analysis of AQP2 protein (bottom). The mean density value of AQP2 was expressed as a relative ratio to that of GAPDH. Each bar represents means ± S.E. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. CHF.

4. Discussion

The present study indicate that V_2R blockade using the nonpeptide antagonist tolvaptan resulted in significant aquaretic effects that were maintained for 28 days in a rat model of myosin-induced CHF. Chronic V_2R blockade had no natriuretic effect and did not activate RAAS, offering the potential improvement from chronic edema and hyponatremia observed in rats with CHF. Furthermore, V_2R blockade did not aggravate LV dilatation and did not influence cardiac structure or survival in CHF.

Aquaretics have exciting therapeutic potential for the management of patients with water excess and consequent dilutional hyponatremia, as in patients with congestive heart failure, cirrhosis, euvolemic hyponatremia, or with inappropriate ADH secretion [15]. There is an obvious need for a potent V_2R antagonist that can be safely administered orally over the long-term in the clinical setting. Initial investigations were carried out using peptide analogues of AVP-receptor antagonists. Previous reports on chronic blockade of vasopressin receptors by peptide V_2-antagonists did not show persistent aquaresis [23,24], and moreover, further research with these compounds was limited by properties such as poor oral bioavailability, short half-lives, partial agonism, and species-specific activity [25]. The focus of developmental research subsequently shifted to nonpeptide AVP antagonists, OPC-21268 and mozavaptan [14] which showed sufficient oral bioavailability and long-term effects with potential clinical value, further encouraging the development of various nonpeptide AVP antagonists. Tolvaptan was developed through a series of structural modification of mozavaptan. AVP binding studies of this agent reported a 29:1 (V_2:V_{1a}) receptor selectivity in cloned human AVP receptors and 250 times more potent to rat V_2R than to rat V_1R [15]. Similar to the results obtained from studies involving mozavaptan, single and multiple oral dose of tolvaptan have demonstrated significant and sustained dose dependent aquaresis in rats [15,20] and V_2R blockade with this agent augments water excretion without changes in renal hemodynamics or sodium and potassium excretion in human heart failure [26] which in turn prompted us to investigate the effect of tolvaptan in a rat model of CHF after EAM.

The majority of studies using V_2R antagonists have been acute in nature, and the acute effects of an intervention do not necessarily predict its long-term efficacy. In the present study, tolvaptan increased urine volume and decreased urine osmolality in a dose-dependent manner. We calculated E-CH_2O instead of CH_2O. Recently, it was reported that E-CH_2O reflected tonicity balance better than the classic CH_2O in the treatment of electrolyte-abnormal patients [27,28]. In agreement with study reported by Hirano et al. [22], tolvaptan markedly elevated E-CH_2O to a positive value in a dose-dependent manner. In our study, urine volume was decreased only slightly at the end of the treatment period and did not return to the control level, as was seen in the experiments with peptide AVP antagonists and with furosemide and hydrochlorothiazide [29,30]. Furthermore, urine osmolality and urinary AVP excretion showed constant changes throughout the study period, indicating that the aquaretic effect of tolvaptan was unchanged during 28 days of repeated admin-

istration. Tolvaptan was effective in both control and CHF rats, its water diuretic effects were maintained over a long period and the renal effects were accompanied by increases in plasma sodium and osmolality without significant changes in HR, blood pressure or plasma potassium. As a result of aquaresis demonstrated in this report, aquaretics have a potential medical benefit for the treatment of edematous conditions in CHF by removing excess water from the body without activating the RAAS or causing serum electrolyte imbalances.

Chronic V_2R blockade was not associated with any natriuretic effect, suggesting negligible effect on the activity of the RAAS. Plasma aldosterone was decreased by the tolvaptan treatment (10 mg/(kg day)). The suppression of aldosterone with tolvaptan treatment might be caused possibly by raising plasma sodium or AVP may inhibit renin secretion by direct action on the juxtaglomerular apparatus [31]. AVP and the RAAS interact in two major ways. Angiotensin II, the physiologically active component of the RAAS, acts centrally to stimulate the release of AVP which in turn, acts on the kidney to inhibit the secretion of renin. Plasma ANP levels were elevated in CHF rats and were not changed by V_2R blockade, in consistent with negligible improvement of LV dysfunction. Our results are in agreement with previous studies conducted with other V_2R antagonist (mozavaptan) in dogs and rats with congestive heart failure induced through rapid ventricular pacing [14], and coronary artery ligation [32], respectively. Tolvaptan treatment increased urinary AVP excretion in both control and CHF rats. Although we did not measure plasma AVP in these animals, V_2R blockade by tolvaptan has been reported to enhance its level in patients with CHF [2,33]. As AVP is removed from the plasma mainly through renal clearance [34], our observation might have reflected the phenomena occurring in its plasma level. It could also be possible to hypothesize that tolvaptan binding to the V_2 receptors in the kidney operates to increase renal clearance of AVP.

In the present study, hemodynamic and echocardiographic analyses demonstrated LV remodeling with increased LVEDP, LVDs, and LVDd and reduced FS in vehicle-treated CHF rats in comparison to control rats, indicating impaired systolic and diastolic function of the myocardium (Table 1). It has been reported that LV dilatation plays a role in the development of CHF and that the degree of LV enlargement is adversely related to survival in post-infarction patients [35]. This is the first study to assess the effect of a V_2R antagonist (tolvaptan) on cardiac function and survival in a rat model of CHF after EAM. Although chronic V_2R blockade had no significant beneficial effects on cardiac geometry or function in CHF, it is important to note that it had no adverse effects on these parameters (Table 1), nor did it adversely affect survival. We could not ascertain significant improvement in the survival rate between CHF groups (vehicle and tolvaptan treatment), as only one rat died in the vehicle treated CHF group between days 28 and 56 but none of the rats died in the tolvaptan treated groups. Moreover, rats used in this study were very less in number (n = 6/group). Further investigation is warranted to show beneficial effect of tolvaptan on survival with increasing number of animal. By contrast, a study using calcium channel blockers showed aggravation of LV dilatation and reduction of survival in a rat model of chronic myocardial infarction [36]. In keeping with the hemodynamic and echocardiographic findings, no significant effects of tolvaptan on cardiovascular structure or function were seen.

One must be careful in extrapolating the results of studies between different animal models of CHF, between different degrees of heart failure and between different doses and types of AVP antagonists. In contrast to the lack of cardiac structural effects seen in this model of CHF, in a high output aortocaval fistula model of CHF, 4 weeks of V_2R blockade by mozavaptan increased plasma AVP significantly but also improved hemodynamic parameters and reduced right ventricular weight [37]. In an earlier study using the post-infarction model, the benefits of dual V_2/V_1R antagonists depended on the degree of cardiac dysfunction, and increased cardiac output and decreased vascular resistance were only seen in rats with extensive infarcts (>50%) [38].

It has been reported that circulating vasopressin levels correlate directly with AQP2 expression, and has been shown that exogenous administration of vasopressin in normal rats markedly increases AQP2 trafficking on the apical membrane of collecting duct cells (short-term regulation) [8]. In fact, an increase in AQP2 protein levels has been noted in rats with CHF and other diseases of impaired water excretion where the circulating vasopressin level are high (long-term regulation) [39,40]. The latter mode of regulation is also associated with a rise in AQP2 mRNA levels and is most likely due to increased transcription of the AQP2 gene [39]. Defects in the long-term regulation of AQP2 expression have previously been implicated in a wide range of water balance disorders, including central diabetes insipidus [11,41], prolonged hypokalemia [42], hepatic cirrhosis and disorders associated with free-water retention and hyponatremia [43]. The results of those studies make it clear that the water retention associated with severe CHF is due, at least in part, to marked augmentation of long-term regulatory processes. This conclusion is supported by our observation that AQP2 expression was increased only in CHF with reduced plasma sodium concentrations. Recently, similar results demonstrating increased AQP2 levels in the kidneys of rats with CHF have been pointed out in a preliminary report [40]. Our studies are consistent with the view that the observed reduction in plasma sodium is secondary to the increase in AQP2 expression. This was further supported by the observation that rats with mild heart failure (LVEDP 7.2 ± 3.9 mmHg) showed no increase in AQP2 expression, nor did they show any reduction in plasma sodium concentrations [39]. The reduction in plasma sodium concentrations in rats with CHF seems to be correlated with the severity of disease.

Recently several investigators have demonstrated that addition of tolvaptan to the standard therapy in patients with heart failure decreased body weight and edema, corrected hyponatremia and appeared to be well-tolerated with no adverse effects on HR, blood pressure, electrolytes, neurohormonal activation or renal function [33,44,45]. Our results are in agreement with their reports that blockade of V_2 receptors by tolvaptan in a rat model of CHF, resulted in significant aquaretic effect, followed by an increase in plasma sodium and osmolality that were not associated with activation of RAAS and significantly down regulated AQP2 protein expression. We propose that AVP V_2R antagonism

seems to be the promising tool in the management of water retention that characterizes heart failure. Further investigation is warranted to define the role of V_2R blockade in a rat model of CHF after autoimmune myocarditis with increasing number of animals.

Acknowledgments

We thank Gurusamy Narasimman, Rajarajan A.T., Sayaka Mito, and Kunisaki Megumi for their assistance in this research work. We express our sincere thank to Dr. Hitoshi Hama and Mr. Mamoru Koike (Kido Hospital, Niigata, Japan) for providing facility and helping in the determination of electrolytes and to Otsuka Pharmaceutical Co. (Tokushima, Japan) for measuring plasma aldosterone and ANP. This research was supported by a Yujin Memorial Grant; The Ministry of Education, Science, Sports and Culture, Japan and by a grant from the Promotion and Mutual Aid Corporation for Private Schools, Japan.

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