Torasemide, a long-acting loop diuretic, reduces the progression of myocarditis to dilated cardiomyopathy

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Abstract

Torasemide is a long-acting loop diuretic that combines the effects of both furosemide and spironolactone. It has been reported that torasemide may block the renin–angiotensin–aldosterone system and therefore it might attenuate myocardial remodeling accompanied by left ventricular dysfunction. However, nothing is known about the effect of torasemide on myocardial remodeling in a rat model in which myosin-induced experimental autoimmune myocarditis might develop into dilated cardiomyopathy. Experimental autoimmune myocarditis was elicited in Lewis rats by immunization with porcine cardiac myosin. Twenty-eight days after immunization, we investigated the effects of torasemide on metabolic and neurohumoral parameters, cardiac fibrosis and remodeling in experimental autoimmune myocarditis rats. Diuresis was increased dose-dependently by torasemide; the urinary potassium and sodium excretion was significantly decreased and increased, respectively. Myocardial functional parameters measured by hemodynamic and echocardiographic studies were significantly improved by torasemide treatment in a dose-dependent manner. The area of fibrosis, myocyte size and the myocardial protein levels of transforming growth factor-beta1, collagen III, and aldosterone synthase were significantly decreased, and the sarcoplasmic reticulum Ca2+ ATPase2 protein level was significantly increased by torasemide treatment. Moreover, the plasma levels of angiotensin II and aldosterone were increased and atrial natriuretic peptide was decreased in a dose-dependent manner. Our results indicate that torasemide treatment significantly improved left ventricular function and ameliorated the progression of cardiac remodeling beyond its renal effects in rats with chronic heart failure after experimental autoimmune myocarditis.

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1. Introduction

Chronic heart failure is a disorder often characterized by decreased cardiac output and arterial pressure, left ventricular dysfunction and activation of sodium- and water-retaining hormones, including regulators of the sympathetic nervous system and the renin–angiotensin–aldosterone system (RAAS). Myocarditis often progresses to dilated cardiomyopathy, a major cause of heart failure. In our study, we used a rat model of myosin-induced experimental autoimmune myocarditis, in which the heart transits from an acute phase (inflammatory myocarditis) to a chronic phase (remodeling and dilated cardiomyopathy). Experimental autoimmune myocarditis of rats mimics human idiopathic giant cell myocarditis (Kodama et al., 1990, 1994). Giant cell myocarditis is a fatal disorder, often leads to heart failure or arrhythmias (Cooper, 2003). Dilated cardiomyopathy is recognized as a significant cause of morbidity and mortality. Treatment strategies of dilated cardiomyopathy vary due to its diverse etiopathology ranging from...
myocardial infarction, myocarditis (bacterial, viral, parasitic and autoimmune) and pressure overload etc. Diuretic therapy is a core element in the treatment of chronic heart failure because diuretics relieve cardiac load by reducing water retention. Torasemide, (1-isopropyl-3-{[4-(3-methyl-phenylamino)pyridine]-3-sulfonyl}urea) is a novel diuretic whose chemical structure combines features of a loop diuretic and Cl⁻ channel blocker (Ghys et al., 1985a; Uchida et al., 1991c; Wittner et al., 1987).

Torasemide is reportedly more effective than furosemide in chronic heart failure with respect to reducing symptoms, admissions and other adverse cardiovascular events (Murray et al., 2001; Spannheimer et al., 1998). Recently, it has been reported that use of torasemide in the TORIC study was associated with lower mortality than furosemide (Cosin and Diez, 2002), which suggests that torasemide has beneficial effects other than diuresis in patients with chronic heart failure. The difference in cardiac death between these two diuretics has been suggested to depend on the anti-aldosteronergic effect of torasemide. It exerts its action at the ascending limb of the loop of Henle, where it interacts with the Na⁺, 2Cl⁻, K⁺ co-transporter localized in the luminal surface (Greger, 1988; van Zwieten, 1992; Wittner et al., 1986). It has been shown that torasemide elicits longer-lasting diuresis than furosemide and other diuretics (Ghys et al., 1985a; Uchida et al., 1991b).

RAAS affects myocardial fibrosis and plays an important role in left ventricular remodeling (Weber and Brilla, 1991a). Recently it has been reported that torasemide, but not furosemide may block RAAS (Goodfriend et al., 1998; Uchida et al., 1991c; Yamato et al., 2003) and therefore it might attenuate myocardial remodeling accompanied by cardiac dysfunction. Interestingly, it has been shown that transcardiac extraction of aldosterone is reduced in chronic heart failure patients treated with torasemide (Tsutamoto et al., 2004) and that torasemide blocks the binding of the hormone to its mineralocorticoid receptor (Goodfriend et al., 1998; Uchida et al., 1991c; Yamato et al., 2003), and even the demonstration is made that cardiac fibrosis was decreased in patients with chronic heart failure treated with torasemide (Lopez et al., 2004). Our animal model, a non-viral autoimmune myocarditis dilated cardiomyopathy with diffusely distributed fibrosis, besides a cellular immunity and inflammation mediated disease (Kodama et al., 1994; Watanabe et al., 2000) offers suitable setting to evaluate the effects of torasemide on metabolic parameters and left ventricular function using hemodynamic and echocardiographic parameters, neurohumoral factors such as plasma angiotensin II, aldosterone and atrial natriuretic peptide (ANP) and protein levels of marker molecules of cardiac remodeling in a rat model of chronic heart failure after experimental autoimmune myocarditis.

2. Materials and methods

2.1. Animals and experimental protocol

All studies were carried out using 8-week-old male Lewis rats weighing about 230–250 g (Charles River Japan Inc., Kanagawa, Japan). Eight-week-old male Lewis rats were injected in the footpads with antigen-adjuvant emulsion according to the procedure described previously (Kodama et al., 1990; Watanabe et al., 2000). The morbidity of experimental autoimmune myocarditis was achieved 100% in rats immunized by this method. Rats immunized with myosin become ill and immobile on day 14, and their activity gradually recovered beginning at the fourth week. Twenty-eight days after immunization, the 54 surviving rats were divided into four groups and received oral administration (p.o.) of torasemide (0.3 mg/kg/day, group T0.3, n = 12; 3 mg/kg/day, group T3, n = 14; 10 mg/kg/day, group T10, n = 13), or vehicle (0.1 mol Na₂CO₃/0.1 mol HCl and saline, group V, n = 15) for 28 days. Untreated age-matched Lewis rats were used as a normal control (group N, n = 10). The doses used in the experiments were determined on the basis of earlier reports (Uchida et al., 1991c, 1994). Throughout the study, all animals were cared for in accordance with the guidelines of our institute (Watanabe et al., 2000).

2.2. Chemicals

Unless otherwise stated all reagents were of analytical grade and were purchased from Sigma (Tokyo, Japan). Torasemide was provided by Taishotoyama pharmaceutical Co., Ltd (Toshima-Ku, Tokyo, Japan).

2.3. Diuretic study

The animals were placed individually in metabolic cages, and urine samples were collected for 5 and 24 h after drug administration on days 1, 7, 14 and 28. The urinary volume and sodium (Na⁺) and potassium (K⁺) concentrations was measured using an electrolyte autoanalyzer (ATWill EA-06, Yokohama, Japan).

2.4. Hemodynamic and echocardiographic studies

Rats were anesthetized with 2% halothane in O₂ 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, peak left ventricular pressure, central venous pressure, left ventricular end-diastolic pressure and the rate of intra-ventricular pressure rise and decline (±dP/dt) were recorded as previously described (Watanabe et al., 2000). 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 left ventricular dimension in diastole and left ventricular dimension in systole. Left ventricular fractional shortening was calculated as diastolic dimension minus systolic dimension divided by diastolic dimension, expressed as a percentage. The study was performed in a blinded manner.
2.5. Histopathological analysis

After the measurement of hemodynamic and echocardiographic parameters, hearts were excised and weighed immediately, and the heart weight to body weight ratio was 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 Azan-Mallory staining. The area of myocardial fibrosis was measured quantitatively by a color image analyzer (CIA-102; Olympus, Tokyo, Japan), using the differences in color (blue fibrotic area opposed to red myocardium) of the photomicrographs of Azan-Mallory stained slides. The results are presented as the ratio of the fibrotic area to the whole area of the myocardium (Watanabe et al., 2000).

Using hematoxylin and eosin sections, myocyte diameter measurements were performed in 10 myocytes selected per field, in 400-fold magnification by light microscopy. Short axis diameters of each myocyte were measured from the hearts of all groups of rats. Each average value was obtained based on the data from 10 myocytes and was used as an independent sampling data.

2.6. Estimation of neurohumoral parameters

Blood samples were collected by heart puncture immediately after echocardiographic measurements, and were transferred into a chilled glass tube containing 0.25 ml of 125 mM EDTA and 25 mM o-phenanthroline for the purpose of subsequent determinations of plasma angiotensin II, aldosterone and ANP by standardized radioimmunoassay (RIA) (Yamaguchi, 1981) and EIA kit, respectively. Plasma K+ concentration was measured using an electrolyte autoanalyzer (ATWill EA-06, Yokohama, Japan).

2.7. Immunohistochemical assay

Formalin-fixed, paraffin-embedded cardiac tissue sections were used for immunohistochemical staining. After deparaffinization and hydration, the slides were washed in Tris-buffered saline (TBS; 10 mM/l Tris HCl, 0.85% NaCl, pH 7.5) containing 0.1% bovine serum albumin (BSA). Endogenous peroxidase activity was quenched by incubating the slides in methanol and 0.6% H2O2 in methanol. To perform antigen retrieval, the sections were pretreated with trypsin for 15 min at 37 °C. After overnight incubation with the primary antibody, i.e., goat polyclonal anti-collagen III antibody (diluted 1:100) (Santa Cruz Biotechnology Inc. CA, USA) at 4 °C, the slides were washed in TBS and horseradish peroxidase (HRP)-conjugated rabbit antigoat secondary antibody was then added and the slides were further incubated at room temperature for 45 min. The slides were washed in TBS and incubated with diaminobenzidine tetrahydrochloride as the substrate, and counterstained with hematoxylin. A negative control without primary antibody was included in the experiment to verify the antibody specificity. Measurement of myocardial immunoreactivity for collagen III was performed in 100 randomly selected fields in heart sections in 400-fold magnification by light microscopy.

2.8. Western blotting

Ventricular homogenates were prepared from rats treated as described above for 28 days and age-matched untreated normal control rats. For the determination of protein levels of sarcoplasmic reticulum Ca2+ ATPase (SERCA2), aldosterone synthase (CYP11B2), transforming growth factor (TGF) β1, and collagen III, equal amounts of protein extracts (30 μg) were separated by 7.5%, 10%, 15% or 7.5% SDS-polyacrylamide gel electrophoresis (Bio-Rad, CA, USA), respectively, and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 1% nonfat dry milk and 1% BSA (Sigma, Saint Louis, USA) in TBS-T (20 mM/l Tris, pH 7.6, 137 mM/l NaCl, and 0.05% Tween). All antibodies were purchased from Santa Cruz Biotechnology Inc. CA, USA aside from CYP11B2 (Chemicon International, CA, USA), and used at a dilution of 1:1000. After incubation with primary antibody, the bound antibody was visualized with the respective HRP-conjugated secondary antibodies (Santa Cruz Biotechnology Inc. CA, USA) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, UK). The level of cardiac glyceraldehyde-3-
phosphate dehydrogenase (GAPDH) was estimated in every sample. Films were scanned, and band densities were quantified with densitometric analysis using Scion Image program (Epson GT-X700, Tokyo, Japan). Finally, Western blot data were normalized with cardiac GAPDH.

2.9. Quantitative RT-PCR to detect matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1)

Total RNA was extracted from the heart tissues with Trizol (Gibco BRL) according to the standard protocol and reverse transcribed. Thereafter, cDNA was amplified using the ABI 7700 sequence-detector system (Applied Biosystems, Foster City, CA) with a set of primers and probes corresponding to MMP-9, TIMP-1 and GAPDH as previously described (Yndestad et al., 2004).

Except in the metabolic caging study, the above protocols were not carried out in normal treated rats because no changes in myocardial functional parameters were observed in them (data not shown).

3. Results

3.1. Clinical course

Four (27%) and two (17%) of 15 and 12 rats in groups V and T0.3, respectively, died between days 28 and 56 (Fig. 1A, Table 1). None of the rats died in groups T3, T10 or N (Fig. 1A, Table 1). The 56-day survival rate was significantly higher in groups N, T3 and T10 than in group V ($P < 0.01$).

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Survival rate (%)</th>
<th>Body weight (g)</th>
<th>Heart weight (g)</th>
<th>Area of fibrosis (%)</th>
<th>CVP (mm Hg)</th>
<th>MBP (mm Hg)</th>
<th>Peak LVP (mm Hg)</th>
<th>LVEDP (mm Hg)</th>
<th>+dP/dt (mm Hg/s)</th>
<th>−dP/dt (mm Hg/s)</th>
<th>HR (beats/min)</th>
<th>LVDd (mm)</th>
<th>LVDs (mm)</th>
<th>FS (%)</th>
<th>EF (%)</th>
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<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>100</td>
<td>411±7.0</td>
<td>1.0±0.05</td>
<td>3.0±0.5</td>
<td>2.75±0.70</td>
<td>328±18</td>
<td>6.8±0.16</td>
<td>3.8±0.24</td>
<td>45.6±2.54</td>
<td>89±1.02</td>
<td>110±2.6</td>
<td>95±4.00</td>
<td>11.1±1.4p</td>
<td>8.1±0.18b</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>15</td>
<td>73b</td>
<td>316±5.9b</td>
<td>1.49±0.09b</td>
<td>39.2±5.9b</td>
<td>6214±383</td>
<td>3884±333</td>
<td>8.1±0.18b</td>
<td>6.8±0.28b</td>
<td>16±1.55b</td>
<td>45.6±2.57</td>
<td>328±18</td>
<td>341±13</td>
<td>16±1.55b</td>
<td>16±1.55b</td>
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</tr>
<tr>
<td>T0.3</td>
<td>12</td>
<td>83</td>
<td>299±6.6</td>
<td>1.09±0.06d</td>
<td>11.7±2.5d</td>
<td>4957±562</td>
<td>5469±733</td>
<td>306±7.0</td>
<td>5.6±0.69</td>
<td>25±2.8</td>
<td>45.6±2.57</td>
<td>328±18</td>
<td>341±13</td>
<td>16±1.55b</td>
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<tr>
<td>T3</td>
<td>14</td>
<td>100d</td>
<td>273±5.6d</td>
<td>3.65±0.22d</td>
<td>10±1.1d</td>
<td>5013±454</td>
<td>5781±635</td>
<td>343±9.0</td>
<td>6.51±0.36d</td>
<td>31±2.3d</td>
<td>45.6±2.57</td>
<td>328±18</td>
<td>341±13</td>
<td>16±1.55b</td>
<td>16±1.55b</td>
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</tr>
<tr>
<td>T10</td>
<td>13</td>
<td>100d</td>
<td>269±3.0d</td>
<td>3.40±0.14d</td>
<td>7.2±1.4d</td>
<td>4895±232</td>
<td>6171±454</td>
<td>316±13</td>
<td>6.47±0.4d</td>
<td>36±0.9d</td>
<td>45.6±2.57</td>
<td>328±18</td>
<td>341±13</td>
<td>16±1.55b</td>
<td>16±1.55b</td>
<td></td>
</tr>
</tbody>
</table>

Results are presented as the mean±S.E.M. H/B, ratio of heart 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; LVDd, left ventricular dimension in diastole; LVDs, left ventricular dimension in systole; FS, fractional shortening; EF, ejection fraction. Group N, age-matched untreated rats; group V, chronic heart failure rats treated with vehicle; groups T0.3, T3 and T10, chronic heart failure rats treated with torasemide (0.3, 3 and 10 mg/kg/day) respectively. *$P < 0.05$, **$P < 0.01$ vs group N; †$P < 0.05$, ‡$P < 0.01$ vs group V.
3.2. Diuretic action

Throughout the experiment, torasemide (3 and 10 mg/kg/day) significantly increased the urine volume during the 5 h post dosing in both normal and rats with chronic heart failure (Fig. 2A, B). At 0.3 mg/kg/day, the urine volume remained constant throughout the study. In rats with chronic heart failure (Fig. 2B), torasemide (3 and 10 mg/kg/day) significantly increased the urine volume (approximately 1.5-fold) in comparison with that in normal rats (Fig. 2A). The 24 h urine volume excretion with torasemide (3 and 10 mg/kg/day) was significantly increased (approximately 1.7-fold) in rats with chronic heart failure in comparison with that in normal rats.

Sodium retention and potassium elevation were observed in group V in comparison to group N. The 5 h urine Na⁺ and K⁺ excretion was significantly increased and decreased, respectively, with torasemide compared to that in group V (Fig. 3A, B), and therefore the urinary sodium to potassium (Na⁺/K⁺) ratio increased (Fig. 3C). The decrease of urinary K⁺ excretion by torasemide was relatively slight compared to the increase in urinary Na⁺ excretion, and plasma K⁺ increased in dose-dependent manner that was not significant compared that in group V (Fig. 3D).

3.3. Effect of torasemide on body weight and neurohumoral parameters

The efficacy of torasemide treatment can be assessed by measuring body weight. Throughout the study period, body weight was significantly decreased in all groups of rats than those in group N rats. Body weight was decreased by torasemide treatment in rats with chronic heart failure on day 7. Following day 7, body weight loss was significantly higher at a dose of 3 and 10 mg/kg/day in comparison with that in vehicle-treated rats (Fig. 1B). The reduction of body weight by torasemide treatment was a consequence of increased urine volume excretion and natriuresis throughout the study period.

The plasma angiotensin II, aldosterone and ANP concentrations were significantly increased in group V in comparison to group N. Treatment with torasemide significantly elevated plasma concentrations of angiotensin II and aldosterone and
decreased ANP concentration in comparison to those in group V (Fig. 4A–C).

3.4. Effect of torasemide on myocardial functions

Although heart rate was not different among the five groups of rats, central venous pressure and left ventricular end-diastolic pressure were significantly higher (3.1 ± 0.6 vs 0.34 ± 0.3 mm Hg \( P < 0.01 \); 11.1 ± 1.4 vs 2.75 ± 0.7 mm Hg \( P < 0.01 \), respectively), and mean blood pressure, peak left ventricular pressure and ±dP/dt were significantly lower in group V in comparison to group N (81 ± 7.2 vs 95 ± 2.3 mm Hg \( P < 0.05 \); 95 ± 4 vs 110 ± 2.6 mm Hg \( P < 0.05 \); 3816 ± 317 vs 6214 ± 383 mm Hg/s \( P < 0.01 \); and 3884 ± 333 vs 5818 ± 333 mm Hg/s \( P < 0.01 \), respectively), indicating systolic and diastolic dysfunction in vehicle-treated rats. Central venous pressure and left ventricular end-diastolic pressure were significantly decreased in groups T3 and T10 compared to those in group V. However, mean blood pressure, peak left ventricular pressure and ±dP/dt were not improved by the treatment. Torasemide improved ±dP/dt dose-dependently, but the effect was significant only in group T10 in terms of −dP/dt. Although hemodynamic parameters tended to be improved in group T0.3 in comparison to group V, the effect did not attain statistical significance (Table 1).

Echocardiographic data revealed that both left ventricular diastolic and systolic dimension were increased in group V compared to group N (8.1 ± 0.18 vs 6.8 ± 0.16 mm \( P < 0.01 \); 6.8 ± 0.28 vs 3.8 ± 0.24 mm \( P < 0.01 \), respectively). In addition, left ventricular systolic function, as assessed by fractional shortening, was also reduced in group V compared to that in group N (16 ± 1.55 vs 45.6 ± 2.57% \( P < 0.01 \)). The increases in both left ventricular diastolic and systolic dimension were significantly attenuated in groups T3 and T10, since the fractional shortening was significantly increased. Although low-dose (0.3 mg/kg/day) torasemide treatment reduced those parameters (left ventricular diastolic and systolic dimension) and increased fractional shortening compared to those in group V, the effects did not attain statistical significance, whereas ejection fraction was significantly increased in all treatment groups (Table 1).

3.5. Effect of torasemide on histopathology and myocardial levels of collagen III assessed by immunohistochemistry

Heart weight and the ratio of heart weight to body weight were significantly larger in group V than in group N rats (1.49 ± 0.09 vs 1.0 ± 0.05 g \( P < 0.01 \); 4.71 ± 0.27 vs 2.51 ± 0.10 g/kg \( P < 0.01 \), respectively). Torasemide treatment significantly reduced heart weight and the ratio of heart weight to body weight in a dose-dependent manner, and heart weight was significantly lower in groups T3 and T10 than in groups V and T0.3, in which it was comparable to that in group N rats (Table 1).

The hearts from group V rats showed massive fibrosis compared to those from group N rats (Fig. 5A). The area of fibrosis was significantly and dose-dependently decreased by torasemide treatment compared to that in group V. Among the four treatment groups, the area of fibrosis was lowest in group T10 (Figs. 5A and 6A, Table 1). The myocyte size in group V was significantly larger than that in group N (Figs. 5B and 6B). Torasemide treatment significantly reduced myocyte size in a
dose-dependent manner compared to those in group V. The myocyte size was significantly lower in groups T3 and T10 than in groups V and T0.3, in which it was comparable to that in group N rats (Figs. 5B and 6B).

Myocardial immunoreactivity for collagen III was little or absent in the hearts of group N. Midventricle sections of group V showed stronger immunoreactivity for collagen III than those of group N (Fig. 5C). Immunohistochemical analysis of the torasemide groups revealed a significant and dose-dependent decrease in the myocardial level of collagen III (Figs. 5C and 6C).

### 3.6. Collagen III, TGF β1, CYP11B2 and SERCA2 protein levels assessed by Western blotting

Western blotting showed that collagen III, TGF β1 and CYP11B2 protein levels were upregulated in vehicle-treated rats (5.4, 2.9 and 2.8-fold, respectively) compared to group N rats. Treatment with torasemide significantly decreased the myocardial levels of collagen III, TGF β1 and CYP11B2 protein. The protein level of SERCA2 in the myocardium was decreased (2.5-fold)

![Western blot images](image-url)
in vehicle-treated rats, and treatment with torasemide prevented the downregulation of SERCA2, with significant effects in groups T3 and T10 compared with vehicle-treated rats (Fig. 7).

3.7. MMP-9 and TIMP-1 mRNA levels assessed by RT-PCR

Rats with chronic heart failure had an upregulated expression of MMP-9 and reduced levels TIMP-1 mRNA in comparison to group N, and the treatment with torasemide significantly reversed the myocardial mRNA levels of MMP-9 and TIMP-1 in rats with chronic heart failure (Fig. 8).

4. Discussion

The results of present study demonstrated that treatment with oral torasemide improved both systolic (+dP/dt, % ejection fraction and % fractional shortening) and diastolic (−dP/dt and left ventricular end-diastolic pressure) function and rate of survival, and caused a diuretic effect along with a reduction in body weight, changes in neurohormonal parameters such as plasma angiotensin II, ANP and aldosterone, as well as decreased myocardial remodeling and decreases of its marker molecules. Left ventricular systolic and diastolic dysfunction is common in patients with dilated cardiomyopathy and is related to cardiac symptoms and prognosis (Rihal et al., 1994). In the present study, hemodynamic and echocardiographic analyses were used to assess the systolic and diastolic function of the myocardium (Table 1). Although improvement in the myocardial functional parameters was not so evident in group T0.3 compared with group V, improvement in neurohormonal factors and cardiac remodeling (fibrosis and hypertrophy) was seen in group T0.3 (Figs. 4 and 5, Table 1).

Torasemide is a novel diuretic which has a potent and long-lasting diuretic action (Uchida et al., 1991b), which is achieved by inhibiting the reabsorption of water and electrolytes in the distal tubules, including loop of Henle (Greger, 1988; Uchida et al., 1991a; Wittner et al., 1986). Although torasemide is classified as a loop diuretic, like furosemide, bumetanide and piretanide, Ghys et al. (1985b) have shown that i.v. injection of torasemide produces less kaliuresis than does furosemide at doses that cause an equivalent level of natriuresis and diuresis in anesthetized rats. In the present study, orally administered torasemide did not increase the urinary K⁺ excretion at the dosages used. Indeed, it increased both urine volume (approximately 1.5-fold in comparison with that in normal rats treated with torasemide) and Na⁺ excretion (Figs. 2B and 3A) in rats with chronic heart failure. Our data support earlier results (Boesken and Kult, 1997; Patterson et al., 1994) in that the change of fractional K⁺ excretion was considerably smaller (Fig. 3B) than that of Na⁺ excretion (Fig. 3A). Along with enhanced diuresis, there was a relevant reduction in body weight (Fig. 1B), increased natriuresis and improvement in the rate of survival confirming the effectiveness of torasemide in rats with chronic heart failure.

Myocardial fibrosis probably plays an important role in both diastolic and systolic dysfunction (Weber et al., 1993) and has adverse clinical consequences that result in increases in deaths caused by progressive heart failure. It has been proposed that increase in myocardial fibrosis during heart failure is due to both increased collagen synthesis by fibroblasts and uncharged or decreased fibrillar collagen degradation (Weber and Eghbali, 1991b). Myocardial fibrosis, the hallmark of dilated cardiomyopathy, is observed in dilated cardiomyopathic hearts as indicated by Azan–Mallory staining and increased its marker molecules (TGF β1 and collagen III) (Figs. 5 and 7). Taking into account of myocardial collagen turnover and half-life in rodents (Laurent, 1987), it is likely that torasemide is stimulating degradation of collagen more than inhibiting its synthesis (Figs. 5 and 7). Although this possibility is not supported by human data (Lopez et al., 2004), assessment of matrix metalloproteinases (MMPs) and their inhibitors would provide more information.

The myocardial extracellular matrix is a complex network and its balance determines the structural integrity of the heart. Alteration in the matrix degradation system caused by inflammatory mediators, oxygen species and neurohormonal reaction leads to an impairment of left ventricular function as seen in myocarditis and inflammatory cardiomyopathy (Rutschow et al., 2006). The imbalance of the matrix degrading system with induced expressions of MMPs and plasminogen activators as well as the reduced expressions of tissue inhibitors of MMPs (TIMPs) leads to a pathologic collagen turnover, with the loss of structural integrity of the heart and an impairment of left ventricular function (Rutschow et al., 2006). Interestingly, we could observe an increase in myocardial mRNA levels of MMP-9 and decrease in TIMP-1 mRNA levels in rats with dilated cardiomyopathy and these changes in mRNA levels of MMP-9 and TIMP-1 were significantly reversed by torasemide treatment.
The results of Fig. 5 are in agreement with the previous studies in which the cardiac collagen metabolism is deeply modified by torasemide (Lopez et al., 2004; Rutschow et al., 2006).

For instance, a numerous findings suggest that aldosterone may play an important role in myocardial fibrosis, which leads to left ventricular remodeling and results in left ventricular dysfunction (Delcayre and Swynghedauw, 2002; Weber and Brilla, 1991a; Weber et al., 2003). Recently, it has been reported that aldosterone is produced in the ventricle of the failing human (Mizuno et al., 2001) and rat hearts (Silvestre et al., 1998). Additionally, CYP11B2 is detected in the hearts of several species, including rats (Silvestre et al., 1999) and humans (Satoh et al., 2002). Interestingly, it has been shown that torasemide, but not furosemide, interferes with secretion and receptor-ligand binding of aldosterone (Goodfriend et al., 1998; Uchida et al., 1991c; Yamato et al., 2003). Furthermore, rats receiving a 7-day course of torasemide had higher plasma aldosterone concentrations (Shinyama et al., 1996), and similar findings have been reported in humans (Yamato et al., 2003). In the present study, we also observed that the plasma angiotensin II and aldosterone concentration were significantly increased (Fig. 4A, B) along with a significant reduction in CYP11B2 protein levels (Fig. 7). The increase in angiotensin II is due to decreased circulatory blood volume, while the increase in aldosterone concentration is due to prevention of binding of circulatory aldosterone to its receptor (Uechi et al., 2003). Therefore, the possibility exists that the ability of torasemide to decrease myocardial fibrosis and its marker molecules (TGF β and collagen III) may also be related to interference with humoral profibrotic factors such as aldosterone (Delcayre and Swynghedauw, 2002; Weber et al., 2003) and angiotensin II (Gonzalez et al., 2002). On the other hand, it has been reported that, in vitro, torasemide, but not furosemide, inhibits angiotensin II-stimulated signaling pathways in vascular smooth muscle cells (VSMCs), whereas furosemide does not (Fortuno et al., 1999; Muniz et al., 2001).

Alternatively, it has been reported that in patients with chronic heart failure, torasemide may stimulate the release of antifibrotic humoral factors such as prostacyclin to a greater extent than does furosemide (Liguori et al., 1999). From our results it can be suggested that torasemide exerts antifibrotic effect through interference with RAAS, which results in improvement of survival rate. This is supported by changes in parameters assessing cardiac function (Table 1). Among the non-renal actions of torasemide that may also involved in the beneficial effects of this compound on left ventricular remodeling in heart failure is the reduction of cardiac sympathetic activity (Kasama et al., 2006). However, we recommend further studies are required to investigate the precise mechanism of the antifibrotic effects of torasemide.

In the failing heart, abnormal intracellular Ca2+ handling may lead to cardiac dysfunction associated with a variety of structural and biochemical abnormalities (Morgan et al., 1990). Several investigators have demonstrated that in cardiac hypertrophy or in the failing heart, Ca2+ uptake by the sarcoplasmic reticulum is decreased in conjunction with a decreased density of SERCA (Cory et al., 1993; Linck et al., 1996). The Ca2+-ATPase of the SERCA is chiefly responsible for regulating cellular Ca2+ concentration in cardiac myocytes during the excitation–contraction cycle. Recently, it has been reported that a novel transgenic rat model with over expression of sarcoplasmic reticulum Ca2+ ATPase improves reticular Ca2+ handling in normal and diabetic rat hearts (Vetter et al., 2002). In this study, we observed a reduction of the level of SERCA2 that was significantly improved by torasemide (3 and 10 mg/kg/day), while low-dose torasemide caused a smaller increment (Fig. 7). This action can be related to the ability of torasemide to block the increase of Ca2+ induced by angiotensin II in VSMCs (Fortuno et al., 1999; Muniz et al., 2001).

Plasma ANP concentration is a useful prognostic indicator in patients with chronic heart failure, and is documented to be elevated in cardiac hypertrophy or failure (Falcao et al., 2004; Silberbach and Roberts, 2001). Vehicle-treated rats had developed cardiac hypertrophy and left ventricular dilation, evidenced by an increase in ANP levels, myocyte size, heart weight, heart weight to body weight ratio, left ventricular diastolic and systolic dimension, and decrease in fractional shortening (Figs. 4 and 5, Table 1). In the torasemide (3 and 10 mg/kg/day) treated rats a significant reduction in those parameters and increase in the fractional shortening have been observed. In the low-dose torasemide group, those parameters (left ventricular diastolic dimension, left ventricular systolic dimension and fractional shortening) were not different from vehicle-treated rats but ANP levels, heart weight and the ratio heart weight to body weight were significantly decreased. These results indicated that torasemide improves myocardial systolic and diastolic function, and attenuates abnormal cardiac hypertrophy. However, the optimal dose of torasemide for the amelioration of chronic heart failure induced by experimental autoimmune myocarditis in rats has yet to be determined. The absence of information regarding comparative effect of torasemide and furosemide or spironolactone in rats with chronic heart failure can be considered as a limitation of the present study.

In conclusion, our work demonstrated that torasemide improved left ventricular function (ejection fraction, fractional shortening, left ventricular contractility and relaxation) and attenuated myocardial remodeling (fibrosis and hypertrophy) in rats with chronic heart failure after experimental autoimmune myocarditis. These effects were associated with inhibited myocardial levels of TGF β1, collagen III and aldosterone synthase and increased levels of SERCA2 protein.

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