Molecular Effects of M17055, Furosemide and Thiazide on Cardiac Hypertrophy of Spontaneously Hypertensive Rats

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ABSTRACT

Although diuretics have been clinically shown to reduce cardiovascular morbidity and mortality, the effects of diuretics on cardiac hypertrophy are poorly understood. In this study, we examined the molecular effects of diuretics on hypertensive cardiac hypertrophy. Spontaneously hypertensive rats (SHR) were given p.o. M17055 (a novel “high ceiling” diuretic) 1.25, 2.5 or 5 mg/kg/day, furosemide 50 mg/kg/day or trichlormethiazide 30 mg/kg/day for 5 weeks. After the treatment, cardiac myosin isoforms were analyzed by gel electrophoresis, and cardiac hypertrophy-related gene expressions were examined by Northern blot analysis. These three diuretics significantly reduced cardiac hypertrophy of SHR. M17055 and furosemide, but not trichlormethiazide, significantly increased the proportion of cardiac V3 myosin of SHR by enhancing the gene expression of β-myosin heavy chain. On the other hand, trichlormethiazide, but not M17055 or furosemide, suppressed the increased cardiac gene expression of skeletal α-actin in SHR. Cardiac collagen type III expression of SHR was decreased only by treatment with M17055. Plasma thyroid hormone levels of SHR were slightly decreased by M17055 and by furosemide and were negatively correlated with cardiac V3 myosin contents. Thus the effects on the gene expression of cardiac contractile proteins and collagen are significantly different among these three types of diuretics, which suggests that these diuretics may have different cardiac actions independent of their diuretic and antihypertensive actions. The increased cardiac V3 myosin induced by M17055 and by furosemide may be partially due to the decreased plasma thyroid hormone.

Because high blood pressure is one of the major risk factors for cardiovascular diseases, the goal of treating hypertensive patients is to prevent morbidity and mortality associated with high blood pressure (The Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure, 1993). Diuretics are recommended as the first-choice agent for the treatment of hypertension (The Joint National Committee on Detection Evaluation and Treatment of High Blood Pressure, 1993) because they have been shown to reduce cardiovascular morbidity and mortality in long-term controlled clinical trials (Dahlof et al., 1991; MRC Working Party, 1992). Despite the clinical evidence for the beneficial cardioprotective effects of diuretics, the effects of diuretics on cardiac hypertrophy are poorly understood.

The adverse metabolic effects, such as hypokalemia and reduced glucose tolerance, that occasionally occur in the long-term use of diuretics limit their use for treatment of hypertension (The Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure, 1993). Thus the development of a new diuretic with fewer metabolic side effects is necessary for the treatment of hypertension. M17055, which is quite different in chemical structure from the loop or thiazide diuretics, belongs to a novel family of diuretics, the quinolinone oxime sulfonic acids (Shinkawa et al., 1992). This novel diuretic not only acts on the thick ascending limb of Henle’s loop but also acts on the distal nephron segments via the inhibition of Na transport, as does amiloride (Shinkawa et al., 1993a; Shinkawa et al., 1993b). Therefore, M17055 shows less kaliuretic and calcic effects than furosemide at a comparable level of natriuresis, which suggests that this compound may be a useful antihypertensive agent (Shinkawa et al., 1993a; Shinkawa et al., 1993b).

In the present study, to elucidate the cardiac effects of diuretics, we administered p.o. M17055, furosemide and trichlormethiazide to SHR and compared the effects of these

ABBREVIATIONS: SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; CMC, carboxymethylcellulose solution; TGF, transforming growth factor; PRA, plasma renin activity; MHC, myosin heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; AT, receptor, angiotensin II type 1 receptor; ACE, angiotensin-converting enzyme; LV, left ventricular.
diuretics on cardiac myosin isoforms and cardiac hypertrophy-associated gene expression. We obtained evidence that these three different types of diuretics have unique molecular effects on hypertrophied heart of SHR.

Materials and Methods

Drugs. M17055 (7-chloro-2,3-dihydro-1-(2-methylbenzoyl)-4(1H)-quinolinone 4-oxime-O-sulfonic acid potassium salt) was synthesized by Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Furosemide was purchased from Sigma Chemical Co. (St. Louis, MO). Tri-chloromethiazide was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Animals. Male SHR and age-matched control WKY were purchased from Charles River Japan (Atsugi, Japan) and were kept in an air-conditioned room with a constant temperature of 23°C and humidity of 55% ± 10% on a 12-h light-dark cycle. They were fed a standard laboratory chow (CE-2; Clea Japan, Tokyo, Japan) and given tap water ad libitum.

Experimental protocol. All procedures were in accordance with institutional guidelines for animal research. For p.o. administration, all drugs were suspended in 0.5% CMC. Twenty-week-old SHR were randomly separated into six groups and treated with 0.5% CMC (vehicle), M17055 1.25 mg/kg/day, M17055 2.5 mg/kg/day, M17055 5 mg/kg/day, furosemide 50 mg/kg/day or tri-chloromethiazide 30 mg/kg/day. Each drug was injected into one of 6 vials into each group. SHR were anesthetized with ether, the abdominal cavity was opened and blood was collected by puncture of the abdominal aorta for the measurement of hematocrit, plasma electrolytes and PRA. For measurement of urinary volume and electrolyte excretions, SHR were individually housed in metabolic cages at weekly intervals to collect urine for 24 h.

After 5 weeks of drug treatment, SHR were anesthetized with ether, the abdominal cavity was opened and blood was collected by puncture of the abdominal aorta for the measurement of hematocrit, plasma electrolytes and PRA. After the collection of blood, the chest cavity was opened and the heart was immediately excised. The left ventricle was rapidly separated from the right ventricle, and the atria were weighed, separated into two portions (for the analysis of myosin isoforms and the measurement of mRNAs) and frozen in liquid nitrogen. The tissues were stored at −80°C until used.

Analysis of myosin isoforms. Extraction of myosin from the ventricular tissues and the separation of myosin into V1, V2 and V3 isoforms were carried out by a minor modification of the method of Hoh et al. (1978). All procedures of myosin extraction were performed at 2°C. In brief, LV tissues were homogenized in 3 mmol/l sodium phosphate buffer (pH 7.4) containing 20 mmol/l NaH2PO4 (pH 7.4), 6 x SSC (1 x SSC = 0.15 mol/l sodium chloride, 0.015 mol/l sodium citrate, pH 7), 5 x Denhardt’s solution (Ficoll, polyvinylpyrrolidone and bovine serum albumin, 1 mg/ml each), 0.1% SDS and 200 µg/ml denatured salmon sperm DNA at 42°C for 4 h and then hybridized in the same solution as the prehybridization solution, containing the radiolabeled oligonucleotide probes, at 42°C for 24 h. After washing with 2 x SSC, the membranes were washed in 2 x SSC containing 1% SDS for 1 h at 55°C for α-MHC, 53°C for β-MHC, 57°C for skeletal α-actin and 51°C for cardiac α-actin. Finally, they were washed in 0.1 x SSC at room temperature for 20 min. For cDNA probes, the conditions of prehybridization and washing of the membranes have been previously described (Kim et al., 1994). After washing, the membranes were exposed to X-ray film (Kodak X-Omat AR5, Eastman Kodak Co., Rochester, NY) between two intensifying screens at −70°C. The density of mRNA bands obtained by autoradiography was measured by Macintosh LC-III computer with an optical scanner (EPSON GT-8000, Seiko, Tokyo, Japan) by using the public domain NIH Image program (Kim et al., 1994). The hybridization signals of specific mRNAs were divided by those of GAPDH mRNA to correct for differences in RNA loading and/or transfer. After autoradiography, the membranes were boiled in 0.1 x SSC containing 1% SDS for 30 min to strip off the hybridized oligonucleotide or cDNA probe and were then rehybridized with other oligonucleotide or cDNA probe.

Miscellaneous measurements. Urinary and plasma concentrations of Na+ and K+ were assayed by flame photometry, and urinary and plasma Cl concentrations were determined by coulometry (Autoanalyzer for electrolytes, model 710, Hitachi, Tokyo, Japan). Plasma and urinary Ca++ and Mg++ concentrations were measured by atomic absorption (Z-8100, Hitachi). PRA was measured by radioimmunoassay with RENIN-Riabead (Dainabot, Co., Ltd., Tokyo, Japan). 

References. Mayer et al., 1984; Shani et al., 1981, as previously described (Kim et al., 1995). The sequences of oligonucleotide probes used were as follows:

α-MHC, 5'-TTGTGGGATAGCAACAGCGA-3';
β-MHC, 5'-CTCTCAGGGCTCACAGG-3';
skeletal α-actin, 5'-GCAACCAGCACCGTGTTC-3';
cardiac α-actin, 5'-TGACGGTGTTAACACACT-3'.

The oligonucleotide probes were labeled with (γ-32P)-dATP (6000 Ci/mmol) at the 5' end by means of T4 polynucleotide kinase.

The cDNA probes used were rat TGF-β1, cDNA, a HindIII/XbaI fragment (Qian et al., 1990); mouse TGF-β3 cDNA (purchased from American Type Culture Collection, Rockville, MD), a 1.3-kb HindIII/XbaI fragment; rat α1(I) collagen cDNA, a 1.3-kb PsiI/BamHI fragment (Genovese et al., 1984); mouse α1(III) collagen cDNA, a 1.8-kb EcoRI/EcoRI fragment (Liu et al., 1985); rat GAPDH, a 1.3-kb PsiI/PstI fragment (Fort et al., 1985). The cDNA probes were labeled with (32P)-dCTP (specific activity, 3000 Ci/mmol, New England Nuclear, Boston, MA) by the random primer extension method (Feinberg and Vogelstein, 1983).

RNA extraction. Total RNA was isolated from the individual ventricles by the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) as previously described (Kim et al., 1994). The RNA concentration was determined spectrophotometrically by absorbance at 260 nm.

Northern blot hybridization. All procedures were carried out as previously described (Kim et al., 1994). We denatured 20 µg of total RNA from the ventricle in 1 M glyoxal and 50% dimethyl sulfoxide at 50°C for 1 h. It was then separated on 1% agarose gel and transferred to a nylon membrane (Gene Screen Plus membrane, DuPont Co., Boston, MA). The 28S and 18S ribosomal RNAs in gels were stained with ethidium bromide to demonstrate the integrity of applied RNA and to verify that the same amount of RNA was applied to each lane. For oligonucleotide probes, hybridization and washing of the membranes were carried out as described previously (Kim et al., 1995). In brief, the membranes were prehybridized in a solution containing 20 mmol/l NaH2PO4 (pH 7.4), 6 x SSC (1 x SSC = 0.15 mol/l sodium chloride, 0.015 mol/l sodium citrate, pH 7), 5 x Denhardt’s solution (Ficoll, polyvinylpyrrolidone and bovine serum albumin, 1 mg/ml each), 0.1% SDS and 200 µg/ml denatured salmon sperm DNA at 42°C for 4 h and then hybridized in the same solution as the prehybridization solution, containing the radiolabeled oligonucleotide probes, at 42°C for 24 h. After washing with 2 x SSC, the membranes were washed in 2 x SSC containing 1% SDS for 1 h at 55°C for α-MHC, 53°C for β-MHC, 57°C for skeletal α-actin and 51°C for cardiac α-actin. Finally, they were washed in 0.1 x SSC at room temperature for 20 min. For cDNA probes, the conditions of prehybridization, hybridization and washing of the membranes have been previously described (Kim et al., 1994). After washing, the membranes were exposed to X-ray film (Kodak X-Omat AR5, Eastman Kodak Co., Rochester, NY) between two intensifying screens at −70°C. The density of mRNA bands obtained by autoradiography was measured by Macintosh LC-III computer with an optical scanner (EPSON GT-8000, Seiko, Tokyo, Japan) by using the public domain NIH Image program (Kim et al., 1994). The hybridization signals of specific mRNAs were divided by those of GAPDH mRNA to correct for differences in RNA loading and/or transfer. After autoradiography, the membranes were boiled in 0.1 x SSC containing 1% SDS for 30 min to strip off the hybridized oligonucleotide or cDNA probe and were then rehybridized with other oligonucleotide or cDNA probe.
pan). Plasma-free T3 and T4 levels were measured by free T3 kit and T4 kit, respectively (Diagnostic Products Co., Los Angeles, CA).

**Statistical analysis** was carried out by using SuperANOVA (Abacus Concepts, Berkeley, CA). The differences in the data on blood pressure, HR and urinary volume and electrolyte excretions were analyzed by two-way analysis of variance, and the differences between the means of each group were determined by the least-squares means test. Statistical significance of the differences in the data after 5 weeks of drug treatment (body weight, LV weight, myosin isoforms, mRNAs, PRA and plasma-free T3 and T4 levels) was analyzed by one-way analysis of variance followed by the Student-Newman-Keuls multiple-range test for multiple comparisons. Differences were considered statistically significant at a value of P < .05.

**Results**

**Effects of diuretics on blood pressure and HR of SHR.** Figure 1A shows blood pressure of SHR treated with vehicle, M17055, furosemide or trichlormethiazide for 5 weeks. Before the start of drug treatment, 20-week-old SHR were already hypertensive (more than 180 mm Hg), whereas the blood pressure of WKY at the same age was less than 130 mm Hg (data not shown). M17055 at the dose of 1.25 mg/kg/day did not significantly lower blood pressure of SHR throughout 5 weeks of treatment. However, M17055 at 2.5 and 5 mg/kg/day decreased blood pressure of SHR in a dose-dependent manner. Furosemide (50 mg/kg/day) decreased blood pressure of SHR to about the same extent as M17055 (5 mg/kg/day) throughout the treatment. The antihypertensive effects of trichlormethiazide (30 mg/kg/day) were smaller than those of furosemide and M17055 (5 mg/kg/day) and were similar to those of M17055 (2.5 mg/kg/day).

As shown in figure 1B, at 23 weeks of age, the HR of SHR (438 ± 12 bpm) was decreased by treatment with M17055 at the dose of 1.25 mg/kg/day (381 ± 17 bpm; P < .05), 2.5 mg/kg/day (385 ± 22 bpm; P < .05) and 5 mg/kg/day (363 ± 25 bpm; P < .01) and by treatment with furosemide (50 mg/kg/day) (386 ± 15 bpm; P < .05). At 25 weeks of age, the HR of SHR (436 ± 11) was also decreased by M17055 (5 mg/kg/day) (368 ± 23 bpm; P < .01). On the other hand, trichlormethiazide did not change the HR of SHR throughout the treatment.

**Effects of diuretics on urinary volume and urinary electrolyte excretions of SHR.** Table 1 shows the values of 24-h urinary volume and electrolyte excretions 4 weeks after the start of drug treatment (at 24 weeks of age). There were no significant differences in 24-h urinary volume or electrolyte excretions among the six groups of SHR before the start of drug treatment (at the age of 20 weeks; data not shown). Treatment of SHR with M17055 (1.25 mg/kg/day) significantly increased 24-h urinary calcium and magnesium excretions but did not increase 24-h urinary volume or urinary sodium, potassium and chloride excretions. However, M17055 at 2.5 and 5 mg/kg/day increased the 24-h urinary volume of SHR. M17055 (5 mg/kg/day) also increased urinary sodium excretion of SHR. Although urinary volume and urinary sodium excretions of SHR treated with furosemide (50 mg/kg per day) were similar to those of SHR treated with M17055 (5 mg/kg/day), urinary calcium and magnesium excretions of SHR treated with furosemide were significantly greater than those of SHR treated with M17055 (5 mg/kg/day). In spite of causing no apparent increase in urinary volume and urinary sodium, potassium, chloride and calcium excretions, trichlormethiazide significantly increased urinary magnesium excretion about as much as M17055. Thus diuretic effects and the pattern of urinary electrolyte excretions in SHR were significantly different among M17055-, furosemide- and trichlormethiazide-treated groups, which confirms that these three types of diuretics have different diuretic actions.

**Hematocrit, plasma electrolyte concentrations and PRA in SHR after 5 weeks of drug treatment.** Five weeks of treatment of SHR with three doses of M17055, with furosemide or with trichlormethiazide did not alter hematocrit and plasma concentrations of sodium, potassium, calcium and magnesium (data not shown). Plasma chloride concentrations of SHR (103.6 ± 0.1 mEq/l; P < .01) or trichlormethiazide-treated SHR. As shown in table 2, treatment of SHR with M17055 at 1.25 or 2.5 mg/kg/day did not increase PRA. On the other hand, M17055 at 5 mg/kg/day, furosemide and trichlormethiazide significantly increased the PRA of SHR.

**Effects of diuretics on body weight and LV weight of SHR.** As shown in table 3, the body weight of vehicle-treated
TABLE 1

Twenty-four-hour urinary volume and urinary electrolyte excretions in SHR after 4 weeks of treatment with vehicle, M17055, furosemide or trichlormethiazide

<table>
<thead>
<tr>
<th></th>
<th>UV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>U&lt;sub&gt;n&lt;/sub&gt;V</th>
<th>U&lt;sub&gt;o&lt;/sub&gt;V</th>
<th>U&lt;sub&gt;c&lt;/sub&gt;V</th>
<th>U&lt;sub&gt;e&lt;/sub&gt;V</th>
<th>U&lt;sub&gt;m&lt;/sub&gt;V</th>
<th>U&lt;sub&gt;n&lt;/sub&gt;V</th>
<th>μEq/100 g</th>
<th>μEq/100 g</th>
<th>μEq/100 g</th>
<th>μEq/100 g</th>
<th>μEq/100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5.95 ± 0.43</td>
<td>365 ± 15</td>
<td>895 ± 30</td>
<td>490 ± 18</td>
<td>6.06 ± 0.58</td>
<td>71.4 ± 3.0</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>M17055 (1.25 mg/kg)</td>
<td>7.36 ± 0.37</td>
<td>366 ± 22</td>
<td>924 ± 14</td>
<td>426 ± 33</td>
<td>9.45 ± 0.88</td>
<td>112.3 ± 11.1</td>
<td>§</td>
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</tr>
<tr>
<td>M17055 (2.5 mg/kg)</td>
<td>8.12 ± 0.45</td>
<td>383 ± 30</td>
<td>927 ± 18</td>
<td>433 ± 40</td>
<td>10.31 ± 0.64</td>
<td>106.8 ± 6.9</td>
<td>§§</td>
<td></td>
<td></td>
<td>§§</td>
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<tr>
<td>Furosemide</td>
<td>9.17 ± 0.63</td>
<td>437 ± 15</td>
<td>973 ± 24</td>
<td>498 ± 22</td>
<td>13.77 ± 0.58</td>
<td>103.2 ± 7.3</td>
<td>§§</td>
<td></td>
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<td>§§</td>
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<tr>
<td>Trichlormethiazide</td>
<td>9.68 ± 0.38</td>
<td>468 ± 32</td>
<td>919 ± 16</td>
<td>537 ± 43</td>
<td>29.08 ± 2.18</td>
<td>130.4 ± 5.9</td>
<td>§§</td>
<td></td>
<td></td>
<td>§§</td>
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</tbody>
</table>

<sup>a</sup> UV, urinary volume; U<sub>n</sub>V, urinary sodium excretion; U<sub>o</sub>V, urinary potassium excretion; U<sub>c</sub>V, urinary chloride excretion; U<sub>e</sub>V, urinary calcium excretion; U<sub>m</sub>V, urinary magnesium excretion. Values are mean ± S.E.M. (n = 10 in each group of SHR).

TABLE 2

PRA in SHR after 5 weeks of drug treatment

<table>
<thead>
<tr>
<th></th>
<th>PRA</th>
<th>ng angiotensin I/h/ml</th>
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<tr>
<td>SHR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>14.0 ± 2.0</td>
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</tr>
<tr>
<td>M17055 (1.25 mg/kg)</td>
<td>12.0 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>M17055 (2.5 mg/kg)</td>
<td>16.1 ± 1.5</td>
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</tr>
<tr>
<td>M17055 (5 mg/kg)</td>
<td>32.3 ± 2.9 ** §§</td>
<td></td>
</tr>
<tr>
<td>Furosemide</td>
<td>22.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Trichlormethiazide</td>
<td>28.4 ± 1.7 ** §</td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>9.47 ± 0.74</td>
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</tr>
</tbody>
</table>

Values are mean ± S.E.M. (n = 10 in each group of SHR and n = 9 in WKY).

SHR was less than that of WKY (P < .01), whereas the LV weight of vehicle-treated SHR, corrected for body weight or not, was significantly greater than that of WKY (P < .01). Five weeks of treatment with M17055, furosemide or trichlormethiazide did not change the body weight of SHR. On the other hand, all three of these diuretics significantly reduced the LV hypertrophy of SHR. M17055 (2.5 mg/kg/day) decreased the LV weight of SHR about as much as trichlormethiazide. M17055 (5 mg/kg/day) and furosemide, whose effects were more potent than those of M17055 (2.5 mg/kg/day) and trichlormethiazide, reduced the LV hypertrophy of SHR to a comparable degree.

Effects of diuretics on LV myosin isoforms of SHR.

As shown in table 2, there was no significant difference between vehicle-treated SHR and WKY with respect to the relative proportion of LV V1, V2 and V3 myosin isoforms. Neither M17055 at 1.25 or 2.5 mg/kg/day nor trichlormethiazide changed the relative ratio of these three myosin isoforms in the left ventricle of SHR. However, M17055 (5 mg/kg/day) and furosemide significantly increased the relative percent of ventricular V3 myosin isoform in SHR, an effect that was accompanied by a significant decrease in the relative percent of V1 myosin.

Effects of diuretics on LV α-MHC, β-MHC and α-actin gene expressions. As shown in figures 2 and 3, there were no significant differences in LV mRNA levels for α-MHC, β-MHC and cardiac α-actin between vehicle-treated SHR and WKY. On the other hand, LV skeletal α-actin mRNA levels of SHR were significantly higher than those of WKY (P < .01). M17055 and furosemide tended to decrease LV α-MHC mRNA levels of SHR, although this result was not statistically significant. Ventricular β-MHC mRNA levels of SHR were significantly increased by M17055 (5 mg/kg/day) (P < .01). Furthermore, furosemide also tended to increase ventricular β-MHC mRNA levels of SHR, and there was no significant difference in β-MHC mRNA levels between M17055 (5 mg/kg/day)-treated and furosemide-treated groups. Ventricular cardiac α-actin mRNA was not changed by treatment with these three diuretics. However, skeletal α-actin mRNA levels of SHR were decreased by trichlormethiazide, but not by M17055 or furosemide.

Correlation of myosin isoforms with α-MHC and β-MHC mRNA levels. There was a strong positive correlation between %V3 myosin and β-MHC mRNA levels in the left ventricles from all six groups of SHR (r = 0.80, P < .001; n = 42). There was also a significant positive correlation between LV %V1 myosin isoform and α-MHC mRNA levels from all six groups of SHR (r = 0.52, P < .001; n = 42).

Effects of diuretics on LV TGF-β and collagen gene expression. As shown in figure 4, LV TGF-β1, TGF-β3 and collagen type I gene expression of SHR was unchanged by the three types of diuretics. However, collagen type III gene expression of SHR was suppressed by M17055 (5 mg/kg/day), but not by furosemide or trichlormethiazide.

Effects of diuretics on plasma T3 and T4 levels. As shown in figure 5A, plasma T3 levels of SHR tended to be decreased by treatment with M17055 (5 mg/kg/day) and furosemide, although this effect was not statistically significant. Figure 5B shows that M17055 (5 mg/kg/day) and furosemide, but not trichlormethiazide, significantly decreased plasma T4 levels of SHR.

As shown in figure 6, both plasma T3 and plasma T4 levels of SHR were negatively correlated with LV %V3 myosin and positively correlated with LV %V1 myosin.

**Discussion**

Despite the clinical importance of diuretics for the treatment of hypertension, little information is available on the effects of diuretics on hypertrophied heart. This led us to examine comprehensively the effects of diuretics on cardiac hypertrophy and gene expression of SHR.

Judged on the basis of electrophoretic mobility on nondenaturing gels, rat ventricular myosin can be resolved into three isomyosins, which have been classified as V1, V2 and V3 (Hoh et al., 1978; Nadal-Ginard and Mahdavi, 1989; Schwartz et al., 1993). These three isomyosins consist of two MHCs (α-MHC and β-MHC) associated with identical light chains. V1 consists of an α-MHC homodimer and has the highest ATPase catalytic activity, whereas V3 consists of a β-MHC homodimer and has the lowest ATPase catalytic ac-
Effect of Diuretics on Cardiac Gene

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>B.Wt.</th>
<th>LV Wt.</th>
<th>LV Wt./B.Wt.</th>
<th>V1 myosin</th>
<th>V2 myosin</th>
<th>V3 myosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>mg</td>
<td>mg/g</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Vehicle</td>
<td>353 ± 4</td>
<td>952 ± 19</td>
<td>2.70 ± 0.04</td>
<td>54 ± 2</td>
<td>24 ± 2</td>
<td>22 ± 0.4</td>
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<tr>
<td>M17055 (1.25 mg/kg)</td>
<td>355 ± 7</td>
<td>930 ± 14</td>
<td>2.62 ± 0.04</td>
<td>56 ± 4</td>
<td>23 ± 1</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>M17055 (2.5 mg/kg)</td>
<td>357 ± 6</td>
<td>880 ± 29</td>
<td>2.46 ± 0.06*</td>
<td>44 ± 7</td>
<td>27 ± 2</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>M17055 (5 mg/kg)</td>
<td>346 ± 7</td>
<td>752 ± 18$§$</td>
<td>2.16 ± 0.04$§$</td>
<td>14 ± 2$§$</td>
<td>22 ± 2</td>
<td>64 ± 4$§$</td>
</tr>
<tr>
<td>Furosemide</td>
<td>352 ± 7</td>
<td>776 ± 20$§$</td>
<td>2.21 ± 0.03$§$</td>
<td>24 ± 5$§$</td>
<td>25 ± 1</td>
<td>51 ± 6$§$</td>
</tr>
<tr>
<td>Trichlormethiazide</td>
<td>346 ± 7</td>
<td>862 ± 7*</td>
<td>2.50 ± 0.04*</td>
<td>54 ± 3</td>
<td>27 ± 2</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>WKY</td>
<td>410 ± 5*</td>
<td>817 ± 11*</td>
<td>1.99 ± 0.03*</td>
<td>44 ± 3</td>
<td>28 ± 2</td>
<td>26 ± 2</td>
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Values are mean ± S.E.M. (n = 6 in WKY; n = 7 in all groups of SHR). * P < .05, § P < .01 vs. vehicle. $ P < .01 vs. trichlormethiazide. ‡ P < .01 vs. M17055 (2.5 mg/kg). # P < .05 vs. M17055 (5 mg/kg).

Fig. 2. Representative autoradiograms of Northern blot analysis of LV mRNAs for α-MHC, β-MHC, cardiac α-actin, skeletal α-actin and GAPDH. W, vehicle-treated age-matched (25-week-old) WKY; V, vehicle-treated SHR; M (1.25), M17055 (1.25 mg/kg/day)-treated SHR; M (2.5), M17055 (2.5 mg/kg/day)-treated SHR; M (5), M17055 (5 mg/kg/day)-treated SHR; F, furosemide (50 mg/kg/day)-treated SHR; T, trichlormethiazide (30 mg/kg/day)-treated SHR. The autoradiograms of two typical different samples are shown for each group. The size of the mRNA band was 7.1 kb for α-MHC, 7.1 kb for β-MHC, 1.7 kb for cardiac α-actin, 1.7 kb for skeletal α-actin and 1.4 kb for GAPDH.

Fig. 3. Bar graph shows LV mRNA levels for α-MHC, β-MHC, cardiac α-actin and skeletal α-actin in WKY and SHR after 5 weeks of drug treatment. W, V, M (1.25), M (2.5), M (5), F and T all mean the same as in figure 2. Each mRNA value in all groups was corrected for GAPDH mRNA value. The mean value of mRNA levels in WKY (W) is represented as 1. Each bar represents mean ± S.E.M. (n = 6 in W; n = 7 in all groups of SHR). * P < .01 vs. V, § P < .05, §§ P < .01 vs. T, ‡ P < .01 vs. M (2.5).

The present study did not enable us to elucidate the mechanism by which M17055 or furosemide increased the ventricular V3 isomyosin (β-MHC) expression of SHR. However, several possible explanations of this mechanism come to mind. First, the increased β-MHC gene expression might be secondary to the decreased hemodynamic load, because M17055 and furosemide lowered the blood pressure of SHR to a greater extent than trichlormethiazide. However, we very recently found that treatment of stroke-prone SHR with AT1 receptor antagonist, ACE inhibitor or calcium channel antagonist did not at all change ventricular β-MHC gene expression, despite their significant antihypertensive effects (Kim et al., 1996). These findings show that the increased ventricular β-MHC expression by M17055 and furosemide was not due to their antihypertensive effects. Second, the increased β-MHC gene expression might be due to the increased PRA, because our recent data show that angiotensin...
II in vivo can stimulate the gene expression of ventricular β-MHC in rats (Kim et al., 1995). This possibility can be also excluded, however, because trichlormethiazide increased the PRA of SHR to a greater extent than furosemide but did not at all affect the cardiac V3 (β-MHC) expression of SHR. Third, the increased β-MHC might be attributable to a change in circulating thyroid hormone levels, because endogenous thyroid hormone is well-known to be the most potent regulator of ventricular isomyosin expression (Chizzonite and Zak, 1984; Gustafson et al., 1985; Lompre et al., 1984; Nadal-Ginard and Mahdavi, 1989), suggest that the increased proportion of ventricular V3 isomyosin of SHR with M17055 and furosemide may be in part mediated by the decrease in plasma thyroid hormone. Finally, we cannot exclude the possibility that the increase in V3 myosin induced by M17055 and furosemide might be due to their direct actions on cardiac myocytes via inhibition of the Na\(^+\), K\(^+\), 2Cl\(^-\)-cotransport system, because this cotransport system is reported to be responsible for cell hypertrophy (Orlov et al., 1992; Tseng and Berk, 1992). However, further study is needed to elucidate the possible contribution of a direct action of M17055 and furosemide to cardiac hypertrophy and gene expression.

Besides MHC, the gene regulation of α-actin (i.e., skeletal and cardiac α-actins) and collagen plays a key role in the modulation of cardiac performance (Chapman et al., 1990; Morgan and Baker, 1991; Nadal-Ginard and Mahdavi, 1989; Schwartz et al., 1993; Weber and Brilla, 1991). Skeletal α-actin is associated with a greater cardiac contractility than cardiac α-actin, which shows that the altered ratio of skeletal to cardiac α-actin in the heart significantly affects cardiac contractility (Hewett et al., 1994). The increased cardiac interstitial collagen deposition, which is mediated at least in

![Fig. 5. Plasma T3 (A) and T4 (B) levels in WKY and SHR. W, V, M (1.25), M (2.5), M (5), F and T mean the same as in figure 2. Each bar represents mean ± S.E.M. \(\ddagger P < .05\), \(\ddagger \ddagger P < .01\) vs. V. § § \(P < .05\), \(§§ P < .01\) vs. T. \(\ddagger \ddagger P < .05\), \(\ddagger \ddagger \ddagger P < .01\) vs. M (2.5).](image)
part by the increased collagen gene expression, causes the increase in cardiac stiffness, thereby leading to the impairment of cardiac diastolic function (Chapman et al., 1996). In addition, TGF-β is assumed to be responsible for cardiac fibrosis by stimulating the production of collagen (Barnard et al., 1990; Eghbali et al., 1990). Therefore, in this study we also compared the effects of diuretics on α-actin, collagen and TGF-β expressions of SHR. It is notable that although trichlormethiazide had much less antihypertensive and diuretic effect than M17055 and furosemide, ventricular skeletal α-actin expression of SHR was significantly suppressed by trichlormethiazide but not by M17055 or furosemide. These observations suggest that thiazide may inhibit ventricular skeletal α-actin gene expression directly, in a manner independent of antihypertensive or diuretic action. Furthermore, despite the similar antihypertensive and natriuretic effects of M17055 (5 mg/kg/day) and furosemide, ventricular collagen type III expression of SHR was decreased only by M17055, which suggests that M17055 has a different cardiac effect from the loop diuretic at the molecular levels. On the other hand, ventricular mRNA levels for TGF-β1 and TGF-β3, which are potent stimulators of collagen production (Barnard et al., 1990), were not altered by any diuretics, which suggests that TGF-β may not be responsible for the decreased cardiac collagen type III expression of SHR induced by M17055. Thus three different types of diuretics—including M17055, loop diuretics and thiazide diuretics—have different cardiac effects at the molecular levels.

The present study did not enable us to elucidate the mechanisms by which trichlormethiazide and M17055 decreased ventricular skeletal α-actin mRNA and collagen type III mRNA, respectively. Our previous data show that AT1 receptor antagonist, ACE inhibitor and calcium channel antagonist all decrease both skeletal α-actin and collagen type III mRNAs in the left ventricle of hypertensive rats, a result that suggests that locally produced angiotensin II or intracellular calcium contributes to the expression of these genes (Kim et al., 1996). Therefore, it cannot be ruled out that these effects of trichlormethiazide and M17055 might be mediated in part by the action on cardiac autocrine/paracrine factors such as angiotensin II. More study is needed to explore this possibility. Furthermore, our recent work showed that the reduction of HR of SHR with atenolol, a β-adrenergic receptor blocker, caused the significant decrease in cardiac collagen type III expression, which indicates the important role of HR in the regulation of cardiac collagen gene expression (Ohta et al., submitted). Thus it is also possible that the reduction of HR by M17055 participates in the decrease in cardiac collagen type III mRNA.

In conclusion, although M17055, loop diuretics and thiazide diuretics significantly reduced cardiac hypertrophy of SHR, these three diuretics had different effects on cardiac gene expression that were independent of their diuretic and antihypertensive actions.

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