Changes in Contractile and Non-Contractile Proteins, Intracellular Ca\textsuperscript{2+} and Ultrastructures During The Development of Right Ventricular Hypertrophy and Failure in Rats

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Whether cardiac hypertrophy is a compensatory response or a cause of decompensation has been an interesting and important controversy in cardiology. The purpose of this study is to assess qualitative and quantitative changes in biological factors involved in the evolution and the development of right ventricular hypertrophy (RVH) and right ventricular failure in response to pressure overload in rats with pulmonary hypertension induced by monocrotaline injection, and to clarify the process from compensation to deterioration in cardiac hypertrophy biochemically and morphologically.

Significant RVH was produced in rats at 2 weeks after single subcutaneous injection of monocrotaline, and signs of right ventricular failure became obvious at 4 weeks as RVH became more severe. In the right ventricle of these rats, we found that: 1) myosin isoenzymes shifted from V1 to V3 both at 2 and 4 weeks; 2) total collagen content increased, and type III and type V collagens increased with a relative decrease in type I collagen at both 2 and 4 weeks; 3) intracellular Ca\textsuperscript{2+} transient recorded from isolated myocytes showed a lower peak and slower descent slope compared to those of control rats; 4) ultrastructural changes observed by scanning electron microscopy at 1 and 2 weeks disappeared gradually as heart failure developed, and degeneration or destruction of mitochondria or sarcoplasmic reticulum became remarkable at 3 and 4 weeks. These findings suggest that cardiac hypertrophy might be an ominous sign of cardiac failure rather than a benign adaptive process, at least in this model.

(Jpn Circ J 1992; 56: 469–474)

CARDIAC hypertrophy is thought to be a compensatory response to overload which eventually progresses to heart failure, but the process from compensation to deterioration remains to be elucidated. Biochemical and biological changes in cardiac hypertrophy or in cardiac failure have been reported separately, but sequential changes have not been reported.

The purpose of this study is to assess qualitative and quantitative changes in contractile protein (myosin isoenzymes), non-contractile protein (collagen), intracellular Ca\textsuperscript{2+} transient, and membranous ultrastructures during the development of right ventricular hypertrophy (RVH) and right ventricular failure due to pulmonary hypertension induced by monocrotaline injection in rats, and to clarify the process from hypertrophy to failure biochemically and morpholog-
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TABLE 1 BODY WEIGHTS, HEART WEIGHTS, BLOOD PRESSURES, AND BLOOD GAS ANALYSIS IN MONOCROTALINE-TREATED AND CONTROL RATS AT 2 AND 4 WEEKS

<table>
<thead>
<tr>
<th></th>
<th>2 weeks</th>
<th>M-treated</th>
<th>4 weeks</th>
<th>M-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>M-treated</td>
<td>Control</td>
<td>M-treated</td>
</tr>
<tr>
<td>BW (g)</td>
<td>283 ± 21</td>
<td>270 ± 17*</td>
<td>406 ± 34</td>
<td>317 ± 70***</td>
</tr>
<tr>
<td>HW (g)</td>
<td>0.91 ± 0.10</td>
<td>0.93 ± 0.13</td>
<td>1.17 ± 0.13</td>
<td>1.23 ± 0.16</td>
</tr>
<tr>
<td>LVW (g)</td>
<td>0.59 ± 0.06</td>
<td>0.60 ± 0.06</td>
<td>0.76 ± 0.08</td>
<td>0.67 ± 0.09**</td>
</tr>
<tr>
<td>RVW (g)</td>
<td>0.16 ± 0.02</td>
<td>0.22 ± 0.07***</td>
<td>0.21 ± 0.03</td>
<td>0.39 ± 0.11***</td>
</tr>
<tr>
<td>RV/LV</td>
<td>0.27 ± 0.03</td>
<td>0.37 ± 0.08***</td>
<td>0.28 ± 0.02</td>
<td>0.59 ± 0.15***</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>(N=20)</td>
<td>(N=20)</td>
<td>(N=20)</td>
<td>(N=20)</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
<td>136 ± 9</td>
<td>136 ± 13</td>
<td>138 ± 9</td>
<td>102 ± 15**</td>
</tr>
<tr>
<td>(N=10)</td>
<td>(N=10)</td>
<td>(N=10)</td>
<td>(N=5)</td>
<td>(N=5)</td>
</tr>
<tr>
<td>PaO2 (mmHg)</td>
<td>92.6 ± 1.1</td>
<td>89.2 ± 1.2</td>
<td>94.9 ± 3.7</td>
<td>68.5 ± 9.5**</td>
</tr>
<tr>
<td>(N=8)</td>
<td>(N=9)</td>
<td>(N=6)</td>
<td>(N=6)</td>
<td></td>
</tr>
</tbody>
</table>

M-treated = monocrotaline-treated; BW = body weight; HW = heart weight; LVW = left ventricular weight; RVW = right ventricular weight; SBP = systolic blood pressure; MBP = mean blood pressure. Values are mean ± S.D. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

Methods

Animal Models

A single dose of monocrotaline 60 mg/kg was injected subcutaneously into male 6-week-old Sprague-Dawley rats. Monocrotaline, a pyrrolizidine alkaloid extracted from Crotalaria spectabilis, produces alterations in pulmonary vessels and causes pulmonary hypertension and RVH. Previous studies suggest that monocrotaline itself has no direct effect on the heart. Rats that received a single subcutaneous injection of saline served as controls. Animals were sacrificed by decapitation at 2 weeks after injection (hypertrophic stage) and at 4 weeks (failing stage).

Analysis of Myosin Isoenzymes

After extraction of myosins from the right ventricular free walls, myosin isoenzymes were analyzed by pyrophosphate gel electrophoresis under non-dissociating conditions according to the procedures described by Hoh et al., with some modifications. After electrophoresis, the density of each myosin band (V1, V2, V3) was determined by a soft laser photodensitometer at a wave length of 620 nm.

Collagen Concentrations

After cardiac tissues were dried and defatted with acetone, extraction of collagen and hydrolysis of collagen to hydroxyproline were carried out by the method of Fischer and Llaurado. Hydroxyproline was measured by the method of Woessner. Hydroxyproline content thus obtained was multiplied by 7.46 to estimate collagen content.

Collagen Types

Collagens were extracted from the right ventricle (RV) with a slight modification of the method of Murata, et al. Acid-soluble collagens extracted by this method consist mainly of types I, III, and V collagens. The type of collagens was determined by SDS-PAGE according to the procedure of Laemmli with some modifications as described by Nakamura, et al. After electrophoresis, the density of α-chain of each collagen (α 2 (I), α 1 + 2 (V), α 1 (III)) was determined by a soft laser photodensitometer at a wave length of 620 nm.

Intracellular Calcium Transient

Single cardiac myocytes were isolated enzymatically using Langendorff's apparatus as described by Powell et al., with some modifications. After loading fura-2/AM to myocytes, the cells were excited by electrical
field stimulation at 1.5 sec intervals (50–100 V/cm), and excitation lights of 340 and 380 nm wavelength were obtained alternatively with Olympus OSP-3 system. The fura-2 fluorescence ratios were calculated by dividing the 340 nm fluorescence after autofluorescence subtraction by the 380 nm fluorescence after autofluorescence subtraction with a personal computer and recorded as calcium transient.

**Ultrastructures**

Three-dimensional ultrastructural changes during the development of RVH and right ventricular failure were observed with scanning electron microscopy (SEM). Specimens for the SEM were prepared by the A-O-D-O method, by which myofibrils were completely removed with cytoplasmic matrices, and intracellular membranous structures such as mitochondria, sarcoplasmic reticulum (SR) or surface caveolae could be clearly disclosed without significant artifact.

**Statistical Analysis**

Results are presented as mean ± SD. Differences between groups were evaluated by unpaired Student's t-test. A p value of 0.05 or less was considered to be statistically significant.

**RESULTS**

**RVH and Right Ventricular Failure**

*Japanese Circulation Journal  Vol. 36, May 1992*

Heart weights, blood pressures and arterial blood gas analysis of both monocrotaline-treated and control rats at 2 and 4 weeks after the injection of monocrotaline and saline are shown in Table I. It is clear that significant RVH developed at 2 weeks in monocrotaline-treated rats and became more severe at 4 weeks. Blood pressures and pO₂ showed no significant changes at 2 weeks, but they decreased significantly at 4 weeks in monocrotaline-treated rats. In addition to these changes, plasma norepinephrine and atrial natriuretic peptide increased significantly and signs of cardiac failure such as ascites, pleural effusion or pericardial effusion were recognized at 4 weeks in this group.

**Myosin Isoenzymes**

Myosin isoenzymes of the RV shifted from V1 to V3 at 2 weeks in monocrotaline-treated rats and these changes became more prominent at 4 weeks. To quantify the
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TABLE II MORPHOLOGICAL CHANGES IN MEMBRANOUS ORGANELLES OF MONOCROTALINE-TREATED RATS OBSERVED BY SEM

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1wk</th>
<th>2wks</th>
<th>3wks</th>
<th>4wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>variety in size</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>clustering</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>wavy form</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>network density</td>
<td></td>
<td>→</td>
<td>↑</td>
<td>↓</td>
<td>→</td>
</tr>
<tr>
<td>flattened SR</td>
<td></td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Surface Caveolae</td>
<td></td>
<td>→</td>
<td>→</td>
<td>↑↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

changes in myosin isoenzymes, the percentage of β heavy chain was measured from the area of each band of V1, V2 and V3 in the electrophoresis using the formula shown in Fig. 1, since V1 and V3 are homodimers and V2 is a heterodimer of α- and β-heavy chains.

Collagen

There was no significant difference in the collagen content of the RV between monocrotaline-treated rats and controls either at 2 weeks or 4 weeks. However, taking account of the right ventricular weight, total collagen content increased in the RV of monocrotaline-treated rats.

Fine separation of α chains of type I, type III and type V collagens was accomplished by the improved non-interrupted SDS-PAGE. The relative amount of α2(1), α1(III) and α1+2 (V) was expressed as percentages of the total sum of areas under the entire trace (Fig. 2). Type III and type V collagens increased in the RV of monocrotaline-treated rats at both 2 and 4 weeks with the relative decrease in type I collagen.

Calcium Transient

Representative calcium transients from myocytes isolated from the RVs of a monocrotaline-treated rat and a control rat are shown overlapped in Fig. 3. Calcium transients from monocrotaline-treated rats showed lower peak and slower descent slope compared to those of control rats.

Ultrastructures

Sequential changes in intracellular membranous ultrastructures such as mitochondria, SR and surface caveolae, observed by SEM, during the development of RVH and right ventricular failure were summarized in Table II. Qualitative and quantitative changes in ultrastructures observed at 1 and 2 weeks (hypertrophic stage) disappeared gradually as heart failure developed, and degeneration or destruction of mitochondria or SR became remarkable during the failing stage.

DISCUSSION

Whether cardiac hypertrophy is a compensatory response or a beginning of decompensation has been an interesting and important controversy in cardiology during the past decades. Research into this subject has been mainly physiological until recently. In the present study, we tried to investigate the subject from the viewpoint of cellular
biology and morphology.
Recent developments in cellular biology have demonstrated existence of 2 isoforms of myosin heavy chain in mammalian cardiac tissues. Those 2 types of myosin heavy chain, α and β give rise to αα and ββ homodimers, which correspond respectively to the V1 and V3 isomyosins with high and low ATPase activity, and to an αβ heterodimer that corresponds to V2 and has an intermediate ATPase activity. The type of myosin isoenzyme appears to be an important determinant of the myocardial contractility. The shift from V1 to V3 observed in the present study is correlated with slower but more efficient myocardial contraction. This change has been thought to play an important role in the adaptation of cardiac muscle to an overload. However, this change itself might be the start of the pathologic process of heart failure, because the present study revealed similar changes during both hypertrophic and failing stages. It is noteworthy that physiological hypertrophy in rats produced by swimming is characterized by the shift of myosin isoenzymes from V3 to V1.

Collagen is the most abundant noncontractile protein in the interstitial tissues of the heart and is important for supporting myocytes and transmitting contraction to the whole heart as well as for gas exchange and other metabolic process of cardiac cells. Increased total collagen content and unchanged collagen concentration in the RV of monocrotaline-treated rats in the present study may be a proper response to pressure overload in order to support the hypertrophied myocardium. The significance of changes in collagen types, i.e. relative increases in type III and type V collagens and relative decrease in type I collagen, in the RV of monocrotaline-treated rats, remains to be elucidated. Type III collagen is known to be associated with embryonic and neonatal development and to increase in dermal injury. Therefore, type III collagen may provide an initial structure and substrate for hypertrophied heart. Many studies including immunohistochemical study from our laboratory indicate that type V collagen may be associated with organ hypertrophy and/or hyperplasia. These changes in collagen content and collagen types were observed during both hypertrophic and failing stages. Qualitative and quantitative changes in the collagen metabolism of the hypertrophied heart may be causally related to myocardial dysfunction during either systole or diastole and may play a deleterious role in the development of heart failure.

The calcium ion plays an important role in cardiac excitation-contraction coupling. Thus, knowledge of the intracellular Ca²⁺ transients in normal and hypertrophied cardiac muscle is essential to understanding the functional ability of the normal and hypertrophied heart. The recent development of the fluorescent Ca²⁺ indicators including fura-2 and of the technique of isolating myocardial cells has represented a significant advance in measurements of intracellular Ca²⁺ transient in a single cardiac myocyte. In the present study, intracellular Ca²⁺ transients of cardiac myocytes from the RV of monocrotaline-treated rats showed a decreased peak and a slow descent slope. The Ca²⁺ transients of working cardiac myocytes appear to be mainly related to the release and uptake of Ca²⁺ by SR. These data suggest that dysfunction of SR might cause both systolic and diastolic dysfunction during the evolution and the development of heart failure.

The ultrastructural changes observed by SEM are not coincident with the biochemical findings described above. Changes observed in intracellular organelles, such as mitochondria, SR and surface caveolae, during the hypertrophic stage (1W and 2W) disappeared gradually during the failing stage (3W and 4W). The function of surface caveolae has not been clarified yet.

In summary, in this model of RVH and right ventricular failure produced in rats by monocrotaline injection, similar changes were observed during both hypertrophic and failing stages in myosin isoenzymes, collagen metabolism and intracellular Ca²⁺ transients, but not in ultrastructural anatomy. These results suggest that hypertrophy might be an ominous sign rather than a benign adaptive process, at least in this subacute model of RVH and heart failure.

REFERENCES
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