POSTNATAL development of the mammalian ventricular myocyte is characterized by a brief period of cellular hyperplasia followed by a prolonged period of physiological hypertrophy (Abel and Newman, 1984; Zak, 1984). Physiological hypertrophy of the myocyte is a normal component of heart maturation, during which both functional and structural development of the myocardium occurs (Bishop, 1984). During the neonatal growth period of the rat, ventricular myocytes cease to divide and become binuclear. All early increases in heart size are due to an approximate 30- to 40-fold increase (hypertrophy) in individual myocyte volume (Bishop, 1984; Zak, 1984). Because the ventricle is composed of several different types of cells, enlargement of the ventricular myocyte occurs, along with concomitant increases in the number of non-myocyte cells (Zak et al., 1979; Zak, 1984). To assess ventricular growth, many laboratories have utilized changes in ventricular DNA content per milligram of tissue (concentration) as a growth indicator (Korecky and French, 1967; Grove et al., 1969; Sen et al., 1974, 1976; Cutilleta et al., 1978). Growth by cellular hypertrophy of the ventricular myocyte population within a static non-myocyte cell population would result in a fall in the DNA concentration. Conversely, if cardiac growth results from simultaneous myocyte hypertrophy and non-myocyte hyperplasia, an unchanged or slight increase in the ventricular DNA concentration would occur. It has been consistently shown that ventricular DNA content slowly increases with age, and in association with a large increase in ventricular mass, the DNA concentration gradually declines (Sen et al., 1974, 1976; Cutilleta et al., 1978).

In contrast, many studies of neonatal ventricular growth in the normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rat have documented that SHR ventricles contain more DNA than do WKY ventricles, yet have a similar DNA concentration (Sen et al., 1976; Cutilleta et al., 1978). This difference in ventricular DNA content between WKY and SHR is found only during the first 3 weeks of development. From the time of birth until the rats are 4 weeks old, the ventricular myocyte:non-myocyte ratio goes from 4:1 to 1:1 (Rakusan, 1984). The increased ventricular DNA content of the SHR during this time may represent either accelerated myocyte or non-myocyte hyperplasia, or both. Alternatively, an increase in the ploidy levels of either type of cell also may occur. Because ventricular homogenates contain the nuclear contents of both myocytes and non-myocytes, a more accurate assessment of the myocyte contribution to total ventricular DNA would be obtained with isolated myocytes. In this report, we demonstrate that WKY and...
SHR myocytes isolated from fetal to 10-month-old animals show a significant age-related increase in nuclear ploidy levels. Further, the ploidy increases in both WKY and SHR are localized rapidly to specific ventricular regions. Such ploidy localizations, compared to conventional morphological and biochemical parameters, have significant implications for ventricular myocyte development and physiology.

Methods

Materials

Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) obtained from Taconic Farms were used in three developmental stages: timed pregnant females, and 4- and 13-week-old males. A group of 13-week-old SHR and WKY were maintained in our AALAC-approved facility until 10 months old. Collagenase (type CLS-II) was obtained from Millipore Corporation. Pyruvate, bovine serum albumin (BSA), all amino acids, and dextrose were obtained from U.S. Biochemicals. HEPES was obtained from Research Organics. Cytospin collection fluid and cytospin filter adapters were obtained from Boyce Scientific. Schiff’s base was obtained from Fluka Chemical Corp. Protein dye reagent was obtained from BioRad Laboratories. Chicken red blood cells (cRBC) were obtained in heparinized saline from Cleveland Scientific Co. All other reagents were obtained from Scientific Products.

Procedures

Myocyte Isolation

Fetal Myocytes: Timed pregnant female WKY and SHR were anesthetized with ether, near-term fetuses were collected, and their hearts were removed with the aid of surgical magnification glasses and placed in ice-cold Hanks’ balanced salt solution (HBSS). Hearts were pooled in groups of four to six, minced with a razor blade, and placed in the adult myocyte perfusion buffer. This perfusion buffer represents a modification of a previously described buffer utilized for hepatocyte isolation (Engelmann and Fierer, 1983), and consisted of a modified Ca++- and Mg++-free HBSS containing 10 mM pyruvate, 10 mM HEPES, 15 mM potassium bicarbonate, 5 mM glutamine, 11 mM dextrose, 1 mM nicotinamide, and 0.4 mM ascorbic acid. Further additions to the buffer were 1 mM adenosine (Claycomb et al., 1980), 1 mM ribose (Zimmer and Ihel, 1984; Zimmer et al., 1984), 1.2 mM MgCl₂, and 2 mM leucine (Gevers, 1984). The final pH and osmolarity after 0.22 μm filtration were 7.4 and 300 mOsmol, respectively. Before use, the buffer was gassed with 95% O₂/5% CO₂ for 1 hour, and collagenase was added to a final concentration of 1 mg/ml. No additional Ca++ was added to the collagenase buffer. Digestion was performed at 37°C with shaking in a water bath for 15 minutes. Released cells were then removed after nondigested fragments settled. Fresh digestion buffer (10 ml) was added to the remaining fragments. A series of six successive incubations was performed; the cells released during the first incubation were discarded. The remaining released cells were isolated by centrifugation at 200 g for 5 minutes and were resuspended in HBSS. The final cell pellet was resuspended in HBSS, the cell number was determined with a hemocytometer, and samples taken for cytospin preparations were used for light microscopy and nuclear ploidy determinations.

Neonatal and Adult Myocytes: Ventricular myocytes from rats more than 4 weeks old were obtained by retrograde perfusion through the aorta, using the perfusion buffer previously described. The buffer was perfused through two hearts simultaneously at 4 ml/min for 5 minutes. During this Ca++-free ‘washout’ period, the heart blanched, ceased beating immediately, and remained slightly distended. After 5 minutes, enzyme-containing buffer was added, and perfusion was continued with constant aeration for 60 to 90 minutes. The collagenase buffer was recirculated with no effect on myocyte viability or morphology. A minimum enzyme perfusion time of 60 minutes was required for young animals, with increasing times required for older animals with hearts containing higher collagen concentrations. Post-perfusion, the softened hearts were removed from the cannulation tubing, the atria and great vessels were removed, and the ventricles were dissociated into individual cells. Ventricular dissociation was performed either with both ventricles combined or with right ventricular free wall (RV), left ventricular free wall (LV), and septal (S) regions individually dissociated. All ventricular dissociations were performed with the aid of surgical magnification glasses to ensure accuracy. Post-perfusion digestion was in the same collagenase digestion buffer containing 1 mg/ml bovine serum albumin (BSA) for 10 minutes at 37°C in a 5% CO₂ incubator with frequent agitation and aspiration. After the post-perfusion digestion, the isolated ventricular myocytes were filtered through 400-μm nylon fibers (Tetko, Inc.), isolated by centrifugation (50 g, for 30 seconds), resuspended in enzyme-free buffer, and allowed to settle by gravity at room temperature (RT) for 10 minutes. The resulting cell populations were resuspended in buffer, cell concentration was determined with a hemocytometer, and samples were taken for cytospin preparations. All cytospin preparations of the isolated myocytes were made within 15 minutes of isolation for subsequent microspectrophotometric analysis. The remaining cells were used within 1 hour of isolation for subsequent biochemical analysis.

Cytospin Preparations

A sample of 10–25 X 10³ cells for cytospin preparation was placed in the specimen chamber of a Shandon Cyto- spin II, diluted with collection fluid (combination of fixatives and Carbowax), and centrifuged at 100 g for 4 minutes. The cytospin preparations were fixed, cell smears of individual myocytes located within a 4-mm circle on alcohol-cleaned slides. The cell preparations were then stained for routine morphological (hematoxylin and eosin, May-Grunwald geimsa) or microspectrophotometric analysis (Feulgen stain).

Ploidy Analysis

Microspectrophotometry of the cytospin preparations of isolated myocytes was performed by the two-wave-length method of Patau (1952), as described by Mendelson (1961), using a Zeiss Type 01 microscope photometer. All measurements were taken with a 100X (N.A. 1.25) planachromatic, oil-immersion objective ensuring that only one nuclei was read at a time. Instrument alignment and linear phototube response were always checked before use. The Patau method (1952) corrects for errors caused by the heterogeneous distribution of stained material. A minimum of 25 cells (1 nuclei/cell) were examined for each ventricular region or per pooled ventricular isolate.
Chicken red blood cells (cRBC) were used as internal controls for each analysis. Feulgen staining procedure was as follows: cells smears were dehydrated in 70% ethanol overnight at room temperature (RT), hydrolyzed in 5 N HCl at RT for 45 minutes, rinsed in distilled H2O, stained in Schiff's reagent for 1 hour at RT, rinsed in fresh 10% potassium-metabisulphite, dehydrated through a graded series of alcohols, and cleared in a xylene and glass coverslip mounted with Permount. All slides were stained with the same batch of acids and Schiff's reagent, to reduce variability with a coefficient of variation of 13%.

Biochemical Assays

Either isolated ventricular myocytes or intact ventricles were homogenized on ice in 0.05 M Tris, 0.15 M KCl (pH 7.3) with a Polytron PT-10 and were sonicated for 30 seconds at 10 mA. A sample was taken for protein analysis and the remaining homogenate was precipitated with 0.5 M perchloric acid (PCA). Protein analysis of total ventricular homogenates was performed by the method of Lowry et al. (1951); isolated myocyte protein content was determined by the method of Bradford (1976). The Lowry method (1951) was used for total ventricular homogenates due to the inability of the Bradford method (1976) to detect collagen accurately. After incubating at 4°C for 15 minutes, the precipitate was isolated by centrifugation at 2000 g for 10 minutes, washed once with 0.5 M PCA, resuspended in 3 ml of 0.3 M potassium hydroxide, and incubated overnight at 37°C for base hydrolysis of the RNA. After overnight incubation, 0.3 ml of 6 M PCA was added, protein and DNA were precipitated at 4°C for 15 minutes, precipitate was isolated by centrifugation at 1000 g for 10 minutes and 1 ml of the supernatant was assayed for RNA at 260 nm (Manro and Fleck, 1966). Yeast transfer RNA standards and calf thymus DNA standards (Sigma) were processed under identical conditions, with no loss of DNA during the overnight base hydrolysis. The remaining DNA/protein precipitate was analyzed for DNA concentration using the modified diphenylamine procedure described by Leyva and Kelly (1974).

Blood Pressure Measurements

In all rats, arterial blood pressure was measured by the tail-cuff method of Friedman and Freed (1949). The pressures were recorded by the same person, at approximately the same time of day; the last body weight and blood pressure measurements were taken the day of each experiment.

Statistical Analysis

All data are presented as the mean ± se. WKY and SHR data were analyzed by two-way analysis of variance and covariance (BMDP2V), and follow-up t-tests of group-by-group differences were evaluated by the Bonferroni method (Wallenstein et al., 1980). All pair-wise comparisons of individual values in Table 3 were performed using Duncan’s new multiple range test.

Results

To assess ventricular growth, we analyzed the total ventricular homogenates of 4-week- and 13-week-old SHR and WKY (Table 1). Similar animal body weights in both strains, with higher heart weights in the 4-week-old SHR (Table 1), document the frequently observed ventricular hypertrophy within SHR prior to established systemic hypertension. When analyzed biochemically, the 4-week-old SHR ventricle had a significantly (P < 0.05) higher total protein content, with no significant increase in DNA content or concentration. By 13 weeks, SHR blood pressures were established as hypertensive. At 13 weeks, SHR also were significantly (P < 0.05) smaller than WKY, yet had similar ventricular weights, resulting in a significantly higher (P < 0.05) heart weight:body weight ratio. Biochemical analysis of the total ventricular homogenate substantiated this hypertrophy in SHR (Table 1), in that the protein content demonstrated a significant (P < 0.05) increase. However, ventricular DNA content and concentration did not differ significantly between SHR and WKY at either 4 or 13 weeks. Although ventricular protein content was increasing more rapidly in the SHR, with only a slight elevation in DNA content relative to the WKY, these data show that hypertrophy is disproportionate to DNA concentration levels.

Mature ventricular myocytes were isolated by the described methods and routinely provided more than 4 × 10^6 myocytes per heart (Table 2). Isolated myocytes retained their rod shape and cross-sections (Fig. 1A) for more than an hour when maintained in Ca++-free buffer. When exposed to trypan blue, more than 95% of the rod-shaped myocytes, as well as 10–15% of the hypercontracted cells, excluded the vital dye. The average yield of rod-shaped myocytes was 90 ± 5% (n = 15) of the initial ventricle isolate, and 70% of these cells were tolerant of exposure to 1 mM Ca++ for 1 hour. Non-myocyte contamination of the myocytes used for biochemical analysis was minimal.

### Table 1

| Physiological Parameters and Biochemical Characteristics of Total Ventricular Homogenates from 4-Week- and 13-Week-Old Animals* |
|----------------------------------|------------------|------------------|------------------|------------------|
| Body wt (g) | Ventricle wt (mg) | HW:BW (mg/g) | BP (mm Hg) | Protein (mg) | RNA (mg) | DNA (mg) |
| 4-Wk WKY | 72.1 ± 4.0 | 241.7 ± 9.3 | 3.41 ± 0.12 | 83.3 ± 4.4 | 80.5 ± 11.1 | 1.09 ± 0.12 |
| 4-Wk SHR | 70.1 ± 3.1 | 262.0 ± 8.5 | 3.76 ± 0.07† | 103.0 ± 4.6 | 193.8 ± 8.7† | 1.12 ± 0.13 |
| 13-Wk WKY | 334.2 ± 11.7 | 895.4 ± 33.1 | 2.65 ± 0.04 | 123.0 ± 7.5 | 152.5 ± 16.1 | 1.32 ± 0.15 |
| 13-Wk SHR | 274.8 ± 8.9† | 879.3 ± 30.4 | 3.20 ± 0.05† | 159.0 ± 3.0† | 198.4 ± 7.4† | 1.73 ± 0.05* |

* Data expressed as mean ± se (n = 11 at 4 weeks and n = 6 at 13 weeks).
† Significantly different from age-matched WKY (P < 0.05).
TABLE 2

Biochemical Characteristics of Myocytes Isolated from 4- and 13-Week-Old Animals*

<table>
<thead>
<tr>
<th></th>
<th>Yield (10^6 cells/heart)</th>
<th>DNA (μg/10^6 cells)</th>
<th>RNA (μg/10^6 cells)</th>
<th>Protein (mg/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Wk WKY (5)</td>
<td>4.53 ± 0.60</td>
<td>7.42 ± 0.48</td>
<td>15.32 ± 0.61</td>
<td>2.30 ± 0.29</td>
</tr>
<tr>
<td>4-Wk SHR (4)</td>
<td>4.74 ± 0.73</td>
<td>6.75 ± 0.44</td>
<td>20.77 ± 1.49†</td>
<td>3.61 ± 0.30†</td>
</tr>
<tr>
<td>13-Wk WKY (4)</td>
<td>4.17 ± 0.34</td>
<td>7.79 ± 0.36</td>
<td>30.23 ± 2.84</td>
<td>3.08 ± 0.20</td>
</tr>
<tr>
<td>13-Wk SHR (4)</td>
<td>4.18 ± 0.38</td>
<td>9.46 ± 0.71†</td>
<td>54.11 ± 2.53†</td>
<td>5.34 ± 0.86†</td>
</tr>
</tbody>
</table>

* Data expressed as mean ± SE (number of animals per group).
† Significantly different from age-matched WKY (P < 0.05).

Analysis always was less than 5%. Histological examination of the myocytes isolated from 4-week-old WKY and SHR demonstrated an adult binucleation index (>80% binuclear), with no intraventricular variation in the binucleation index. As seen in Figure 1A, variations in nucleation state from mononuclear to polynuclear can be found in isolated, mature myocytes.

Biochemical analyses of myocytes pooled from both ventricles of rats at 4 and 13 weeks of age are shown in Table 2. At 4 weeks, both protein and RNA analysis of SHR myocytes showed significant increases (P < 0.05) relative to WKY. No significant difference in DNA content was detected when myocytes were pooled from both ventricles. By 13 weeks, SHR myocytes also demonstrated significant increases in protein (P < 0.05) and RNA (P < 0.05) relative to those from WKY. A significant increase (P < 0.05) in DNA content of the SHR myocytes was seen when pooled ventricular myocytes were used.

Microspectrophotometric analysis of isolated fetal, 4-week- and 13-week-old pooled ventricular myocytes from WKY and SHR are shown in Figure 2. Myocytes from fetal hearts were morphologically identified from non-myocyte cell types by both their larger size and eccentric nucleus (Fig. 1B). Only cells demonstrating this morphology were read microspectrophotometrically, because intracellular myofibrillar structures characteristic of fetal myocytes were unstained by the Feulgen reaction. Fetal myocytes were considered to represent the diploid (2C) value. The use of fetal myocytes as diploid standards is validated when compared to the cRBC internal standard. The cRBC have a preestablished DNA content of 2.5 pg/nucleus (Owens and Schwartz, 1982, 1983) that permits extrapolation so that the DNA content/nucleus of the fetal myocytes (=4.5 pg/nucleus) can be determined. In addition, the very tight grouping of the relative dye concentrations substantiates the uniform nature of the fetal myocyte nuclear DNA content (Fig. 2). No significant difference (P > 0.05) was observed in the mean relative dye concentrations of fetal myocytes from WKY and SHR (9.965 vs. 9.470). By 4 weeks of age, however, increases in the percentage of polyploid nuclei were seen in both WKY and SHR. There was a 3-fold greater percentage of polyploid nuclei in the SHR (Fig. 2). Further increases in the percentage of polyploid nuclei were seen in both WKY and SHR at 13 weeks. As was seen at 4 weeks, a 2-fold greater percentage of polyploid nuclei was found in the SHR (Fig. 2). Because the ploidy distribution of myocyte nuclei at 13 weeks in both SHR and WKY was heterogeneous, and represented myocytes

* FIGURE 1. Light micrograph of myocytes isolated from mature (panel A) or fetal (panel B) rats. Fetal myocytes (panel B) were distinguished from nonmuscle cell types by their larger size, eccentric nucleus, and occasional binuclear cell. Mature myocytes (panel A) demonstrated the typical rod-shape cell morphology with prominent cross-striations and various nucleation states. Final magnifications are 450x (panel A) and 865x (panel B). Hematoxylin and cosin stain.
pooled from all regions of both ventricles, possible intraventricular regional variations may have been obscured.

To examine this question of regional differences in ventricular myocyte ploidy levels, we separated the hearts into discrete regions after enzyme perfusion. The right ventricular free wall (RV), left ventricular free wall (LV), and intervening septum (S) were dissociated into single cell suspensions of myocytes isolated from each region. The myocytes isolated from each region then were analyzed microspectrophotometrically for nuclear ploidy changes.

At 4 weeks, strikingly unique ploidy distributions were found in both WKY and SHR on a regional basis (Fig. 3). The LV myocytes in both WKY and SHR were almost exclusively (>93%) diploid, whereas RV and S contained predominantly (>80%) polyploid nuclei. Figure 4 documents the persistent nature of this ventricular polyploid regionalization, in that, when 10-month-old WKY and SHR ventricles were examined, polyploidization again was predominant (>85%) in the RV and S. However, although the LV of both WKY and SHR retained their dominant (>85%) diploid characteristics with age, a significant (P < 0.05) increase in the percentage of polyploid nuclei was found in SHR that was not seen in WKY (Fig. 4).

To substantiate biochemically the regional ploidy differences detected microspectrophotometrically, the nucleic acid content of myocytes (<5% non-myocytes) isolated from separate ventricular regions of 13-week-old WKY and SHR was determined (Table 3). Consistent with our ploidy analysis, the LV myocytes from both WKY and SHR contained a significantly (P < 0.05) lower DNA content relative to the RV and S myocytes. As previously shown for pooled myocytes from the entire SHR ventricle (Table 2), a higher RNA content was found in myocytes isolated from each region, yet only in LV and S myocytes of the SHR did the increase reach significance (P < 0.05). The ratio of RNA to DNA content (RNA:DNA) suggests that LV myocytes of the SHR
were transcriptionally more active ($P < 0.02$) than the LV myocytes of the WKY, yet were similar in DNA content.

**Discussion**

Analysis of myocyte growth and development has been, until recently, confined largely to morphological, physiological, and total ventricular biochemical methods (Gray, 1984; Zak, 1984). In the current studies, analysis of heart growth by conventional total ventricular homogenization demonstrated characteristic age-related increases in total protein and nucleic acid contents (Table 1). Although the elevated blood pressure (BP) of the 4-week-old SHR failed to reach statistical significance, the higher BP of the SHR relative to the WKY at this age is consistent with previous observations that the 'prehypertensive' state of the SHR is associated with an elevated BP that may begin in utero (Gray, 1984). Bruno et al. (1979) have suggested that the 'prehypertensive' stage is absent in SHR. In contrast to previous studies of ventricular growth (Sen et al., 1974, 1976; Cutilleta et al., 1978), the total RNA content of the SHR ventricle was not significantly greater than that of the WKY. This variation may reflect strain and/or methodological differences, in that the standard orcinol method of RNA analysis is subject to interferences from both DNA and protein (Almog and Shirey, 1978). These interfering substances are eliminated during the RNA base hydrolysis/DNA-protein precipitation procedure used in this report. At 4 weeks, total ventricular homogenates of the SHR ventricle biochemically demonstrated significant protein increases with nonsignificant differences in DNA content compared to WKY. The elevated protein content of the SHR ventricles, when contrasted to the similar ventricular wet weights of the SHR and WKY, suggests that tissue hydration and/or cellular protein composition varies. Gray (1983, 1984) has described a significant ventricular hypertrophy, on a dry weight basis, in newborn and 10-day-old SHR at a time when ventricular wet weights and body weights were similar to those of WKY. In addition, Anversa et al. (1984a) have shown morphometrically that 4-week-old SHR myocytes have a significantly greater percentage of their cytoplasm filled with myofibrils compared to WKY. These authors also reported that early postnatal SHR myocyte growth is disproportionately greater in their cellular diameter compared to WKY. Taken together, these observations suggest that accelerated myocyte growth of the SHR has occurred during the first 4 weeks of life. Although these data substantiate previous publications on ventricular hy-

**Table 3**

<table>
<thead>
<tr>
<th>Nucleic-Acid Characteristics of Myocytes Isolated from Separate Ventricular Regions of 13-Week-Old Animals*</th>
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<tr>
<td><strong>RNA (μg/10⁶ cells)</strong></td>
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</tr>
<tr>
<td>WKY-LV</td>
</tr>
<tr>
<td>WKY-RV</td>
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<tr>
<td>WKY-S</td>
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<tr>
<td>SHR-LV</td>
</tr>
<tr>
<td>SHR-RV</td>
</tr>
<tr>
<td>SHR-S</td>
</tr>
</tbody>
</table>

* Data expressed as mean ± SE (n = 4).
† Significantly different from RV or S value ($P < 0.05$).
‡ Significantly different from similar WKY region ($P < 0.05$).
pertrophy (Sen et al., 1974, 1976; Cutilleta et al., 1978), they do not directly address the specific contribution of the myocyte component of the ventricle to these growth-related changes.

Using the continually evolving technique of ventricular myocyte isolation (Alischuld, et al., 1980; Wittenberg and Robinson, 1981; Farmer et al., 1983; Bihler et al., 1984), populations of myocytes free from contaminating non-myocyte cell types can now be morphologically and biochemically analyzed more accurately. When myocytes isolated from 4- and 13-week-old animals were analyzed biochemically (Table 2), SHR myocytes showed significant increases in both protein and RNA content relative to those from WKY. These differences were found in 4-week-old SHR animals, prior to any sustained hypertension. Although comparable data on isolated WKY and SHR myocytes have not been published previously, Katz et al. (1984) and Farmer et al. (1983) have reported that 10% myocytes isolated from 200–250 g stock rats contained approximately 4 mg of protein. Contrasted to the protein content of the myocytes from younger 4-week-old and similar 13-week-old SHR in the present study, the myocyte hypertrophy of the SHR is further substantiated.

Although multiple morphological and biochemical parameters of the developing rat ventricular myocyte have been documented, relatively few reports on changes in myocyte nuclear ploidy content have been described (Grove et al., 1969; Grimm et al., 1970; Stere and Anthony, 1977; Adler and Sandritter, 1980; Kasten et al., 1981). Grove et al. (1969), using 12- to 13-week-old female Sprague-Dawley rats, analyzed tissue sections of only the left ventricle by microspectrophotometry for changes in myocyte nuclear ploidy levels. They reported that less than 2% of the left ventricular myocyte nuclei were polyploid through 18 weeks of age. Although ventricular myocyte isolation methodologies have improved during the last decade, only an abstract by Kasten et al. (1981) has appeared on the analysis of nuclear ploidy levels of isolated ventricular myocytes. Using a cytofluorometric method, Kasten et al. (1981) reported only 5% of the ventricular myocyte nuclei were polyploid in 8-week-old rats. Despite the relative consistency in the reports of Grove et al. (1969) and Kasten et al. (1981), it may be significant that both used rat liver nuclei as controls, which somewhat jeopardizes interpretation of these results (Stere and Anthony, 1977). Because of the well-documented polyploidization of liver parenchymal cell nuclei (Brodsky and Uryvaeva, 1977), a more appropriate control cell for nuclear ploidy studies would be one with a well-established, consistent DNA value. In this regard, we have chosen the chicken red blood cell as our internal control cell and fetal myocytes as our diploid (2C) standard. The cRBC control cell is ideally suited for the cytospin method of cell smear preparation that was used for the isolated myocytes. The cRBC also has a well-defined DNA content (2.5 pg/nucleus) (Owens and Schwartz, 1982, 1983) that enables estimation of myocyte nuclear DNA content when stained together.

When pooled ventricular myocytes isolated from fetal, 4-, and 13-week-old animals were analyzed on this basis for nuclear ploidy changes (Fig. 2), a pronounced shift toward higher ploidy levels was found. When 4-week-old ventricular myocytes were analyzed, a gradual increase in tetraploid (4C) and octaploid (8C) cell nuclei was found. By 13 weeks, the ventricular myocyte nuclei from both WKY and SHR showed a decreasing percentage of 2C nuclei with increasing polyploidation. Our data contradict the conclusions of Grove et al. (1969) that few rat ventricular myocyte nuclei are polyploid. Because these authors examined only myocyte nuclei in the left ventricular free wall, the myocyte population from other areas of the ventricle were assumed to be similar in their ploidy levels. Although similar conclusions were drawn by Kasten et al. (1981), who used isolated myocytes, it is possible that methodological, standardizational (i.e., liver cell nuclei as standards), and regional differences influenced their data analysis. However, our data substantiate the observations by Stere and Anthony (1977) that most rat cardiac muscle cell nuclei are polyploid. They used cardiac fibroblasts that were taken from the same tissue section as 2C standards. Although ploidy analysis of the total ventricular isolate (Fig. 1) supports the findings of Stere and Anthony (1977), the exact region of the ventricle examined by these authors was not given. Because the ventricular myocytes used in this portion of the study represented a random distribution of cells from both ventricles and septum, the left ventricular predominance in cell numbers compared to the right ventricle (Anversa et al., 1980) may have obscured regional differences.

When myocytes isolated from separate regions of the ventricles from 4-week-old SHR and WKY were analyzed (Fig. 3), regional variation was observed with respect to nuclear ploidy levels. In both WKY and SHR, LV myocytes remained predominantly 2C, whereas RV and S myocytes were highly polyploid. These data clarify the reports by Grove et al. (1969) that LV myocytes are predominantly diploid. Although our data on regional variations in nuclear ploidy cannot be compared directly to the results of Stere and Anthony (1977), the authors' claim that 90% of rat myocyte nuclei are tetraploid is not completely supported in this report. This discrepancy may reflect the analysis of tissue sections of nuclei by the plug method from a ventricular region containing predominantly polyploid nuclei (i.e., RV and S), compared to the whole, intact nucleus of...
isolated cells in the current study. The mean relative dye concentrations of both LV and S myocyte nuclei from SHR were significantly higher than those of WKY at 4 weeks. The biochemical analysis of the DNA content of myocytes isolated from separate ventricular regions of 13-week-old animals substantiated the ploidy regionalization, in that LV myocytes contained a significantly lower DNA content compared to RV and S myocytes (Table 3). When ventricular myocytes isolated from 10-month-old animals were analyzed for regional ploidy differences (Fig. 4), it was found that the ploidy regionalization established by 4 weeks of age was maintained in both SHR and WKY. A similar ventricular ploidy pattern was found in aged American Wistar rats (data not shown).

The broad 2C and 4C peaks of the SHR myocytes may be related to increased transcriptional activity of the genome required to maintain the myocyte protein hypertrophy (Zak and Rabinowitz, 1979). Taylor and Tang (1985) recently reported that RNA polymerase activity in myocyte nuclei is 80–90% higher than in non-myocyte nuclei. The transcriptionally active chromatin is in an open conformation (Igo-Kemenes et al., 1982) and, therefore, more accessible to binding of the Feulgen stain, resulting in broader peaks. The basis for this stems from extensive studies, indicating that expansion of the chromatin template is frequently associated with an increase in the acid lability of the chromatin and increased staining. This has been described as the chromatin activation reaction that is indicative of a heightened metabolic state (Martin et al., 1984). The increased RNA transcriptional activity of the SHR was biochemically documented in isolated myocyte (pooled and regional) RNA content of the 13-week-old animals.

Although the ventricular myocytes cease mitotic activity by the time the rat is 2 weeks old (Zak, 1984), development of binuclear myocytes occurs concomitantly, and near adult binucleation levels are found by the time the animals are 4 weeks old (Anversa et al., 1980; Farmer et al., 1983; Clubb and Bishop, 1984). Korecky et al. (1979) have reported that both interventricular and intraventricular binucleation ratios in the rat are similar. Since we have also found this to be true in WKY and SHR, it indicates that regional analysis of the ventricular myocytes would not preferentially select cells with distinctly different binucleation ratios. Therefore, nearly all ventricular myocytes of the rat are "polyploid" due to their binucleation. Because myocytes undergo a normal physiological hypertrophy during ventricular growth, the normal nuclear:cytoplasmic ratio can be best maintained by binucleation. In contrast to the rat myocyte, the human ventricular myocyte is predominantly mononuclear, yet highly polyploid (Pfitzer, 1971). In both rat and human myocytes, the amount of genetic material required for the maintenance of cellular integrity is increased, but by different mechanisms. Similarly, Owens and Schwartz (1982, 1983) and Schwartz (1984) have reported age-related increases in the ploidy of aortic smooth muscle cells (SMC) in SHR. Although the SMC remain mononuclear, up to 30% of the cells become polyploid by 1 year of age.

During the normal aging process, the LV myocyte of the SHR has been shown to undergo additional (pathological) cellular hypertrophy beyond the "normal" physiological level (Zak, 1984). Owens and Schwartz (1982, 1983) have reported that the age-related increase in SMC hypertrophy is accompanied by increased polyploidation. The present results similarly suggest that ventricular myocytes may respond in the hypertensive state with an increase in nuclear ploidy levels. The mechanisms controlling this are unknown, but evidence in the literature (Sen et al., 1974, 1976; Yamori et al., 1979; Tarazi et al., 1983) suggests that myocyte hypertrophy may be independent of increased workload. In this regard, even at 4 weeks, before any sustained hypertension, pooled ventricular myocytes of SHR contained more polyploid nuclei than did those of WKY (Fig. 2). When regional ventricular ploidy differences of 4-week-old WKY and SHR were analyzed (Fig. 3), the increased ploidy levels were located only in the LV and S of the SHR. Oberpriller et al. (1983) have recently reported an age- and hypertension-mediated increase in the binucleation and ploidy levels of atrial myocytes. After left coronary artery ligation, the predominant method of polyploidization in atrial myocytes was binucleation, with a small increase in the nuclear ploidy level. These data, as well as ours, suggest that hypertension-stimulated hypertrophy of the myocardium may increase the genetic material needed for subsequent cellular growth, and that this may be accomplished by either polyploidization of individual nuclei, multinucleation, or both.

The striking regional differences in myocyte nuclear ploidy levels, to our knowledge, have never been reported. Because both WKY and SHR demonstrated a similar myocyte ploidy regionalization, with polyploidy being highest in RV and S regions that show limited hypertrophy, our results suggest that maintenance of a diploid nuclear content in the LV myocyte nuclei is required for a rapid hypertrophic response, which may not be unique to the SHR. Normal growth of the RV in WKY and SHR has been shown to be similar in magnitude and duration (Pfeffer et al., 1979; Anversa et al., 1984a), and results in greater than 80% of the maximum RV mass being established by 13 weeks of age. RV hypertrophy is detected only in aged or senescent SHR (Weiss and Lundgren, 1978; Pfeffer et al., 1979). Biochemical analysis of myocytes isolated from separate ventricular regions of 13-week-old animals suggest that RNA transcription is not significantly elevated in the RV of the SHR. In addition, the nuclear ploidy levels of the SHR RV myocytes
were not significantly different from those of 4-week-old WKY. In contrast, only approximately 55–60% of the maximum LV mass is seen by 13 weeks in the SHR and WKY (Pfeffer et al., 1979). RV hypertrophy experimentally induced by either exercise/endurance training or abdominal aorta/coronary artery stenosis results in only a 20–30% increase in RV mass after extended periods of time (Anversa et al., 1979, 1983, 1984a; Loud et al., 1984). In sharp contrast, short-term abdominal aortic stenosis produces a 92% increase in LV-free wall mass after only 8 days (Anversa et al., 1979). Since the current results show that RV myocyte nuclei are polypliod while LV nuclei remain diploid, the rapid response of the LV to both growth (hyperplasia) and hypertrophic stimuli may be directly related to these fundamental genetic differences, and the rapid polyploidy of RV nuclei may retard their hyperplastic and hypertrophic responses to stimuli. Myocyte nuclear ploidy variations were suggested by the morphological observations of Stere and Anthony (1979) who reported tremendous size variation of nuclei (7.8–39 μm long) in tissue sections. We have also observed variations in nuclei size of isolated myocytes; RV myocyte nuclei often are greatly enlarged, relative to the LV nuclei. Anversa et al. (1983) also have reported that the RV nuclei occupy a greater volume percentage of the cell than LV nuclei, a percentage that is accentuated by RV hypertrophy. A recent report by Goldberg et al. (1984) also suggests that tetraploid SMC have reduced replicative capacity in vitro, which mimics the in vivo data of Barrett et al. (1983) to the effect that atherosclerotic plaques contain predominantly diploid cells. Goldberg et al. (1984) propose that diploid cells possess a growth advantage over tetraploid cells when exposed to an unusual growth stimulus.

Based on the total ventricular DNA content and ploidy analysis of the myocytes isolated from 4-week-old animals, these data suggest that SHR ventricles contain fewer myocytes. Cellular RNA content was higher in isolated SHR myocytes—yet, total ventricular RNA was similar to that of WKY. Nuclear ploidy levels, were 15% higher in 4-week-old SHR myocytes isolated from the entire ventricle—yet, total ventricular DNA content was similar to that of WKY. If one assumes that non-myocyte cell numbers are the same in WKY and SHR, these data suggest that the SHR ventricle contains fewer myocytes. Although the isolation procedure did not produce different cell yields from the WKY and SHR, the procedure does not isolate the entire myocyte population contained in both ventricles. As is shown for myocyte ploidy, regional differences may also exist, as suggested by the ease with which myocyte populations can be isolated from within each ventricle. Oparil et al. (1984) recently reported that the newborn SHR ventricle contains more myocytes than the WKY—yet, by 15 days of age, the myocyte number is similar. During this 2-week neonatal growth period, therefore, SHR myocyte hyperplasia would have been retarded relative to WKY. In agreement with these authors, we also suggest that the early postnatal growth period of the SHR ventricular myocyte is altered in some manner so that accelerated maturation of the myocyte occurs. Because maturation of the ventricular myocyte is accompanied by loss of mitotic activity (Zak, 1984), accelerated maturation of the SHR myocyte would yield a smaller myocyte population in the SHR. This accelerated myocyte maturation may reflect the biochemical abnormalities seen in the early neonatal SHR such that ventricular DNA content is elevated relative to WKY (Sen et al., 1974; Cutilleta et al., 1978, data not shown). This elevation may represent the more rapid binucleation process seen in the SHR (Oparil et al., 1984), or the accelerated development of polyploidy as seen in this report, or both.

Without a sufficiently large myocyte population, the hypertrophic growth of the myocyte may fail to maintain the structural and functional integrity of the SHR ventricle with a prolonged work load. To compensate for this deficiency, an age-related increase in the extracellular matrix (i.e., collagen) of the SHR ventricle is required (Sen et al., 1976; Sen and Bumpus, 1979; Gilligan and Spector, 1984). The resulting increase in the percentage volume of the SHR ventricle occupied by extracellular material may also affect the diffusion of oxygen, nutrients, or both, to the myocytes (Tomanek and Hovanec, 1981; Tomanek et al., 1982; Loud and Anversa, 1984). This oxygen/nutrient deficiency may lead to necrosis and cell death, with resulting fibrosis that compounds the volume of extracellular material. In this regard, we have morphometrically detected an age-related increase in the percent volume of the SHR ventricle occupied by interstitial material (Vitulo, Engelmann, and Gerrity, in preparation). Concomitant with this increase in extracellular material, the capillary density of the ventricle decreases with age, suggesting that neovascularization does not occur. The decrease in capillary density, increase in extracellular material, and myocyte population deficiency could all contribute to the age-related increase in ventricular fibrosis and cellular necrosis seen in 18- and 24-month-old SHR (Pfeffer et al., 1979; Tomanek et al., 1984; Vitulo, Engelmann, and Gerrity, in preparation). A recent report by Yonekura et al. (1985) has described a regional variation in myocardial energy substrate uptake in the hypertrophied left ventricle. As a result of cardiac hypertrophy, the use of free fatty acids by the LV-free wall (normal energy substrate) declines, and is associated with an increase in glucose utilization. Glucose utilization as an energy substrate is indicative of a more anaerobic environment (Opie, 1984) that may have developed in the older SHR LV. The significant increase in cellular mass of LV myocytes appears to be largely independent of nu-
clear ploidy levels. Owens and Schwartz (1983) reported that there also was an increase in SMC cellular mass in the Goldblatt hypertension model that was independent of a change in cellular ploidy. An analysis of the cellular and molecular biology of isolated myocytes is required to address the hypothesis that, although cells from the LV of WKY and SHR contain predominantly diploid nuclei, myocytes from SHR are larger. The age- and hypertension-mediated increases in nuclear ploidy of the SHR may also be associated with cellular structural and functional changes detected in vivo. Further analysis of the molecular, biochemical, and morphological pattern of ventricular myocyte growth and of its relationship to nuclear status clearly is required for a complete understanding of this process.

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