Right Ventricular Hypertrophy Secondary to Pulmonary Hypertension Is Linked to Rat Chromosome 17
Evaluation of Cardiac Ryanodine Ryr2 Receptor as a Candidate

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Background—Fischer 344 (F344) rats are relatively resistant to hypoxia-induced right ventricular (RV) hypertrophy compared with the Wistar-Kyoto (WKY) strain. These 2 strains were used to examine the genetic basis for the differential response.

Methods and Results—Male F2 offspring from an F344×WKY intercross were exposed to hypoxia (10% O2) for 3 weeks, and pulmonary artery pressure and cardiac chamber weights were measured. Genomic DNA was screened by use of polymorphic microsatellite markers across the whole genome (excluding the sex chromosomes). A quantitative trait locus (QTL) for RV weight was identified on rat chromosome 17 (lod score 6.5) that accounted for 22% of the total variance of RV weight in the F2 population and was independent of pulmonary artery pressure. The peak was centered over marker D17Rat41, close to Chrm3, with a 1-lod support interval of 5 cM. Comparison of homologous regions in mice and humans suggested that Ryr2, the cardiac isoform of the ryanodine receptor, colocalizes with our QTL. A panel of somatic cell hybrids and fluorescence in situ hybridization mapped Ryr2 close to the gene Chrm3 within our QTL. [3H]Ryanodine binding to cardiac membranes from the parental strains showed a 21% reduction in Bmax in the WKY compared with the F344 strain, with no difference in Kd.

Conclusions—These data provide the first demonstration of a QTL linked to the RV response to hypoxia-induced pulmonary hypertension. The Ryr2 receptor gene lies within this QTL and merits further investigation as a candidate for this differential RV response. (Circulation. 2001;103:442-447.)

Key Words: genetics ▪ hypotrophy ▪ hypoxia

The normal adult pulmonary circulation is a low-pressure, low-resistance system with little or no resting vascular tone. The thin walls of pulmonary vessels and the relatively thin-walled right ventricle (RV) (compared with the left ventricle [LV]) reflect this low hemodynamic stress. Pulmonary artery pressure is increased by chronic hypoxia (eg, in patients with obstructive airway disease or people living at high altitude), and this leads to RV hypertrophy and premature death.1,2

The initial rise in pulmonary artery pressure during exposure to hypoxia is due to vasoconstriction. Chronic hypoxia leads to structural remodeling of pulmonary vessels and RV hypertrophy, features that are common to a number of species, including the rat.3 A number of pathological abnormalities have been described in the pressure-overloaded hypertrophied RV, which can impair myocardial hypertrophy, including polymerization of microtubules in cardiac myocytes4 and stimulation of interstitial collagen deposition.5 However, the biochemical processes involved in the hypertrophic myocardial response to pressure overload remain unclear.

In the present study, we report the differential susceptibility of the heart to pressure overload of 2 inbred strains of rats, the development of which has allowed us to perform a genetic study that implicates the cardiac ryanodine Ryr2 receptor in the hypertrophic response of the RV to hypoxia-induced pulmonary hypertension.

Methods

Animals and Genetic Crosses

Inbred Wistar-Kyoto rats (WKY, Charles River, UK) and Fischer 344 (F344) rats (Olac, UK) were used. Reciprocal mating of the
parental strains. WKY (male) × F344 (female) and WKY (female) × F344 (male), produced the first-generation F1 hybrid animals, and brother-to-sister mating produced an F2 segregating population. Male F2 progeny (n = 130) were selected for genotype and phenotype analysis. This number of animals is sufficient to detect 4 quantitative trait loci (QTLs) of additive effect (each explaining one fourth of the difference in RV weight between the WKY and F344 lines) with 80% power when all the progeny are genotyped and interval mapping is used to analyze the data.6

Animals were fed a regular diet (3.3% fat, 21.3% protein, and 58% carbohydrate) and maintained at a constant temperature (20°C to 22°C) with a 10-hour on/14-hour off light cycle. All procedures were conducted in accordance with Imperial College of Science, Technology, and Medicine guidelines.

**Phenotyping Procedure**

Male rats aged 12 weeks were placed in a normobaric hypoxic chamber for 21 days.7 Mean pulmonary artery pressure and systemic blood pressure were measured in the anesthetized animal (pentobarbital sodium, 60 mg/kg IP) via cannulas placed in the pulmonary artery (via the right jugular vein) and left carotid artery, respectively.8 Arterial blood was taken for estimation of hematocrit before the heart was removed and blotted. The RV was dissected free and weighed. The LV and septum were weighed together.

**Genomic DNA Preparation**

The liver and the kidneys were snap-frozen in liquid nitrogen and stored at −80°C until genomic DNA was extracted by use of a Nucleon genomic DNA extraction kit (Scotlab).

**Genotyping**

Polymerase chain reaction (PCR) amplification was used to genotype the F2 animals at 157 polymorphic microsatellite loci as described below.9 The PCR primer pairs (from Research Genetics or Genosys Biotechnologies) produced products that differed by 0.5% between the 2 strains, and the PCR conditions were optimized to permit visualization of the specific PCR products with ethidium bromide in agarose gel.9

In the primary screen, 55 F2 animals constituting the top and bottom quartiles of the cumulative frequency distribution of RV weight were genotyped across all the autosomes. The remaining 76 F2 animals were then genotyped by using markers for regions showing evidence of linkage (lod score > 1.5) in the primary screen.

**Linkage and Statistical Analysis**

The statistical significance of an interstrain difference in parental phenotype was determined by the Mann-Whitney U test. The significance level was set at P < 0.01.

The Mapmaker/EXP 3.0 and Mapmaker/QTL 1.1 computer packages were used to construct genetic linkage maps and to localize QTLs relative to the position of the microsatellite markers.10 Maximum lod scores supporting the presence of a QTL at each 1-cM interval along the genetic map were computed by assuming either a free, additive, dominant, or recessive mode of inheritance. The lod scores were then represented graphically in relation to chromosome position. In addition, an estimate of the fraction of the total variance explained by a particular QTL was obtained. The effect on the RV weight of alleles at the marker locus associated with the highest lod score was evaluated by 1-way ANOVA. To reduce type 1 errors, the lod score thresholds for significant linkage were set as follows: free genetics model, 4.3; dominant and recessive models, 3.4; and codominant model, 3.3.11 These thresholds correspond to a 0.05 probability of a false-positive in a genome scan.

**Somatic Cell Hybrid and FISH Analysis**

A panel of 15 (rat × mouse) somatic cell hybrids segregating rat chromosomes was used as described previously12 with the use of Southern blotting with a 0.76-kb cDNA probe derived from rat Ryr2 mRNA (gift from Prof F.A. Lai, University of Wales College of Medicine, Cardiff) to detect the Ryr2 gene.

Fluorescence in situ hybridization (FISH) was performed as described elsewhere.13 A 287-bp biotin-labeled probe was generated by PCR with use of the cloned cDNA as template and the following primers: forward 5′-GAGACAGACTACGGAGTTAC and reverse 5′-GAGAGGAGAGATGTCTCAAG.

**Ryanoine Binding Studies**

Sarcoplasmic membranes were prepared by homogenizing, on ice, the free wall of the RVs from individual normoxic rat hearts. The binding protocol was a modification of one described previously.14 Cardiac homogenate proteins (230 μg) were incubated for 60 minutes at 37°C with 0.1 to 50 nmol/L [9,21(n)-3 H]ryanodine (specific activity 1.85 to 3.7 TBq/mmol, 7.4 MBq/mL; Amersham Pharmacia Biotech) in 1 mL binding buffer containing 1 mol/L KCl, 25 mmol/L HEPES (pH 7.2), 0.3 mol/L sucrose, 0.1 mg/mL BSA, 1 mmol/L ATP, and 100 μmol/L CaCl2. To compare ryanoine binding sites in WKY and F344 hearts, we used the [Ca2+] (10 -1 mmol/L) that gave the highest Bmax in preliminary experiments. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled ryanoine (Sigma). The incubations were terminated by adding 5 mL ice-cold buffer, followed by rapid filtration through Whatman GF-B glass-fiber filters presoaked in 4% polyethyleneimine. Each filter was washed 3 times with 5 mL ice-cold buffer and air-dried. The radioactivity remaining on the filter was determined with use of an LKB liquid scintillation counter.

**Results**

**Hypoxia-Induced Pulmonary Hypertension and RV Hypertrophy**

Adult WKY and F344 rats have similar pulmonary artery pressures at rest when breathing air, but WKY have a lower systemic blood pressure and a greater cardiac mass (Table 1). Exposure to hypoxia (Fio2, 10%) for 21 days was associated with an increase in mean pulmonary artery pressure and RV weight in both strains but significantly more in the WKY strain. LV mass also increased more in the WKY strain despite a further fall in systemic blood pressure (Table 1). However, the LV measurement included the septum, which hypertrophies with the RV. Histology of the hypertrophied ventricles shows that the main cellular change is myocyte hypertrophy rather than proliferation of nonmyocyte cells or other cell types.

**TABLE 1. Phenotypic Differences Between F344 and WKY Strains**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Condition</th>
<th>BW, g</th>
<th>PAP, mm Hg</th>
<th>BP, mm Hg</th>
<th>RV, mg</th>
<th>LV, mg</th>
<th>RV/LV</th>
<th>Hct, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>F344 (n=12)</td>
<td>Normal air</td>
<td>290 (240–321)</td>
<td>16 (14–17)</td>
<td>121 (107–130)</td>
<td>139 (115–145)</td>
<td>536 (458–559)</td>
<td>0.25 (0.24–0.27)</td>
<td>46 (45–47)</td>
</tr>
<tr>
<td>F344 (n=23)</td>
<td>Hypoxia</td>
<td>238* (210–260)</td>
<td>27* (25–28)</td>
<td>112 (102–133)</td>
<td>207* (194–225)</td>
<td>553 (491–555)</td>
<td>0.41* (0.36–0.44)</td>
<td>70* (69–71)</td>
</tr>
<tr>
<td>WKY (n=12)</td>
<td>Normal air</td>
<td>250 (248–310)</td>
<td>15 (14–15)</td>
<td>91* (89–106)</td>
<td>179* (163–217)</td>
<td>599 (532–678)</td>
<td>0.31* (0.28–0.34)</td>
<td>45 (44–46)</td>
</tr>
<tr>
<td>WKY (n=28)</td>
<td>Hypoxia</td>
<td>270 (255–290)</td>
<td>38*‡ (36–39)</td>
<td>78*‡ (70–89)</td>
<td>417*‡ (383–460)</td>
<td>765*‡ (714–796)</td>
<td>0.52*‡ (0.51–0.59)</td>
<td>71* (69–71)</td>
</tr>
</tbody>
</table>

Data are median (interquartile range). BW indicates body weight; PAP, pulmonary artery pressure; BP, systemic blood pressure; and Hct, hematocrit.

*P < 0.01 vs normal-air group within same strain; † P < 0.01 vs F344 normal air group; and ‡ P < 0.01 for hypoxic WKY vs hypoxic F344.
excessive collagen deposition (data not shown). The rise in hematocrit was the same in the 2 strains, making it unlikely that the strain difference is due to variation in oxygen sensing.

We examined the following phenotypes in a WKY×F344 intercross: (1) RV weight, (2) RV/body weight ratio, (3) RV weight/LV weight ratio, (4) LV weight alone, and (5) pulmonary artery pressure. Because the septum hypertrophies as part of the RV response, expressing RV as a ratio of LV+septum weight reduced the interstrain differences in RV hypertrophy. For these reasons and because the animals were all studied at the same age, we focused on uncorrected RV weight.

F₁ (n=27) and male F₂ (n=130) animals from a WKY×F344 intercross showed an intermediate rise in pulmonary artery pressure and RV mass compared with the parental strains (Figure 1). The average of the phenotypic variances of the 3 nonsegregating generations (WKY, F344, and F₁) was used to estimate the environmental variance of RV weight. The proportion of the total F₂ variance that was due to genetic variation, expressed as heritability, was calculated according to the following formula: (total F₂ variance−environmental variance)×100/total F₂ variance; the result was found to be 64%. Comparison of the variances in the segregating generations with those in the nonsegregating generations indicated the involvement of at least 3 or 4 genes in the interstrain difference of the hypertrophic response of the RV.

### Linkage of RV Weight to Rat Chromosome 17 QTL

A genetic linkage map for each of the 20 rat autosomes was constructed with genotypic data from the male F₁ rats and a panel of 157 polymorphic microsatellite markers (the X and Y chromosomes were not screened). The best map order for the markers was determined by multipoint linkage analysis, and the genetic distances between markers were calculated by using the Haldane mapping function. We estimate that our markers cover >99% of the rat autosomes on the basis of the recent reevaluation of the genome size, with an average intermarker distance of 20 cM.

A genetic linkage map for rat chromosome 17 was generated with the data from 15 markers for all 130 F₂ progeny. Using the Mapmaker/QTL program, we found a QTL on rat chromosome 17 that had a major affect on RV weight (Figure 2). This QTL was supported by a lod score of 6.5, accounted for 22% of the total variance of RV weight in the F₂ population, and was independent of pulmonary artery pressure (lod score 1.6). The peak was centered over the marker D17rat41, close to Chrm3, with a 1-lod support interval of 5 cM. The linkage persisted when RV weight was expressed as a ratio of body weight (lod score 4.5) to LV+septum weight (lod score 3.7).

The results of 1-way ANOVA of the cosegregation of RV weight with D17Rat41 are shown in Table 2. This locus appears to affect RV weight in a dominant fashion, inasmuch as the F₂ animals homoygous for the WKY (WW) or F344 (FF) allele had the largest and smallest mean RV weights,

**Figure 1.** RV weight in parental, F₁, and F₂ generations. After 21 days in normobaric hypoxic chamber (FIO₂ 10%), RV weight is greater in WKY strain. F₁ (n=27) and male F₂ (n=130) animals showed intermediate RV mass.

**Figure 2.** Genetic linkage map of rat chromosome 17 showing RV weight QTL. Multipoint lod score plots were computed assessing either free, dominant, codominant, or recessive genetic model. Representation of genetic linkage map of rat chromosome 17 was produced using Mapmaker/EXP 3.0 and data obtained from analysis of 157 polymorphic microsatellite markers in 130 F₂ progeny of WKY×F344 cross. Distances between markers are given in centimorgans. Linkage with RV weight was supported by lod score of 6.5 for both dominant and free genetic model, 4.8 for an additive model, and 0.9 for recessive model. AT₁ indicates angiotensin type 1.
respectively, and the mean values for the heterozygotes (WF) were indistinguishable from those of the homozygous F344 group. Rats homozygous for the WKY allele at the marker D17Rat41 have a RV weight $\sim 52.4$ mg greater than those homozygous for the F344 allele ($P<0.001$).

No other QTLs for RV weight and no QTLs for LV weight or pulmonary artery pressure were identified in this cross that met our stringent criteria (see Linkage and Statistical Analysis) (Figure 3). Even though the maximal distance between markers was never $>31$ cM, it is possible that we may have missed genetic factors with an autosomal-recessive or sex-linked pattern of inheritance or autosomal-dominant loci of small effect.

Localization of Ryr2 to Rat Chromosome 17

Inspection of the mouse and human genetic maps homologous to our QTL on rat chromosome 17 suggested that Ryr2, the gene encoding the cardiac form of the ryanodine receptor, is in the region of interest. The rat Ryr2 gene was first assigned to this rat chromosome by using a panel of 15 (rat x mouse) somatic cell hybrids segregating rat chromosomes. A 0.76-kb rat Ryr2 cDNA probe detected 6 fragments when rat genomic DNA was digested with the restriction enzyme PstI (5.0, 4.2, 3.5, 3.0, 2.6, and 1.7 kb) (data not shown). The 2 most intense bands (5.0 and 3.5 kb), as well as the 2.6- and 1.7-kb bands, were easily distinguishable from the bands obtained with digested mouse genomic DNA (8.9, 8.1, 5.8, 4.2, 4.0, and 2.8 kb). The 4 unique rat fragments segregated together with rat chromosome 17 (data not shown), as did other genes previously assigned to this chromosome, eg, Chrm3 (17q12.1) and Tpl2 (17q12.1-q12.2). To confirm the position of Ryr2 within rat chromosome 17, FISH was performed with the use of a biotin-labeled 289-bp rat Ryr2 cDNA probe. Double spots (2 labeled sister chromatids) were found only on rat chromosome 17 (Figure 4). The fluorescent signals were located in the distal half of 17q, namely, in the region 17q12.2-q12.3. Because the human homologues CHRM3, RYR2, and TPL2 are separated (CHRM3 and RYR2 map to human chromosome 1,17,18 whereas TPL2 maps to human chromosome 1019), the most likely order of the rat genes on rat chromosome 17 is as follows: 17qcentr-Chrm3-Ryr2-Tpl2-17qtel.

Ryanodine Binding Studies

To explore the role of Ryr2 in our rat cross, we looked for evidence of a difference in functional expression of this channel in the 2 strains. Ryr2 is the predominant form of the ryanodine receptor in myocardium. Therefore, we performed ligand-binding studies with the use of [3H]ryanodine and homogenates of the RV containing sarcoplasmic reticular
membranes. There was a significant 21% reduction in $B_{\text{max}}$ for ryanodine binding to cardiac membranes from normal WKY compared with F344 animals, with no significant difference in $K_\text{d}$ (Figure 5).

**Discussion**

QTLs contributing to the regulation of adult cardiac mass and blood pressure have been reported on several rat chromosomes, including 1, 20 3, 21 and 8. 22 In these cases, the cardiac mass phenotype may be acting as a surrogate for the pressure load. On the other hand, genetic loci for cardiac mass that are independent of the influence of blood pressure have been reported on several rat chromosomes, including 1, 20 3, 21 and 8. 22 In these cases, the cardiac hypertrophy in several species, including the rat, there is a reduction of receptor density and Ryr2 mRNA that correlates with a decrease in $\text{Ca}^{2+}$ transient. 29, 30 However, it has been unclear whether these changes are primary events or a consequence of the hypertrophic process. In support of the former is the observation that administration of FK-506 (tacrolimus), an immunosuppressant that binds to FKBP12.6, has been associated with the development of hypertrophic cardiomyopathy in children. 31, 32 FKBP12.6 itself is known to bind to and modulate channel gating of the Ryr2 receptor. 33, 34 Knockout mice lacking either FKBP12 or Ryr2 die during embryogenesis, principally because of disruption of heart tube development.

In conclusion, we have described segregation and linkage data that implicate a gene on rat chromosome 17 in the hypertrophic response of the RV to hypoxia-induced pulmonary hypertension. We have also presented the first evidence that Ryr2 is in the vicinity of the QTL, and we clearly demonstrate a difference between F344 and WKY in the binding of [3H]ryanodine to myocardial homogenates. Significantly, these differences in binding are evident before exposure to hypoxia. It is of considerable interest that our previous study examining the genetic factors controlling normal cardiac mass in these 2 strains identified a QTL on rat chromosome 3 but not on chromosome 17, 9 highlighting the differences between the genetic mechanisms controlling normal cardiac growth and pathological hypertrophy. It is interesting to speculate that the greater cardiac hypertrophy in the WKY strain results from an attempt of cardiac myocytes to compensate for a lower abundance of ryanodine receptors compared with those in the myocardium of F344 rats. If true, mechanisms to improve cardiac Ryr2 activity and increase contractile efficiency may, paradoxically, reduce the stimulus to hypertrophy and the potential to develop cardiac failure.

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References


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