Age- and Training-Dependent Development of Arrhythmogenic Right Ventricular Cardiomyopathy in Heterozygous Plakoglobin-Deficient Mice

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Background—Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited disorder that causes sudden death and right ventricular heart failure in the young. Clinical data suggest that competitive sports may provoke ARVC in susceptible persons. Genetically, loss-of-function mutations in desmosomal proteins (plakophilin, desmplakin, or plakoglobin) have been associated with ARVC. To test the hypothesis that reduced desmosomal protein expression causes ARVC, we studied the cardiac effects of heterozygous plakoglobin deficiency in mice.

Methods and Results—Ten-month-old heterozygous plakoglobin-deficient mice (plakoglobin+/−) had increased right ventricular volume, reduced right ventricular function, and spontaneous ventricular ectopy (all P<0.05). Left ventricular size and function were not altered. Isolated, perfused plakoglobin+/− hearts had spontaneous ventricular tachycardia of right ventricular origin and prolonged right ventricular conduction times compared with wild-type hearts. Endurance training accelerated the development of right ventricular dysfunction and arrhythmias in plakoglobin+/− mice. Histology and electron microscopy did not identify right ventricular abnormalities in affected animals.

Conclusions—Heterozygous plakoglobin deficiency provokes ARVC. Manifestation of the phenotype is accelerated by endurance training. This suggests a functional role for plakoglobin and training in the development of ARVC. (Circulation. 2006;114:1799-1806.)

Key Words: adherens junctions ■ arrhythmia ■ cardiomyopathy ■ conduction ■ death, sudden ■ desmosomes ■ exercise

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is one of the most common inherited cardiomyopathies and a prevalent cause of ventricular arrhythmias and sudden death in young adults, especially in competitive athletes.1–3 ARVC often presents with ventricular tachycardias of right ventricular origin and causes right ventricular enlargement and dysfunction.1,2 Genetic studies have identified mutations in junctional proteins in patients with ARVC, eg, in plakophilin, plakoglobin, desmoglein, and the plakoglobin binding site of desmplakin.4–9 Most of the mutations are predicted to cause loss of function. Genetically, ARVC is therefore considered a disease of the desmosome.5

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Plakoglobin forms part of the mechanical intercellular connections organized in the adherens junctions and desmosomes.10 Homozygous plakoglobin mutations are associated with Naxos disease, an autosomal-recessive form of ARVC characterized by additional palmo-plantar hyperkeratosis and woolly hair.4,11 Expression of dysfunctional, mutated proteins will diminish mechanical adhesion of cardiomyocytes,12,13 analogous to the effect of plakoglobin deficiency in keratinocytes.13–15 Diminished mechanical adhesion between cardiomyocytes could result in preferential right ventricular...
dysfunction resulting from the thinner wall of the right ventricle, which may be more sensitive to reduced mechanical stability.

To investigate whether desmosomal dysfunction can be the cause of ARVC, we studied heterozygous plakoglobin-deficient mice (plakoglobin<sup>+/−</sup>). We found that plakoglobin deficiency provokes the full functional phenotype of ARVC. Aggravation of ARVC by endurance training supports the hypothesis that susceptibility to mechanical cardiac strain contributes to ARVC in this model.

**Methods**

**Transgenic Mice**

Heterozygous plakoglobin-deficient mice (plakoglobin<sup>+/−</sup>) and their wild-type siblings (bred by P.R. and H.W. at the Cardiovascular Research Center, Berlin, Germany) were studied. All functional analyses were performed by researchers blinded to genotype in age- and sex-matched littermate pairs of mice. All experiments were approved by the Institutional Review Board at the University of Muenster.

**Cardiac Morphology and Function In Vivo**

Sedated mice (diazepam 17.5 mg/kg body weight IP or oxygenated isoflurane 1.5% by inhalation, 37°C body temperature) were studied on a murine echocardiography system (Phillips Sonos 5500, Philips, Hamburg, Germany; 15-MHz linear transducer, 12-MHz Doppler transducer<sup>16–19</sup>). Cardiac dimensions and function were assessed by M-mode, 2-dimensional, and Doppler echocardiography. Right ventricular measurements derived from a clinical study protocol were performed by researchers blinded to genotype in age- and sex-matched littermate pairs of mice. Methodology of ARVC was approved by the Institutional Review Board at the University of Muenster.

**Histology, Immunohistochemistry, and Electron Microscopy**

Cardiac tissue was fixed in 3.7% formalin and stained with hematoxylin and eosin and Goldner’s trichrome staining. Cardiomyocyte diameter was measured in 100 right and left ventricular cells per heart at the level of the nucleus. For immunohistochemistry, 5-μm paraffin sections were dewaxed and rehydrated in a series of alcohols, autoclaved (0.01 mol/L citrate buffer, 45 minutes), and incubated with a plakoglobin antibody (1:1500 in 0.6% bovine serum albumin, Transduction Clone 15, San Diego, Calif). Sections were then incubated with a rabbit anti-mouse bridging antibody (1:30 in phosphate-buffered saline, 45 minutes; Dako, Glostrup, Denmark) and a polyclonal mouse APAAP complex (1:100 in RPMI, 60 minutes; Dianova, Hamburg, Germany) and developed for 25 minutes in new fuchsin solution containing naphthol-bis-phosphate and levamisole. Sections were counterstained with hematoxylin and eosin and mounted with Kayser’s glycerin gelatin. For electron microscopy, small right and left ventricular tissue samples were fixed in 0.1 mol/L cacodylate-buffered 4% glutaraldehyde for 4 hours at 4°C, postfixed in 1% osmium tetroxide, stained en bloc in 2% uranyl acetate, and embedded in Epox. Semithin sections for light microscopy were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM 201 transmission electron microscope.

**Electrophysiology in Isolated, Langendorff-Perfused Hearts**

The heart was excised and immediately perfused on a Langendorff apparatus.<sup>17,21</sup> The atroventricular node was ablated to allow for slow ventricular rhythms, and 3 monophasic action potential cathe- ters were simultaneously recorded from the left ventricular free wall, right ventricular free wall, and septal epicardium. Initially, sponta- neous rhythm after atroventricular block was observed. This inter- vention can provoke spontaneous arrhythmias in susceptible hearts.<sup>16,21</sup> Thereafter, pacing was performed at 100-, 150-, and 200-ms pacing cycle length to measure steady-state action potential durations and activation times, followed by programmed right ventricular endocardial stimulation using up to 2 premature stimuli.<sup>16</sup> The protocol was repeated during continuous infusion of orciprena- line (1.6 μmol/L).

**Myocardial Glucose-Uptake Positron-Emission Tomography**

Myocardial glucose uptake was measured in anesthetized mice (inhaled isoflurane 1.5%, 37°C body temperature). Sixty minutes after intravenous injection of 10 MBq [18F]fluor-desoxy-glucose, the emitted positrons were measured with a high-resolution small- animal positron-emission tomography camera (quadHIDAC, Oxford Positron Ltd, Oxford, England) for 15 minutes.<sup>25</sup> Images were reconstructed into a matrix with a voxel size of 0.4 mm<sup>3</sup>. Myocardial glucose metabolism was quantified by dividing myocardial uptake of [18F]fluor-desoxy-glucose in the left ventricle by the injected dose.

**Endurance Training**

Four to 6 pairs of littermate mice swim simultaneously in a 30×50-cm-large, 20-cm-deep container containing 35°C water and a floating rotating wheel. Swimming duration was increased from 5 to 90 minutes per day over the 8-week training period (6 d/wk). Daily training intensity was quantified using a semiquantitative activity score defined as follows: running on the floating wheel=2, swimming=1, and floating on the water=0.

**Right Ventricular Gene Expression Analysis**

Gene expression analyses were performed in right ventricles taken from 5 severely affected plakoglobin<sup>+/−</sup> mice and their wild-type littermates (n=4 pairs of trained mice, n=1 pair of 10-month-old mice). Each right ventricular specimen was analyzed separately. Mouse pairs were selected by marked right ventricular enlargement in the plakoglobin<sup>+/−</sup> sibling on echocardiography. Tissue was rapidly extracted and stored in liquid nitrogen. Amplification and labeling of the RNA samples followed usual protocols (Affymetrix, Santa Clara, Calif). Total RNA was quantified by UV spectroscopy and analyzed on a LabChip (BioAnalyzer, AGILENT Technologies, Santa Clara, Calif). In the presence of an oligo(dT)<sub>24</sub> primer containing a T7 RNA polymerase promoter (TIBMOL Biol, Berlin, Germany), 1 to 3 μg total RNA was used for double-stranded cDNA synthesis (SuperScript Transcriptase, Life Technologies, Inc, Carls- bad, Calif). Labeled cRNA was prepared from double-stranded cDNA by in vitro transcription (GeneChip RNA transcript labeling kit, Affymetrix). After cleanup (Qiagen, Hilden, Germany), the biotin-labeled cRNA was fragmented by alkaline treatment (40 mmol/L Tris-acetate [pH 8.2], 100 mmol/L potassium acetate, and 50 mmol/L magnesium acetate) at 94°C for 35 minutes. We hybridized 15 μg of each cRNA sample to Affymetrix RAE 430A GeneChip arrays (22 500 probe sets). Probe arrays were washed and scanned at 3-μm resolution with the Affymetrix GeneChip System ConfoScan 2500. Data were analyzed with the Affymetrix GeneChip Operating Software, normalized to a global intensity of 500. Significance analyses of microarrays and hierarchical clustering were used for statistics.
Statistical Analysis
All measurements and analyses were performed by researchers blinded to genotype. All variables are reported as mean±SEM. Continuous variables were compared by use of Student t test. Both paired and unpaired tests were used to compare results in littermate pairs. The results of paired and unpaired testing were congruent in this study. Two-way ANOVA and regression analyses were used to assess the effect of genotype and age/training on functional parameters. Fisher exact test and χ² test were used to compare categorical variables. Two-sided values of P<0.05 were considered significant. SPSS (version 12; SPSS, Inc, Chicago Ill) was used to compute statistics.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

Age- and Training-Dependent Right Ventricular Enlargement in Plakoglobin+/− Mice
At the age of 10 months, right ventricles were enlarged in plakoglobin+/− mice (Figure 1A and 1B and Table). Endurance training (8 weeks of daily swimming) caused premature right ventricular dilatation and dysfunction in plakoglobin+/− mice at 5 to 6 months of age (Figure 1 and Table). Training intensity was not different between plakoglobin+/− and wild-type mice. Training intensity correlated with training-induced right ventricular enlargement in plakoglobin+/− mice (Figure 2B). There was no difference in left ventricular size or function between genotypes. Right heart size (Table) and function (Figure 1C and 1D) were not different between genotypes at earlier ages. Cardiac glucose uptake as assessed by positron-emission tomography was normal in plakoglobin+/− mice (3-month-old mice [n=8 per genotype]: plakoglobin+/− 3.8±1.6% versus wild-type 3.5±1.9% of injected dose, P=0.77; trained mice [n=12 per genotype]: plakoglobin+/− 3.8±1.3% versus wild-type 3.2±1.2%, P=0.18). There were no excess deaths in plakoglobin+/− mice in this study.

Spontaneous Ventricular Arrhythmias in Freely Roaming Plakoglobin+/− Mice
Ten-month-old plakoglobin+/− mice showed increased spontaneous ventricular ectopy (Figure 3; during normal activity, plakoglobin+/− 0.64±0.3 ventricular premature beats [VPBs] per hour versus wild-type 0.35±0.2 VPBs per hour, P=0.48; during stress, plakoglobin+/− 2.19±0.6 VPBs per hour versus wild-type 1.26±0.5 VPBs per hour; P=0.14). Ventricular arrhythmias were not increased in younger plakoglobin+/− mice.

Spontaneous Ventricular Tachycardias of Right Ventricular Origin in 10-Month-Old and Trained Plakoglobin+/− Mouse Hearts
Spontaneous ventricular tachycardias were frequent in 10-month-old and trained plakoglobin+/− hearts (Figure 4). Arrhythmias were self-terminating in all but 2 plakoglobin+/− hearts and in all wild-type hearts. Arrhythmia duration was 12.2±6.7 seconds (minimal duration, 0.3 seconds; maximal duration, 132 seconds) in 21 plakoglobin+/− hearts and 3.2±0.7 seconds (minimal duration, 0.4 seconds; maximal duration, 6.4 seconds) in 8 littermate wild-type hearts. In
plakoglobin \(^{+/−}\) hearts, ventricular arrhythmias originated from the right ventricular free wall or septum, as evident from the site of earliest activation in the first tachycardia beat, but never from the left ventricular free wall (Figure 5). Spontaneous arrhythmias in wild-type hearts originated in equal proportions from the 3 recording sites.

**Right Ventricular Conduction Slowing in 10-Month-Old and Trained Plakoglobin \(^{+/−}\) Mice**

Right ventricular activation times were prolonged in 10-month-old and trained plakoglobin \(^{+/−}\) mouse hearts, ie, the conditions associated with ventricular tachycardias (Figure 5B). Left ventricular activation times and right and left ventricular action potential durations were not different between genotypes (Table).

**Normal Right Ventricular Structure in Old and Trained Plakoglobin \(^{+/−}\) Hearts**

There was no sign of increased fibrosis, cardiomyocyte hypertrophy (Table), or replacement of myocardium with fibrofatty tissue in right ventricles of affected plakoglobin \(^{+/−}\) mice \((n=4\) ten-month-old mice, \(n=10\) trained plakoglobin \(^{+/−}\) mice) compared with wild-type littersmates. Desmosomal and adherens junctions’ structure was not different between genotypes (electron microscopy; Figure 6). Connexin43 distribution and density were not altered in immunohistochemical images stained for connexin43 \((n=8\) plakoglobin \(^{+/−}\) hearts selected by marked phenotype and wild-type littersmates; data not shown).

**Right Ventricular Gene Expression Profiling in Plakoglobin \(^{+/−}\) and Wild-Type Mice**

Training induced expression of several hypertrophy-associated genes (Figure 2A). The expression patterns between wild-type and plakoglobin \(^{+/−}\) samples were not different, even when different statistical approaches, eg, combination of littermates (significant analysis of microarrays: 1 class response) or calculation with logarithmic or linear expression values, were used. Hierarchical clustering...
of the 10 samples using all “expressed” genes (~8800 gene tags) separated between untrained control mice and trained mice but not between genotypes (Figure 2A, right). Expression data are accessible at http://www.ncbi.nlm.nih.gov/projects/geo/ (GEO number GSE4120).

Discussion

Age- and Training-Dependent Development of ARVC in Plakoglobin+/− Mice

The present study demonstrates for the first time that reduced expression of the junctional protein plakoglobin is sufficient for the development of an ARVC-like phenotype. These data strongly support the pathophysiological concept derived from genetic abnormalities of junctional proteins in ARVC patients4–6,24,25 that decreased expression and function of junctional proteins is a key element in the genetic predisposition to ARVC. Furthermore, endurance training can worsen right ventricular function and provoke right ventricular arrhythmias in this murine model of reduced plakoglobin expression. These data support the recommendation that patients with ARVC should avoid endurance training.26 Different individual degrees of physical activity could contribute to incomplete penetrance or atypical manifestation of ARVC in patients carrying mutations in desmosomal proteins.7

Similar to patients with ARVC,1,2 old and trained plakoglobin+/− mice developed ventricular arrhythmias of right ventricular origin. This is of special note because Naxos disease, the clinical entity with mutations in the plakoglobin gene, is an autosomal-recessive form of ARVC.4 The most prominent electrophysiological abnormality was conduction slowing in the right ventricle (Figure 5), consistent with prolonged QRS duration in the signal-averaged ECG in ARVC patients.27 In contrast, cardiac repolarization was not altered in plakoglobin+/− mice. It is usually assumed that right ventricular conduction slowing in ARVC, including Naxos disease,28,29 is due to anatomic barriers (focal fibrofatty replacement)1 or to markedly reduced cardiac connexin43 expression. Much to our surprise,1,28,29 we found neither fibrofatty replacement of right ventricular myocardium nor reduced connexin43 expression or altered gene expression profiles in affected right ventricles from plakoglobin+/− hearts. Hypothetically, this discrepancy could have 3 explanations. First, the histological changes found in ARVC patients—often analyzed postmortem—may develop later than the functional changes, concordant with occasional reports that the clinical cardiac manifestations of Naxos disease may develop without histological abnormalities in the heart in younger patients.7,28 Second, the structural abnormalities found in ARVC patients may be due to genetic abnormalities other than those resulting in plakoglobin deficiency. Third, abnormal function of mutated junctional proteins (as opposed to reduced levels of normal
proteins in our model) may cause the structural changes in ARVC.

Further studies are required to elucidate the relation between functional and structural changes in different models and populations of ARVC.

Comparison With Other Models of Disrupted Myocardial Intercellular Connections

Our findings contrast to those in laminin-receptor 1–deficient mice, which develop fibrofatty replacement of the right ventricles, although their cardiac electrophysiology has not been studied. Heart-directed or inducible disruption of N-cadherin causes loss of intercalated disks, marked reduction in connexin43 expression, and ventricular arrhythmias. In contrast to N-cadherin, plakoglobin is a linker protein that connects the cytoplasmic portion of the desmosomes and adherens junctions to the intracellular mechanical structure formed by actin and intermediate filaments. Concordant with this functional difference, the functional consequences of N-cadherin deficiency differ from those of plakoglobin deficiency: Inducible disruption of N-cadherin reduces left ventricular function, and plakoglobin deficiency causes right ventricular dysfunction (Figure 1). Heart-directed N-cadherin deficiency causes inducible ventricular arrhythmias; plakoglobin deficiency causes spontaneous ventricular arrhythmias (Figures 3 and 4).

Plakoglobin Deficiency: A Functional Defect That Causes ARVC?

Reduced plakoglobin expression could hypothetically reduce adhesive forces in the heart, comparable to the effect of plakoglobin deficiency in keratinocytes, which may be attributed to a reduced number of functional desmosomes and to reduced mechanical contacts between desmosomes...
and the intracellular cytoskeleton.\textsuperscript{12,13} These changes could potentially predispose to right ventricular enlargement secondary to passive distension of the right ventricular wall and to slow right ventricular conduction secondary to reduced mechanical contact between cardiomyocytes.

The physiological response to endurance training includes an upregulation of plakoglobin.\textsuperscript{14,34} The deficiency of 1 plakoglobin allele appears to be sufficient to cause the phenotypic changes we report here. This suggests that the intact allele is not sufficient for normal cardiac plakoglobin function. In addition to an abnormal response to myocardial contractile and electrophysiological function without affecting myocardial structure, desmosomal contact is not sufficient for normal cardiac plakoglobin function. In addition to an abnormal response to myocardial contractile and electrophysiological function, \textit{H11003} plakoglobin allele appears to be sufficient to cause the phenotypic changes we report here. This suggests that the plakoglobin allele appears to be sufficient to cause the

**Conclusions**

Deletion of 1 plakoglobin allele profoundly alters right ventricular contractile and electrophysiological function without affecting myocardial structure, desmosomal contact appearance, or connexin43 expression. Further studies are warranted to delineate the subcellular mechanisms by which reduced plakoglobin expression causes right ventricular enlargement and arrhythmias and to investigate the cause for the structural abnormalities in ARVC. Because ARVC is pheno-

typically and genetically a heterogeneous disease, this will require studies in different models and populations of ARVC.
Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited cardiomyopathy and a prevalent cause of sudden death in the young. The disease is characterized by dilatation and dysfunction of the right ventricle. Ventricular tachycardias of right ventricular origin are often its first manifestation. The most prominent histopathological finding is fibrofatty replacement of right ventricular myocardium. Genetic studies have consistently demonstrated defects in genes coding for desmosomal proteins (plakophilin 2, desmoglein, plakoglobin, and others) in affected families and patients, suggesting that ARVC is a disease of the desmosome. The present article reports the first functional proof of this concept: Mice with heterozygous deletion of the desmosomal protein plakoglobin develop right ventricular dilatation, dysfunction, and arrhythmias. The present study has additional clinically relevant aspects. First, physical training accelerated the development of ARVC phenotype in these mice. This surprising finding requires further study but highlights the concept that ARVC is due to functionally altered intercellular connections. Finally, plakoglobin mutations have so far been found only in patients with Naxos disease, an autosomal-recessive form of ARVC. The present study suggests that deletion of 1 plakoglobin allele may be sufficient to develop the phenotype of ARVC. Together with the published genetic data, the present study suggests that desmosomal dysfunction is a key factor in the development of right ventricular dysfunction.
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