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PRE-CLINICAL RESEARCH

Load-Reducing Therapy Prevents Development of Arrhythmogenic Right Ventricular Cardiomyopathy in Plakoglobin-Deficient Mice

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Objectives We used a murine model of arrhythmogenic right ventricular cardiomyopathy (ARVC) to test whether reducing ventricular load prevents or slows development of this cardiomyopathy.

Background At present, no therapy exists to slow progression of ARVC. Genetically conferred dysfunction of the mechanical cell–cell connections, often associated with reduced expression of plakoglobin, is thought to cause ARVC.

Methods Littermate pairs of heterozygous plakoglobin-deficient mice (plako$^{+/-}$) and wild-type (WT) littermates underwent 7 weeks of endurance training (daily swimming). Mice were randomized to blinded load-reducing therapy (furosemide and nitrates) or placebo.

Results Therapy prevented training-induced right ventricular (RV) enlargement in plako$^{+/-}$ mice (RV volume: untreated plako$^{+/-}$ 136 ± 5 μl; treated plako$^{+/-}$ 78 ± 5 μl; WT 81 ± 5 μl; $p < 0.01$ for untreated vs. WT and untreated vs. treated; mean ± SEM). In isolated, Langendorff-perfused hearts, ventricular tachycardias (VTs) were more often induced in untreated plako$^{+/-}$ hearts (15 of 25), than in treated plako$^{+/-}$ hearts (5 of 19) or in WT hearts (6 of 21, both $p < 0.05$). Epicardial mapping of the RV identified macro–re-entry as the mechanism of ventricular tachycardia. The RV longitudinal conduction velocity was reduced in untreated but not in treated plako$^{+/-}$ mice ($p < 0.01$ for untreated vs. WT and untreated vs. treated). Myocardial concentration of phosphorylated connexin43 was lower in plako$^{+/-}$ hearts with VTs compared with hearts without VTs and was reduced in untreated plako$^{+/-}$ compared with WT (both $p < 0.05$). Plako$^{+/-}$ hearts showed reduced myocardial plakoglobin concentration, whereas β-catenin and N-cadherin concentration was not changed.

Conclusions Load-reducing therapy prevents training-induced development of ARVC in plako$^{+/-}$ mice. (J Am Coll Cardiol 2011;57:740–50) © 2011 by the American College of Cardiology Foundation

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an important cause of sudden death in young athletes (1–5). Dysfunction of the mechanical cell-cell junction seems to play a central role in ARVC: mutations in genes encoding the mechanical cell-cell contact proteins plakophilin-2, plakoglobin, desmocollin-2, desmoglein-2, and desmoplakin are found in ARVC patients (6–12). These proteins are localized in

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special junctions of the area composita (constituting >90% of the intercalated disk area), which consists of desmosomal and fascia adherens components (13,14).

Transgenic mouse models have verified the biological relevance of mechanical cell–cell contact dysfunction for ARVC: heterozygous deletion of plakoglobin is sufficient to provoke an ARVC phenotype comprising right ventricular (RV) enlargement and dysfunction and inducible ventricular tachycardias (VTs) without cardiac fibro-fatty infiltration (15). Heterozygous deletion of desmoplakin causes a biventricular cardiomyopathy with myocardial deposition of fat and fibrous tissue (16), and transgenic expression of desmoglein causes biventricular myocardial necrosis (17). Recent analyses in myocardial tissue from ARVC patients extend these findings and suggest that reduced immunohistological plakoglobin light intensity might be a sensitive and specific marker for ARVC in patients (4).

Despite progress in the prevention of sudden cardiac death in ARVC patients by antiarrhythmic drugs and implanted defibrillators (18,19), there is at present no treatment available that slows or prevents development of the disease (1,20,21). Clinical observations suggest that endurance training, especially when performed at a competitive level, might accelerate the manifestation of ARVC in susceptible individuals (22), and current recommendations suggest that ARVC patients should abstain from high-level sports activity (23). Likewise, endurance training can accelerate the development of ARVC in heterozygous plakoglobin-deficient mice (plako$^{+/-}$) (15), suggesting that chronically increased volume load might contribute to development of ARVC in susceptible patients. Therefore, we tested whether reducing ventricular pressure and volume load can prevent or slow training-induced development of ARVC.

Abbreviations and Acronyms

ARVC = arrhythmogenic right ventricular cardiomyopathy
Cx43 = connexin43
PCR = polymerase chain reaction
P-Cx43 = phosphorylated connexin43
plako$^{+/-}$ = heterozygous plakoglobin-deficient mice
RV = right ventricular
VT = ventricular tachycardia
WT = wild-type

Methods

Animal model and study design. We studied young adult plako$^{+/-}$ mice and their wild-type (WT) littermates (15). After a baseline echocardiography, all mice underwent 7 weeks of endurance training by daily swimming, initiated at 5 min/day and increased to 90-min/day swimming duration (15). Mice were randomized to a load-reducing therapy consisting of the loop diuretic furosemide (4 mg/kg/day continuous treatment) and nitrates (isosorbide dinitrate 20 mg/kg for 15 h/day alternating with molsidomine 8 mg/kg for 9 h/day to avoid nitrate tolerance) (24) or to sham therapy (water) (Fig. 1). These agents were chosen because they are clinically available and reduce ventricular load: chronic therapy with nitrates lowers RV pressure (24,25), and furosemide reduces pulmonary capillary wedge pressure and volume load (24,25).

All drugs were administered via drinking water. Individual daily water intake was monitored, and drug concentra-

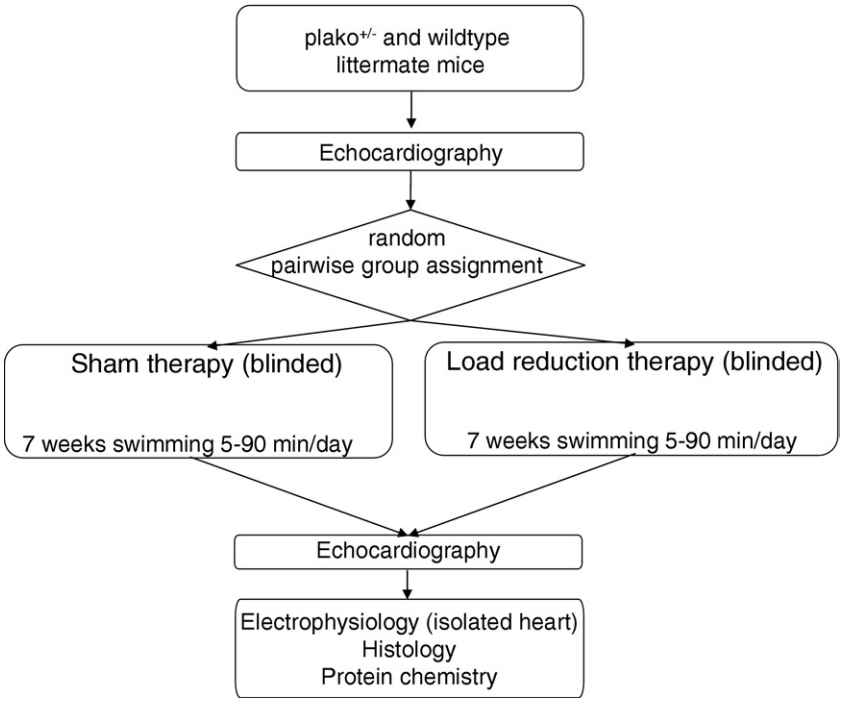


Figure 1 Flow Chart of the Study Protocol

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Table 1 Echocardiographic Parameters in Heterozygous Plako$^{+/-}$ and WT Mice Before and After Training Without and With Pre-Load–Reducing Therapy

	Before Training (7 Weeks)				After Training (15 Weeks)			
	WT		Plako$^{+/-}$		WT		Plako$^{+/-}$	
	Sham (n = 10)	Treated (n = 11)	Sham (n = 29)	Treated (n = 26)	Sham (n = 10)	Treated (n = 11)	Sham (n = 29)	Treated (n = 26)
Heart rate (beats/min)	427 ± 3	423 ± 3	425 ± 2	427 ± 2	422 ± 4	425 ± 3	422 ± 3	428 ± 3
RV parameters								
RV area 4-chamber view, mm^2	5.4 ± 0.4	5.3 ± 0.4	5.3 ± 0.3	5.4 ± 0.3	5.7 ± 0.5*	6.0 ± 0.3*	**8.8 ± 0.3*†‡**	5.9 ± 0.2*
RV area long-axis view, mm^2	3.8 ± 0.3	4.0 ± 0.3	3.9 ± 0.2	3.9 ± 0.2	4.3 ± 0.3*	4.5 ± 0.2*	**6.4 ± 0.2*†‡**	4.4 ± 0.1*
RV area short-axis view, mm^2	3.7 ± 0.3	3.9 ± 0.3	3.9 ± 0.2	3.9 ± 0.2	4.1 ± 0.3*	4.2 ± 0.3*	**6.1 ± 0.2*†‡**	4.2 ± 0.2*
RV diastolic volume (μl)	64 ± 1	66 ± 1	68 ± 0	66 ± 0	76 ± 1*	78 ± 1*	**138 ± 1*†‡**	78 ± 0*
LV parameters								
LV end-diastolic diameter (mm)	3.69 ± 0.07	3.66 ± 0.07	3.68 ± 0.05	3.59 ± 0.04	3.91 ± 0.06*	3.85 ± 0.07*	3.90 ± 0.04*	3.85 ± 0.04*
LV end-systolic diameter (mm)	1.99 ± 0.06	1.99 ± 0.04	1.96 ± 0.03	1.95 ± 0.02	2.09 ± 0.05*	2.08 ± 0.04*	2.10 ± 0.03*	2.06 ± 0.03*
LV fractional shortening (%)	46 ± 1	46 ± 1	47 ± 1	46 ± 1	47 ± 1	46 ± 1	46 ± 0	46 ± 0
Cardiac output (ml/min)	9.7 ± 0.5	8.8 ± 0.4	9.1 ± 0.4	9.1 ± 0.4	10.1 ± 0.4	10.0 ± 0.5*	10.8 ± 0.5*	10.3 ± 0.5*
LV mass (mg)	80 ± 4	80 ± 4	81 ± 3	75 ± 2	105 ± 5*	102 ± 3*	105 ± 2*	99 ± 2*

Values are mean ± SEM. Echocardiographic parameters in heterozygous plakoglobin-deficient (plako$^{+/-}$) and wild-type (WT) mice before and after training without and with preload-reducing therapy (Sham/Treated). *$p < 0.05$ before versus after training (paired *t* test); †$p < 0.05$ untreated plako$^{+/-}$ versus WT mice; ‡$p < 0.05$ untreated versus treated plako$^{+/-}$ mice (**bold** numbers, analysis of variance). All information is given for paired measurements before and after therapy. Data are expressed as mean ± SEM.

RV = right ventricular; LV = left ventricular.

tions in the drinking water were adapted accordingly. Therapy was well-tolerated and reduced blood pressure, measured as mean of 5 tail-cuff measurements/mouse on 2 independent occasions (n = 6 mice, mean ± SEM), from 98 ± 2/66 ± 1 mm Hg (mean pressure 77 ± 1 mm Hg) to 87 ± 5/60 ± 3 mm Hg (mean pressure 69 ± 3 mm Hg, $p < 0.05$). Therapy also reduced RV pressure measured as maximal trans-pulmonary Doppler flow velocity from 2.8 ± 0.3 m/s to 2.3 ± 0.2 m/s (18% difference, n = 13 mice, p = 0.03, mean ± SEM, all measurements performed during light sedation).

After training, follow-up echocardiography was performed; hearts were isolated for electrophysiological studies and immediately shock-frozen or fixated in formalin for protein chemistry and histological studies. All functional tests were blinded to genotype and therapy.

Echocardiography. All mice underwent echocardiography (VEVO 770 and 2100, Visualsonics, Toronto, Ontario, Canada, or HP5500, Philips Medical Systems, Andover, Massachusetts) during light sedation (oxygenated isoflurane 1.5% by inhalation) following validated protocols for left ventricular (LV) and RV size and function (15).

Electrophysiological studies. Mice were anesthetized (urethane 2 g/kg bodyweight IP) and heparinized, and their hearts were excised. Preparation time was between 3:00 and 4:30 min from excision of the heart to initiation of perfusion on a modified Langendorff apparatus (15,26,27). A 2-F octapolar catheter (interelectrode distance 500 μm) was inserted through the tricuspid orifice into the RV, and a 247-point multielectrode (13 × 19 grid, interelectrode distance 300 μm) was positioned over either the RV or LV. The heart was stimulated from the center of the multielectrode or from the tip of the octapolar catheter at twice diastolic threshold with steady-state pacing at 100-ms cycle length or an 8-pulse drive train at a pacing cycle-length of 100 ms followed by a single premature stimulus. The coupling interval of the premature stimulus started at 60 ms and was reduced in steps of 2 ms until the effective refractory period was reached.

Unipolar electrograms from the multielectrode were recorded at a 2-kHz sampling rate. The metal aortic cannula was used as reference. Signals were analyzed with a custom-made analysis program and manually validated (28). The time of maximal negative dV/dt in the QRS complex in the unipolar electrograms was defined as local activation. Sustained VT was defined as either monomorphic VT of more than 1-s duration or any arrhythmia requiring defibrillation or termination of the experiment. We counted spontaneous arrhythmias and arrhythmias induced by a single RV extra stimulus (15).

Western blots. Hearts were snap-frozen in liquid nitrogen. Left ventricular tissue was homogenized (3,000 rpm, 45 s) with Mikro-Dismembrator S (Sartorius) and resuspended in lysis buffer (50 mmol/l tris(hydroxymethyl)amino methane pH 7.4, 150 mmol/l sodium chloride, 0.5% Triton-X-100), supplemented with a protease inhibitor cocktail tablet (Complete Mini, Roche, Indianapolis, Indiana). Homogenates were centrifuged (2,500 rpm, 5 min). Total protein lysate (30 μg) was loaded onto a 12% sodium dodecyl sulfate–polyacrylamide resolving gel (1-mm-thick) and electrophoresed (60 min, 200 V). Gels were blotted to a nitrocellulose membrane (Protran, Whatman, Piscataway, New Jersey) by means of a wet transfer system (60 min, 100 V). Membranes were incubated overnight at 4°C with mouse-derived anti-plakoglobin (BD TL [BD Transduction Laboratories, San Jose, California] clone 15, 1:2,000), anti-β-catenin (BD TL clone 14, 1:1,000), anti-N-cadherin (BD TL 32, 1:5,000), and anti–glyceraldehyde-3-phosphate

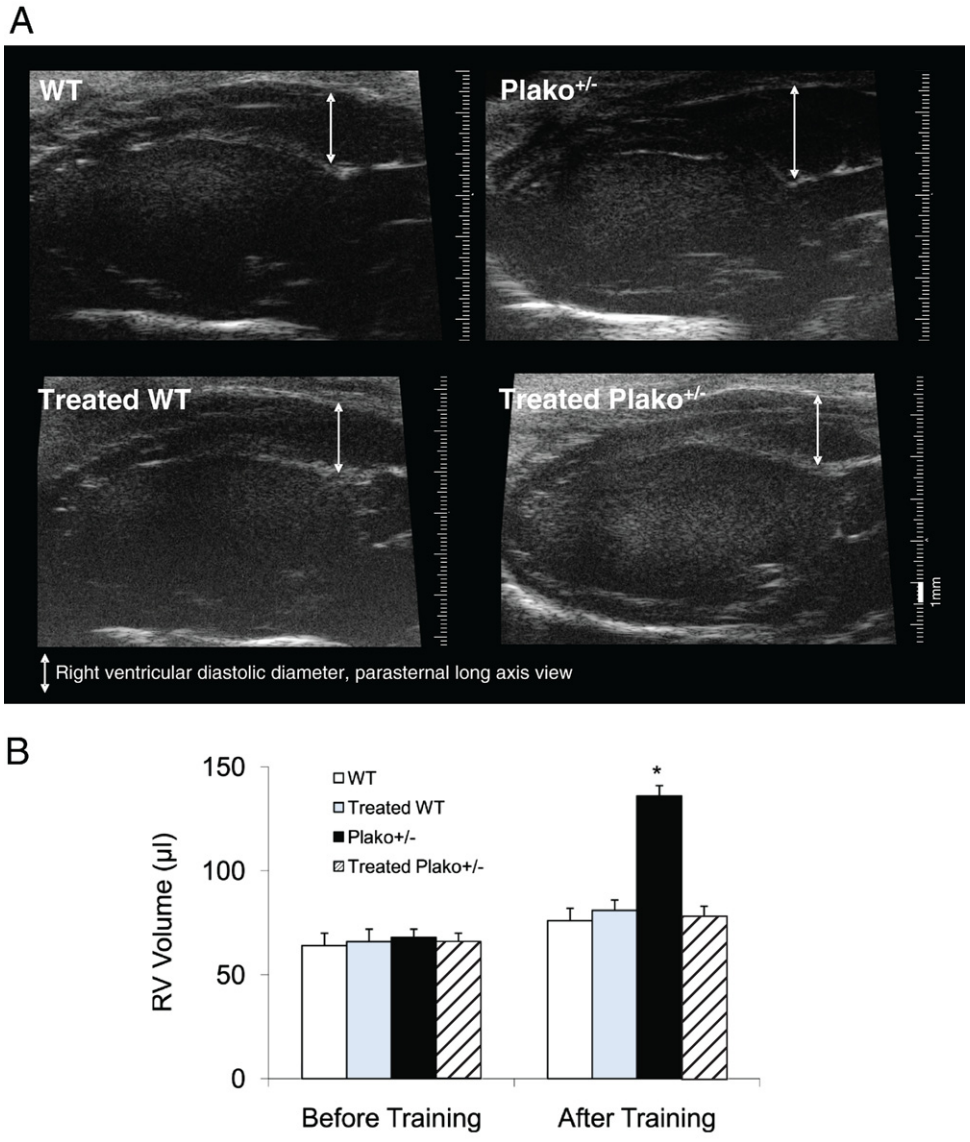


Figure 2 **Training-Induced RV Enlargement Is Prevented by Preload-Reducing Therapy in Heterozygous Plako$^{+/-}$ Mice**

(A) Representative echocardiographic long-axis views of the heart of 4 mice after endurance training: wild-type mouse without therapy (WT), treated wild-type mouse (treated WT), heterozygous plakoglobin-deficient mouse without therapy (plako$^{+/-}$), and treated plako$^{+/-}$ mouse (treated plako$^{+/-}$). Right ventricular (RV) diameters are indicated by **arrows**. **(B)** Mean RV volume in the 4 study groups. Training increased RV size in untreated plako$^{+/-}$ mice, therapy prevented RV enlargement. All data given as mean ± SEM.

dehydrogenase (Ambion, AM4300, 1:20,000). The membranes were then incubated with the polyclonal rabbit anti-mouse Ig, HRP-linked, secondary antibody (Dako, 1 h). Detection was performed with ECL plus (GE Healthcare, Amersham, Piscataway, New Jersey).

Membrane proteins were isolated (29). Protein (50 μg) was added to a 12% sodium dodecyl sulfate–polyacrylamide resolving gel (1-mm-thick) and electrophoresed (75 min, 160 V). Gels were blotted to a pre-equilibrated PVDF membrane (Immobilon-P, Millipore, Bedford, Massachusetts) by means of a semi-dry system (60 min, 12 V, 500 mA). Post-blot membrane was incubated with rabbit-derived anti-calnexin (1:2000 dilution in block buffer) or pan-cadherin (rabbit polyclonal, Sigma C3678, Sigma, St. Louis, Missouri) and anti-connexin43 (Cx43) rabbit-derived primary antibody (1:800 dilution in block buffer, 4 h each), anti-plakoglobin antibody (mouse monoclonal clone PG 5.1, Progen #61005, Progen Biotechnik, Heidelberg, Germany), or anti-β-catenin antibody (BD mouse monoclonal, #610153) antibodies.

Immunohistology. Immunofluorescence microscopy was performed as described (13,14). We used monoclonal anti-

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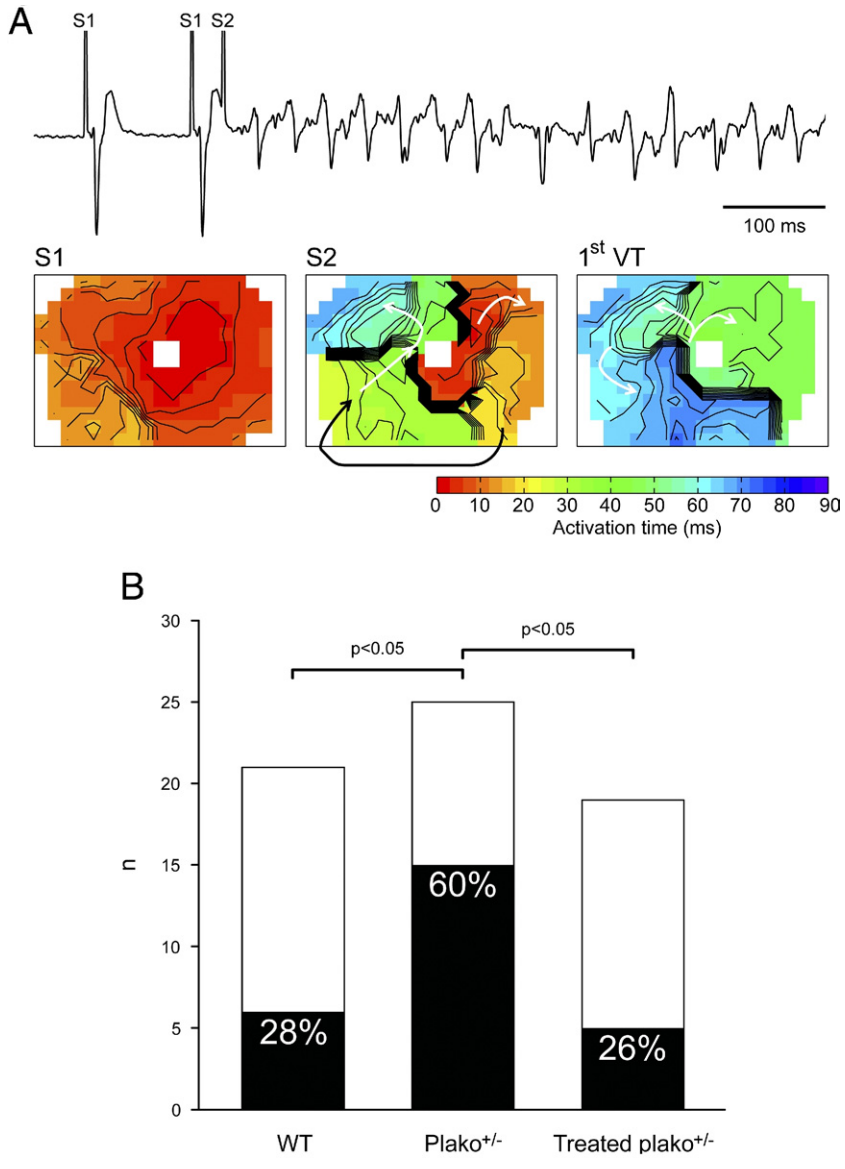


Figure 3 **Inducibility of Macro–Re-Entrant VTs Is Prevented by Preload-Reducing Therapy in Trained Plako$^{+/-}$ Mouse Hearts**

(A) Tracing **(top)** and activation map **(bottom)** of a polymorphic ventricular tachycardia (VT) in a trained, untreated plako$^{+/-}$ mouse after a single premature stimulus (**S2**, coupling interval 30 ms) at the RV epicardium. **Colors** show scale activation time, **lines** indicate 2-ms isochrones. The single premature stimulus was followed by an inhomogeneous activation pattern showing functional conduction block. Activation circled around the area of activation block **(thick black line)** and re-entered at the center and left of the electrode grid. In the map of the S2 activation of the first beat of the VT, the same isochrones appear in the **left upper corner**. **(B)** Sustained VTs occurred more often in untreated than in treated plako$^{+/-}$ or WT mouse hearts. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; other abbreviations as in Figure 2.

bodies specific for desmoplakin (murine mAb, clone DP447, Progen Biotechnik), β-catenin (rabbit antibodies, Sigma, Taufkirchen, Germany), plakoglobin (murine AB, clone PG 5.1., Progen Biotechnik), and plakophilin-2 (murine mAB, mix of clones PP2/62, PP2/86, and PP2/150, Progen Biotechnik).

Immunohistochemistry was performed on freshly fixed heart tissue with a monoclonal mouse anti-Cx43 antibody (Chemicon #3067, 1:250, Chemicon, Temecula, California) and a biotin-labeled goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, California) followed by visualization with peroxidase-coupled streptavidin and 4′,6-diamidino-2-phenylindole. Nuclei were counterstained with hematoxylin.

Quantitative polymerase chain reaction (PCR). Total ribonucleic acid isolated from hearts of plako$^{+/-}$, embryonal plako$^{-/-}$, and WT hearts was reverse transcribed with the Multi-Scribe RT kit (Applied Biosystems, Carlsbad, California) and random hexamers. Subsequently, quantitative PCRs were conducted in triplicate with SYBR-Green I

Table 2 Electrophysiological Parameters of WT, Trained Treated, and Trained Untreated Heterozygous Plako$^{+/-}$ Mice

	WT	Plako$^{+/-}$	Plako$^{+/-}$ Treated
RV longitudinal CV S1 (cm/s)	53.1 ± 1.3	44.0 ± 1.8*†	52.5 ± 2.2
RV longitudinal CV S2 = 50 ms (cm/s)	50.4 ± 2.0	41.4 ± 1.4*†	50.8 ± 1.4
RV transversal CV (cm/s)	30.8 ± 1.7	29.5 ± 1.7	30.9 ± 1.8
RV transversal CV S2 = 50 ms (cm/s)	26.3 ± 2.6	25.8 ± 1.7	28.8 ± 2.4
RV ERP (ms)	38 ± 3	35 ± 3	34 ± 3
LV longitudinal CV (cm/s)	55.6 ± 2.8	56.4 ± 3.8	55.7 ± 3.5
LV transversal CV (cm/s)	29.2 ± 2.2	28.9 ± 2.4	30.3 ± 1.9
LV ERP (ms)	47 ± 5	46 ± 3	42 ± 5
ERP RV catheter (ms)	37 ± 4	34 ± 4	35 ± 5
VT-induced during RV pacing	6/21 (29%)	15/25 (60%)‡§	5/19 (26%)
VT-induced during LV pacing	1/19 (5%)	2/20 (10%)	1/16 (6%)

Values are mean ± SD or n (%). Electrophysiological parameters of WT (untrained and trained), trained treated, and trained untreated heterozygous plako$^{+/-}$ mice. Data expressed as mean ± SEM. *$p < 0.001$ versus WT; †$p < 0.01$ versus treated plako$^{+/-}$ mice; ‡$p < 0.05$ versus WT; §$p < 0.05$ versus treated plako$^{+/-}$ mice (red numbers).

CV = conduction velocity in cm/s; ERP = effective refractory period; S1 = pacing with fix cycle length, S2 = 50 ms programmed stimulation applying a single extra stimulus at 50-ms coupling interval; VT = sustained ventricular tachycardia; other abbreviations as in Table 1.

master mix (Applied Biosystems) and 10 ng of complementary deoxyribonucleic acid as template in a Stratagene Mx3000P cycler (Stratagene, Amsterdam, the Netherlands). Relative expression compared with WT tissue was calculated with the ΔΔCt method. Two housekeeping genes (hypoxanthine phosphoribosyltransferase 1 and 60S acidic ribosomal protein PO) were assayed. Intron-spanning gene-specific primers were designed with the Primer3 software (30).

Statistical analysis. Values are shown as mean ± SEM. One-way analysis of variance followed by a Holm-Sidak test to correct for multiple group testing was used to compare numerical parameters between study groups. Paired *t* test was used to assess the effect of therapy on numerical parameters within each group. Because there was no effect of therapy on cardiac size or function in WT mice, we combined WT measurements for most of the tissue analyses. Categorical variables were compared by Fisher exact test. A statistical significance was assumed at a 2-sided $p < 0.05$.

Results

Load-reducing therapy prevents RV enlargement and induction of VTs in trained plako$^{+/-}$ mice. Training caused mild LV hypertrophy without altering LV function in both WT and plako$^{+/-}$ mice (Table 1). Training slightly affected RV parameters in WT mice and treated plako$^{+/-}$ mice but markedly increased RV size in untreated plako$^{+/-}$ mice (Fig. 2, Table 1) (compare Kirchhof et al. [15]). Although therapy completely prevented training-induced RV enlargement (Fig. 2, Table 1), training-induced LV hypertrophy was not affected (Table 1).

In isolated beating perfused hearts, sustained VTs occurred more often in untreated plako$^{+/-}$ hearts than in treated plako$^{+/-}$ hearts or WT hearts (Fig. 3). Mean cycle length of VTs was 46 ± 3 ms in untreated plako$^{+/-}$ hearts (n = 11), 44 ± 3 ms in treated plako$^{+/-}$ hearts (n = 7), and 37 ± 2 ms in WT hearts (n = 7, p = 0.02 for WT vs. all plako$^{+/-}$ hearts combined). Premature stimulation from the LV (i.e., from a site distant to the suspected pathology) rarely resulted in sustained VTs in either group (27). Effective refractory periods were not statistically different between study groups (Table 2). Induction of VTs was associated with functional conduction block and macro-reentry in untreated plako$^{+/-}$ hearts (example in Fig. 3, upper panels). To further investigate whether anatomic factors (enlargement of the RV) or functional effects (conduction slowing in RV myocardium) caused prolongation of activation, high-density ventricular activation maps during pacing from the center of the multielectrode were reconstructed and conduction velocities were computed. Longitudinal RV conduction velocity was reduced in untreated plako$^{+/-}$ compared with treated plako$^{+/-}$ or WT hearts. Treated mouse hearts showed normal longitudinal conduction velocities. Transverse conduction velocity was not altered (Fig. 4, Table 2).

Reduced phosphorylation of Cx43 in myocardium from plako$^{+/-}$ hearts after training is improved by therapy. Plakoglobin, β-catenin, and plakophilin-II seemed normally localized in treated and untreated plako$^{+/-}$ hearts (12 sections/heart, 3 hearts) (Fig. 5A) (15). The absolute light intensity of plakoglobin was not different between groups (12 sections and 1,600 to 2,400 images/group, not shown). When β-catenin was used as reference protein, immunohistological plakoglobin light intensity was reduced in plako$^{+/-}$ hearts as expected (plakoglobin/β-catenin ratio: WT 0.64 ± 0.2; plako$^{+/-}$ 0.43 ± 0.1; treated plako$^{+/-}$ 0.45 ± 0.08, mean ± SEM; 12 sections/heart, 3 hearts, 1,600 to 2,400 images/group; p = 0.01 WT vs. plako$^{+/-}$).

Western blots confirmed reduced plakoglobin expression in LV homogenates (Fig. 5B), whereas β-catenin and N-cadherin concentration was not significantly altered (Fig. 5B). Furthermore, we did not find a significantly different plakoglobin or β-catenin concentration in LV

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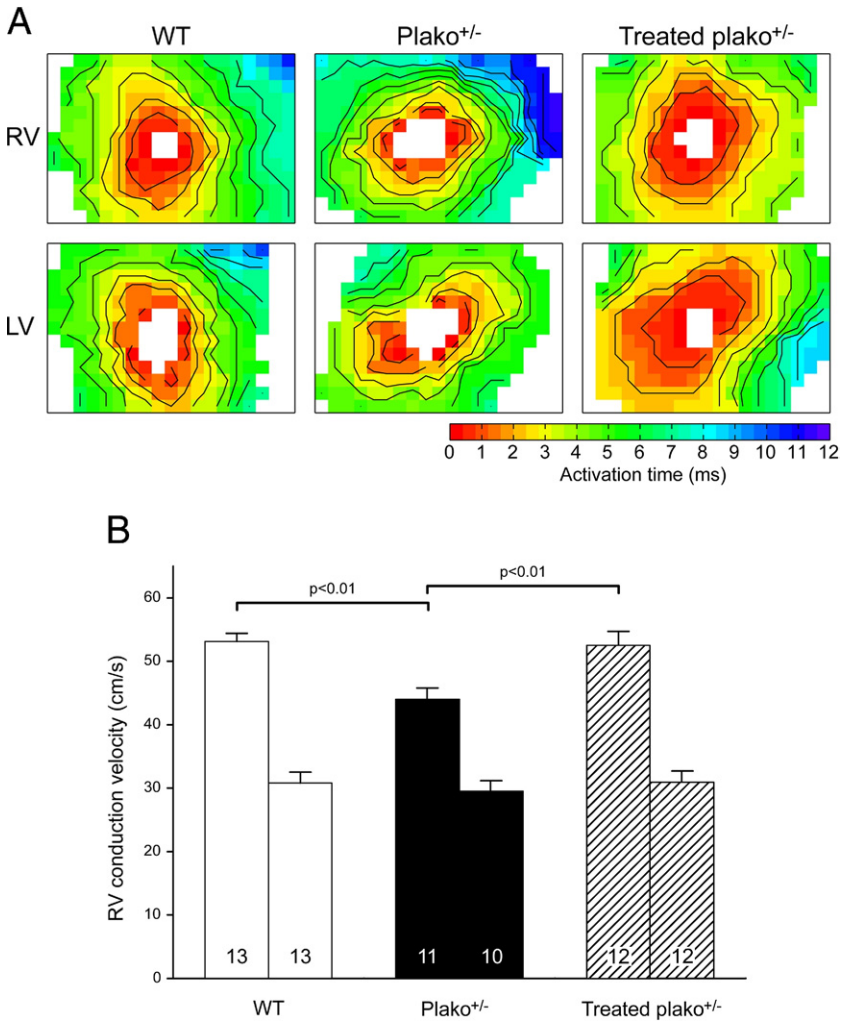


Figure 4 **Pre-Load–Reducing Therapy Prevents Training-Induced Longitudinal RV Conduction Slowing in Plako$^{+/-}$ Mouse Hearts**

(A) Activation patterns after central stimulation from the right ventricle (RV) and left ventricle (LV) of a trained WT, an untreated plako$^{+/-}$, and a treated plako$^{+/-}$ mouse heart. Conduction slowing is indicated by closely spaced isochrones. Conduction slowing was observed in the RV activation maps of trained, untreated but not in trained, treated plako$^{+/-}$ mice. Color scale shows activation time, lines are 1-ms isochrones. **(B)** Mean RV conduction velocities in the longitudinal **(open bars)** and transversal **(hatched bars)** direction to the fiber orientation. Longitudinal RV conduction velocity was reduced in untreated plako$^{+/-}$ mice compared with either WT or treated plako$^{+/-}$ mouse hearts after training, both during fix frequent pacing and at premature stimulation (S2 = 50 ms). Number is indicated in the **bars**. All data given as mean ± SEM. Abbreviations as in Figures 2 and 3.

membrane preparations between treated and untreated plako$^{+/-}$ hearts (plakoglobin untreated 0.4 ± 0.1, treated 0.9 ± 0.5; β-catenin untreated 1.6 ± 0.4, treated 2.4 ± 0.6; mean ± SEM; both $p > 0.2$; $n = 4$ to 6/group).

Histological examination of myocardial slices from untreated plako$^{+/-}$ and WT mice did not reveal abnormalities (Figs. 5A and 6A) (15). Target genes of the wnt signaling pathway were neither differentially expressed in plako$^{+/-}$ hearts (PCR ratio compared with WT: peroxisome proliferator-activated receptor gamma 1.22; cyclin D1 1.12; collagen 1a 1.41; collagen 3a 1.17) nor in embryonal plako$^{-/-}$ hearts (Day 10 to 11 after conception, PCR ratio compared with WT: peroxisome proliferator-activated receptor gamma 0.93; cyclin D1 0.71; collagen 1a 0.78; collagen 3a 0.73).

Localization of Cx43 at the short ends of cardiomyocytes was not different between groups (Fig. 6A). Connexin43 concentration was lower in untreated plako$^{+/-}$ hearts than in WT hearts, and this effect was attenuated in treated plako$^{+/-}$ hearts: Three consecutive Western blots consistently showed a lower level of non-phosphorylated Cx43 and phosphorylated connexin43 (P-Cx43) in untreated plako$^{+/-}$ mice compared with WT hearts (examples in Figs. 6B and 6C). The Cx43 and P-Cx43 concentrations were significantly lower in untreated plako$^{+/-}$ hearts than in WT, whereas we found no statistically significant difference between treated plako$^{+/-}$ and WT hearts (Fig. 6D, 3 blots). Individual blots showed similar trends in RV myocardium (Fig. 6C), albeit with higher intraindividual variability. The

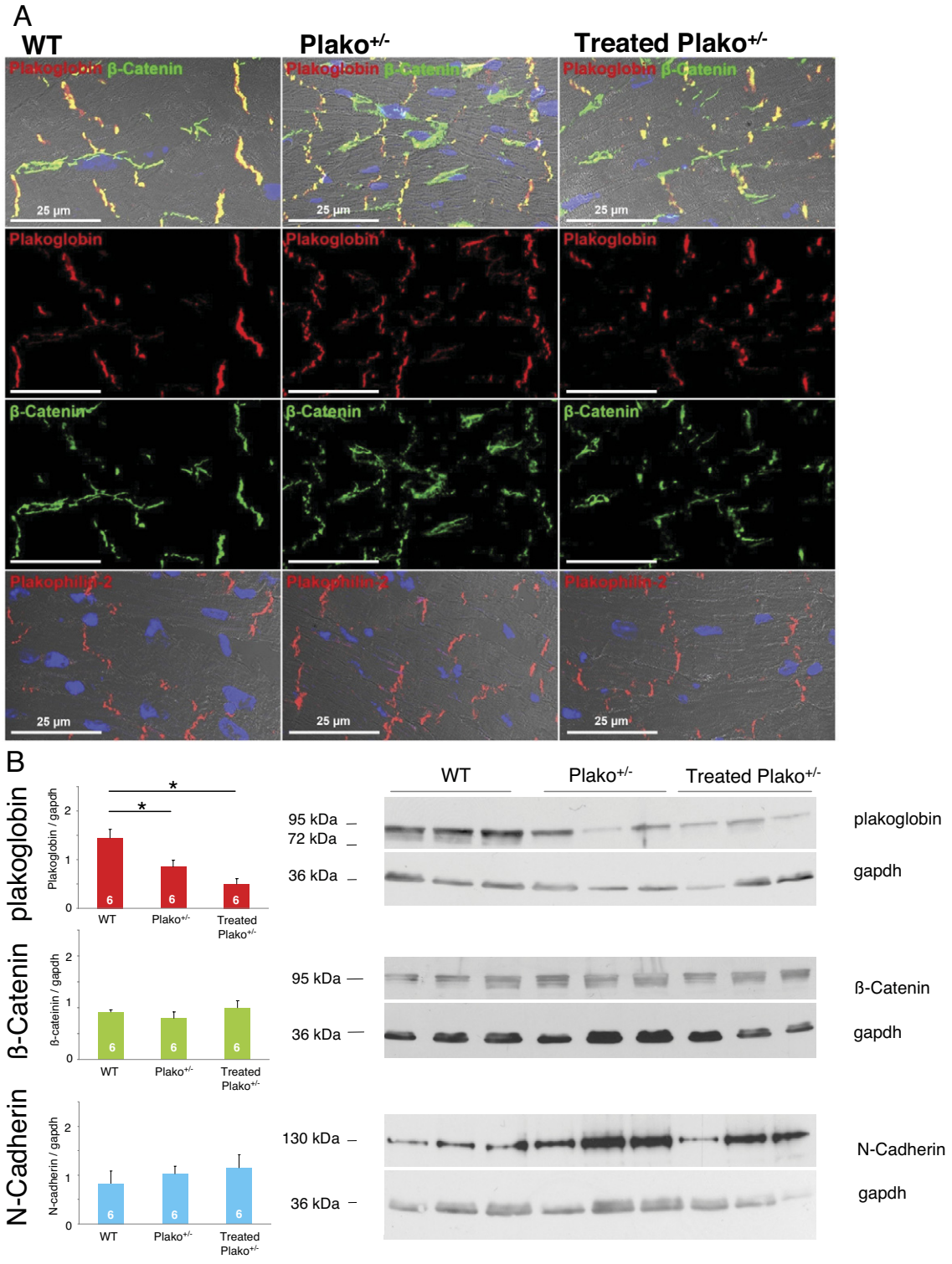


Figure 5 **Localization and Concentration of Cell Adhesion Proteins**

(A) Normal localization of area composita proteins at cell–cell interfaces. Shown are immunofluorescence micrographs of RV sections of a WT, an untreated plako$^{+/-}$, and a treated plako$^{+/-}$ mouse heart. The β-catenin **(green)** and plakoglobin **(red)** localize in area composita junctions at the short cell axes, irrespective of study group. Both proteins localize in intercalated disk areas in addition to often laterally situated small capillaries. By contrast, plakophilin-2 **(red)** is restricted to the intercalated disk regions. **(B)** Reduced myocardial concentration of plakoglobin but not β-catenin or N-cadherin. Shown are mean (± SEM) protein levels in LV myocardium **(left panel)** and representative blots of plakoglobin, β-catenin, N-cadherin, and the respective GAPDH loading control **(right panel)**. Abbreviations as in Figures 2 and 3.

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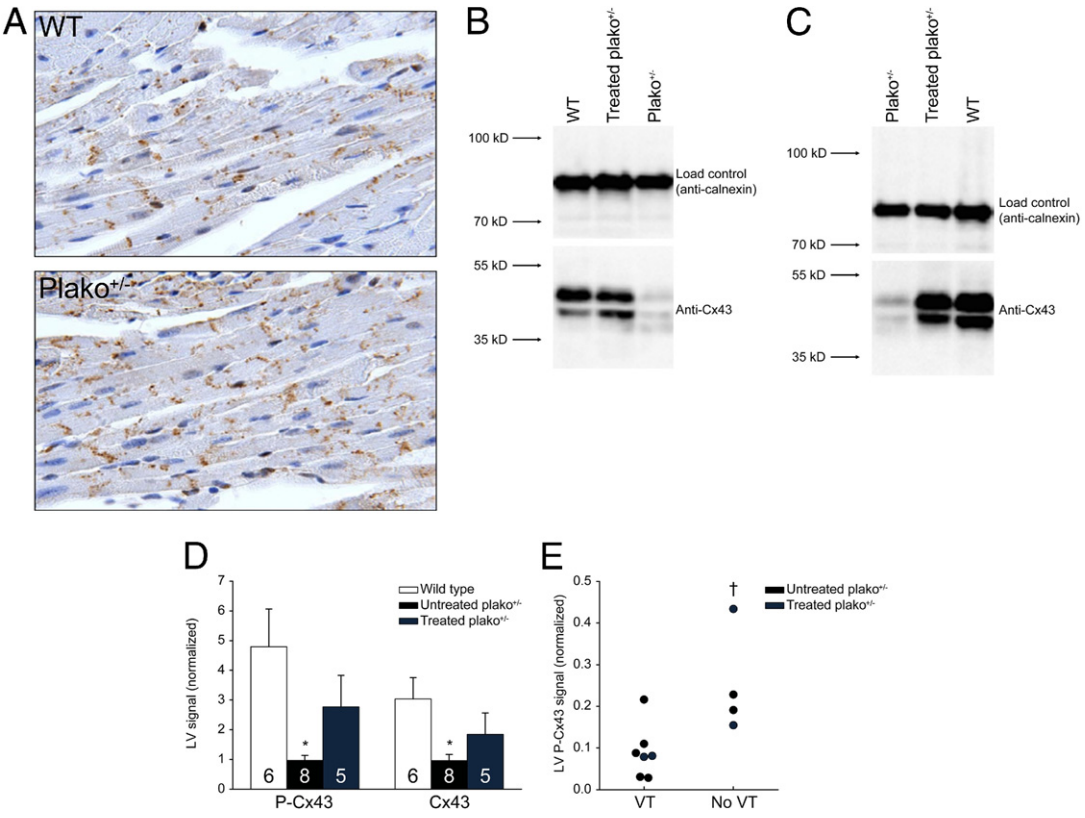


Figure 6 **Reduced Cx43 Concentration in Plako$^{+/-}$ Mouse Hearts**

(A) Connexin43 was normally located in plako$^{+/-}$ mouse hearts. **(B, C)** Representative Western blot of Cx43 in WT, plako$^{+/-}$, and treated plako$^{+/-}$ LV **(B)** and RV **(C)** tissue. **(D)** Quantitative comparison of the mean concentration levels of phosphorylated connexin43 (P-Cx43) and non-phosphorlyated Cx43 (Cx43) in LV tissue normalized to the housekeeping protein calnexin and expressed as a fraction of the mean value of untreated plako$^{+/-}$ mice (mean of 3 blots). Both P-Cx43 and Cx43 were reduced in untreated plako$^{+/-}$ hearts. **(E)** P-Cx43 concentration in plako$^{+/-}$ hearts with and without VT. P-Cx43 concentration was lower in plako$^{+/-}$ hearts with than without VTs (mean of 1 blot performed specifically to compare hearts with and without VT). Columns show mean ± SEM; n is indicated in the columns; *$p < 0.05$ versus WT; †$p < 0.05$ versus hearts with VTs. F: Bar graph of mean LV Cx43 and P-Cx43 expression levels in the 3 study groups. Abbreviations as in Figures 2 and 3.

P-Cx43 concentration was higher in a separate blot in tissue from plako$^{+/-}$ mice without VTs compared to plako$^{+/-}$ mice with VTs (Fig. 6E).

Discussion

Main findings. We report for the first time that a therapy aimed at reducing RV load prevents development of ARVC in a model with reduced expression of the gene encoding the mechanical cell–cell contact protein plakoglobin. Therapy completely prevented the development of RV enlargement and conduction slowing and normalized inducibility of VTs, thereby rendering treated plakoglobin-deficient animals phenotypically indistinguishable from their trained WT littermates. In untreated animals, conduction slowing was associated with reduced concentration and phosphorylation of Cx43. Our data suggest that load reduction could be a simple and effective way to prevent or slow the development of ARVC in genetically susceptible hearts.

Training-induced dilation of the RV might confer ARVC. Endurance training can provoke or accelerate the development of ARVC or RV arrhythmias (2,22,23,29,31). Our study suggests that increased RV load during training periods contributes to the accelerated expression of ARVC in susceptible persons (e.g., in endurance athletes carrying a genetic predisposition for the disease).

Therapy prevents RV enlargement and increased Cx43 expression in trained plako$^{+/-}$ mice. Conduction slowing was associated with reduced Cx43 expression in this study, whereas there were no histo-morphological changes in affected RVs. Cx43, the main protein that forms gap junctions and confers electrical coupling between cardiomyocytes, co-localizes with desmosomal proteins in the area composita region (32). It has been suggested that mechanical dysfunction of cell–cell contacts (e.g., caused by reduced plakoglobin gene expression) interferes with formation of gap junctions and reduces electrical cell–cell coupling (33). Indeed, patients with ARVC might contain less Cx43 at the myocardial intercalated disks (4,33). Our study provides

further support of this hypothesis: training dilated the RV, provoked arrhythmias, and reduced Cx43 content in plako$^{+/-}$ hearts. It is conceivable that reduced mechanical contacts between cells resulted in lesser binding of connexins to the cell–cell contacts, thereby promoting conduction slowing.

Decreased myocardial plakoglobin in ARVC. Myocardial plakoglobin content might be a sensitive and specific marker of ARVC in patients (4). Reduced concentration of plakoglobin with normal localization was confirmed by Western blots and immunohistology in this study, although we did not identify obvious alterations in β-catenin, N-cadherin, or plakophilin-II. Further studies are warranted to characterize the composition of cell adhesion complexes in ARVC in more detail. Taken together with published clinical (4) and experimental findings (15), our study supports the hypothesis that reduced myocardial plakoglobin concentration might constitute a key component and possibly a "final common pathway" in the development of ARVC.

Reduced concentration and phosphorylation of Cx43 explains longitudinal conduction slowing in plako$^{+/-}$ hearts. Although statistically robust, the observed changes in conduction velocity were modest. However, the combination of reduced conduction velocity and increased RV and LV size enhances the arrhythmogenic substrate for re-entry. Transverse conduction velocity was not altered in plako$^{+/-}$ hearts in this study, possibly because gap junctions are preferentially expressed in the longitudinal interfaces of myocardial fibers (Fig. 6A) (26). The observed longitudinal conduction delay will cause conduction heterogeneity and can thereby explain functional reentry and induction of ventricular arrhythmias (Fig. 3) in untreated plako$^{+/-}$ hearts.

ARVC develops without activation of the wnt pathway in this model. Unlike observations in most but not all (33) patients with ARVC, fibro-fatty replacement of RV myocardium is not found in plakoglobin-deficient mice, and gene expression is not different from WT tissue in affected hearts (Fig. 2A in Kirchhof et al. [15]). These surprising observations led to a speculation that fibro-fatty replacement could be a prominent but not a required finding in ARVC (15), possibly driven by nuclear translocation of plakoglobin and suppression of the wnt signaling pathway (16)—which might occur when mutations in desmoplakin or desmoglein reduce desmosmal binding of plakoglobin but not when plakoglobin is reduced generally, as in the present model. In line with this hypothesis, we did not find alterations of wnt signaling in plakoglobin-deficient mice.

Study limitations. We did not investigate whether load-reducing therapy has beneficial effects when ARVC has already developed or whether such a therapy has effects when fibro-fatty replacement is present. Therapy-induced increased myocardial Cx43 concentration and phosphorylation might suggest an effect on electrical conduction even in such conditions, but this requires further study. Although reduced expression of plakoglobin appears as a specific and sensitive molecular change in ARVC patients (4), the findings of this experimental study require confirmation in other models and in patients.

Conclusions

Load-reducing therapy consisting of furosemide and nitrates completely prevents the development of an ARVC-phenotype in heterozygous plakoglobin-deficient mice. Our data invite further validation of such an intervention in other models of ARVC and in patients.

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