CALL FOR PAPERS | Exercise Training in Cardiovascular Disease: Mechanisms and Outcomes

Accelerated cardiac remodeling in desmoplakin transgenic mice in response to endurance exercise is associated with perturbed Wnt/β-catenin signaling

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Martherus R, Jain R, Takagi K, Mendaikhan U, Turdi S, Osinska H, James JF, Kramer K, Purevjav E, Towbin JA. Accelerated cardiac remodeling in desmoplakin transgenic mice in response to endurance exercise is associated with perturbed Wnt/β-catenin signaling. Am J Physiol Heart Circ Physiol 310: H174–H187, 2016. First published November 6, 2015; doi:10.1152/ajpheart.00295.2015.—Arrhythmogenic ventricular cardiomyopathy (AVC) is a frequent underlying cause for arrhythmias and sudden cardiac death especially during intense exercise. The mechanisms involved remain largely unknown. The purpose of this study was to investigate how chronic endurance exercise contributes to desmoplakin (DSP) mutation-induced AVC pathogenesis. Transgenic mice with overexpression of desmoplakin, wild-type (Tg-DSPWT), or the R2834H mutant (Tg-DSPR2834H) along with control nontransgenic (NTg) littersmates were kept sedentary or exposed to a daily running regimen for 12 wk. Cardiac function and morphology were analyzed using echocardiography, electrocardiography, histology, immunohistochemistry, RNA, and protein analysis. At baseline, 4-wk-old mice from all groups displayed normal cardiac function. When subjected to exercise, all mice retained normal cardiac function and left ventricular morphology; however, Tg-DSPR2834H mutants displayed right ventricular (RV) dilation and wall thinning, unlike NTg and Tg-DSPWT. The Tg-DSPR2834H hearts demonstrated focal fat infiltrations in RV and cytoplasmic aggregations consisting of desmoplakin, plakoglobin, and connectin 43. These aggregates coincided with disruption of the intercalated disks, intermediate filaments, and microtubules. Although Tg-DSPR2834H mice already displayed high levels of p-GSK3βSer9 and p-AKT1Ser473 under sedentary conditions, decrease of nuclear GSK3-β and AKT1 levels with reduced p-GSK3-βSer9, p-AKT1Ser473, and p-AKT1Ser308 and loss of nuclear junctional plakoglobin was apparent after exercise. In contrast, Tg-DSPWT showed upregulation of p-AKT1Ser473, p-AKT1Ser308, and p-GSK3-βSer9 in response to exercise. Our data suggest that endurance exercise accelerates AVC pathogenesis in Tg-DSPR2834H mice and this event is associated with perturbed AKT1 and GSK3-β signaling. Our study suggests a potential mechanism-based approach to exercise management in patients with AVC.

glycogen synthase kinase 3-β; protein kinase B

NEW & NOTEWORTHY

We demonstrate that the overexpression of desmoplakin (DSP) in murine heart causes translocation of plakoglobin (JUP) in cytoplasm at baseline. Upon exercise, mutant DSP sequesters JUP at the cellular insoluble part. This was associated with reduced protein kinase B (AKT) activation perturbing Wnt/β-catenin signaling, potentially underlying exercise-induced acceleration of arrhythmogenic cardiomyopathy.

ARRHYTHMOGENIC VENTRICULAR cardiomyopathy (AVC) is an inherited myocardial disease clinically manifested by right ventricular (RV) and/or left ventricular (LV) dilatation and dysfunction, ventricular arrhythmias, heart failure, syncope, and sudden cardiac death (SCD) (22). The most reported pathological feature of AVC is fibrofatty replacement of the RV myocardium, and the most common clinical feature is ventricular arrhythmia, hence its name (23). Mutations underlying AVC have predominantly been identified in genes encoding desmosomal proteins with a wide variety of mutations, including compound and digenic heterozygosity (36). Incomplete penetrance and variable expressivity is commonly seen in AVC, which suggests a possible role of additional genetic modifiers and external factors in the clinical manifestation and progression of AVC (11, 24, 31). Individuals with AVC performing regular or intense, burst sports activities have been reported to display palpitations, syncope, and SCD more frequently, and these symptoms tend to occur at a younger age than in patients with AVC who were not athletically inclined (7, 10). Moreover, high levels of exercise such as is performed by endurance athletes or even in otherwise healthy subjects can provoke RV remodeling and dysfunction (6, 16). However, management of physical exercise in patients with AVC remains controversial due to the uncertain mechanism(s) responsible for the disease onset and progression and its response to intense exercise (30).

Desmoplakin (DSP) is a key structural element of desmosomes expressed in cardiac and epithelial tissues, providing the scaffold for cross-linking of plakoglobin or γ-catenin (junctional plakoglobin, JUP), plakophilin, and cadherins at the NH2-terminal domain and anchoring the intermediate filament protein desmin at its COOH-terminal domain (18). Besides its role as a structural component in providing mechanical integrity to the heart muscle, DSP is one of the key proteins in DSP-cytoskeletal dynamics, cell-cell electrical coupling, and nuclear signaling pathways. Suppres-
sion of DSP in cardiomyocytes was found to lead to nuclear localization of JUP and a strong decrease in Wnt/β-catenin signaling through blocking of transcription factor (Tcf)/lymphoid enhancer-binding factor 1 transcription factor activity, thereby increasing expression of adipogenic and fibrogenic genes (8). DSP has also been shown to exert a regulatory function on the formation of microtubules and regulate connexin43 membrane localization (27, 32).

Fig. 1. A: exercise protocol used in the study. x-Axis represents the number of days exercised. y-Axis represents the total running distance in meters, speed in m/min, and slope/inclination. B: Western blotting of mouse heart lysates fractionated into soluble and insoluble fractions. NTg, nontransgenic; Tg-DSPWT, mice overexpressing human wild-type desmoplakin (DSP); Tg-DSPR2834H, mice overexpressing human mutant DSP-R2834H protein. Nos. underneath bands indicate levels expressed in relative density (RD) units compared with RD/H11005 in NTg controls. /H9252-Actin used as a loading control.

![Fig. 1](image_url)
Our previous report demonstrated that transgenic mice with cardiac-specific overexpression of human DSP with a COOH-terminal R2834H mutation (Tg-DSPR2834H) recapitulated the human AVC phenotype by 6 mo of age (37). The basis of this is that the histidine substitution of arginine at the position that usually facilitates glycogen synthase kinase (GSK) 3 recruitment to the C-tail of DSP results in disruption of intercellular junction integrity (2). Whether endurance exercise contributes to AVC pathogenesis has yet to be investigated. Herein, we demonstrate the effects of exercise on AVC disease onset and progression in 4-wk-old mice subjected to daily treadmill exercise for up to 12 wk. Furthermore, possible mechanisms of disease progression in response to endurance exercise were investigated.

MATERIALS AND METHODS

Animals and endurance exercise. Transgenic C57/BL6 mice expressing the human cardiac-specific DSP isoform, wild-type (Tg-DSPWT), or mutant R2834H (Tg-DSPR2834H) under control of the α-myosin heavy chain promoter, were recreated as previously described (37). Animals were maintained in housing under barrier conditions with standard chow and water ad libitum. Mice were randomly divided into the following two groups: sedentary and those undergoing the exercise regimen. The exercise regimen was started in mice isolated from the exercise group at the age of 4 wk, after the first baseline echocardiograms were completed. The exercise regimen included involuntary treadmill running 5 days/wk on an Exer 3/6 treadmill (Columbus Instruments, Columbus, OH). During this regimen, treadmill speed, distance, and inclination were gradually increased from 5 m/min, 50 m, 0° at day 1 to 16 m/min, 850 m, 10° at day 40, respectively (Fig. 1A). The maximum speed to which the mice were exposed was set at 90% of the speed at which the animals reached their maximal ability. An initial warm-up period was incorporated into each exercise session. After day 40, mice were subjected to running three times per week with the same parameters as on day 40. After 12 wk of exercise, mice were euthanized under pentobarbital anesthesia by 1.5% oxygenated isoflurane by inhalation. Echocardiography was performed using a Visualsonics Series 2100 high-resolution imaging system, with a 38 MHz Microfocus transducer probe as described previously (21). The imaging was optimized for mouse cardiovascular imaging with frame rates >300 frames/s. Measurements included LV end-diastolic diameter (in parasternal long-axis, M-mode, and short-axis view), left atrial (LA) and RV diameter (in the short-axis view), and fractional shortening (FS). LV and RV measurements were done at end diastole, and LA measurements were done just before the opening of the mitral valve. During analysis, if the endocardial borders were not well visualized or there was artifact, the images were discarded. Each echocardiogram was analyzed in the mouse echocardiography laboratory by two readers (Jain and James) after acquisition of the images.

Echocardiogram. After anesthesia, subcutaneous needle electrodes were placed in both front and rear left legs in all mice in similar positions to obtain consistent recordings across all mice. A single lead electrocardiogram (ECG) was recorded on a BIOPAC ECG system (Biopac, Goleta, CA) for 30 s at a sampling rate of 200 Hz and 1,000 gain using AcqKnowledge 3.9.2 software (Biopac). The voltage was set on a scale of 1 mv/division, and duration was set at 0.7 s/division. All recorded ECGs were imported into the Sigma Scan Pro 5.0 software (Systat Software, San Jose, CA) for further analysis.

Heart tissue fractionation, RNA and protein expression analysis. Cardiac tissue was dissected and cut into small pieces. RNA was isolated using Trizol reagent (Invitrogen, Paisley, UK). First-strand RT-PCR was performed using Qucript (Quanta Biosciences, Gaithersburg, MD) starting from 500 ng total RNA. QPCR was performed using a Power SYBR-Green master mix (Life Technologies, Foster City, CA) in 20 µl reactions on an ABI7500 (Life Technologies). In all experiments β-actin was used as the reference gene.

Protein isolates were prepared using T-PER reagent (Thermo Scientific, Rockford, IL) supplemented with phenylmethanesulfonyl fluoride (Sigma Aldrich, St. Louis, MO) to 10 µM/lit and complete plus Phosphostop protease inhibitors (Roche, Indianapolis, IN) according to the manufacturer’s instructions. For the soluble phase, supplemented T-PER (20 µM/lit of tissue) and zirconia/silica beads (BioSpec, Bartlesville, OK) were added before the sample was mixed on a Bertin Precellys 48/96 extraction (Mikro system, Montigny-le-Bretonneux, France) during three cycles of 45 s each at 5,000 revolutions/min with 20-s pauses. Homogenates were centrifuged at 10,000 g for 5 min at 4°C. After the supernatant containing the “soluble phase” was collected, the insoluble phase was prepared by adding supplemented T-PER solution containing 9 M urea at one-half the volume used for the soluble phase. For cytoplasmic and nuclear protein fractionation, the NE PER kit (Thermo Scientific, Waltham,

| Table 1. Echocardiography of 16-week-old mice from sedentary and exercise groups |
|---------------------------------|----------|----------|----------|
|                                 | NTg      | Tg-DSPWT | Tg-DSPR2834H |
| n                               | Sedentary| Exercise | Sedentary | Exercise | Sedentary | Exercise | Sedentary | Exercise |
| EF, %                           | 50.58 ± 6.63 | 47.98 ± 8.44 | 45.35 ± 8.09 | 50.35 ± 7.53 | 49.61 ± 5.8 | 47.69 ± 7.35 | 25.62 ± 2.66 | 23.82 ± 4.95 |
| FS, %                           | 24.75 ± 4.07 | 24.84 ± 6.24 | 22.41 ± 4.17 | 25.2 ± 4.16 | 4.06 ± 0.34 | 4.22 ± 0.41 | 3.06 ± 0.31 | 3.21 ± 0.45 |
| LVID,d, mm                      | 4.48 ± 0.43 | 4.44 ± 0.56 | 4.18 ± 0.81 | 4.19 ± 0.41 | 0.93 ± 0.13 † | 0.88 ± 0.16 | 0.79 ± 0.13 † | 0.86 ± 0.12 |
| LVID,s, mm                      | 3.34 ± 0.41 | 3.31 ± 0.65 | 3.26 ± 0.71 | 3.12 ± 0.42 | 1.28 ± 0.16 † | 1.31 ± 0.24 | 1.22 ± 0.2 | 1.27 ± 0.12 |
| IVS,d, mm                       | 0.67 ± 0.12 | 0.77 ± 0.14 † | 0.93 ± 0.13 † | 0.98 ± 0.12 | 0.92 ± 0.09 | 0.98 ± 0.12 | 0.89 ± 0.12 | 0.89 ± 0.12 |
| IVS,s, mm                       | 1.07 ± 0.16 | 1.18 ± 0.16 | 1.28 ± 0.16 † | 1.31 ± 0.24 | 0.93 ± 0.13 † | 0.98 ± 0.12 | 0.92 ± 0.09 | 0.98 ± 0.12 |
| LV mass, g                      | 88.3 ± 15.17 | 98.83 ± 27.01 | 90.23 ± 27.09 ‡ | 99.78 ± 14.36 | 90.41 ± 21.17 | 96.19 ± 20.91 | 91.91 ± 20.51 | 96.53 ± 20.18 |
| LVPW,d, mm                      | 1.91 ± 0.15 | 0.93 ± 0.2 | 0.92 ± 0.09 | 0.98 ± 0.12 | 0.91 ± 0.15 | 0.93 ± 0.2 | 0.89 ± 0.12 | 0.89 ± 0.12 |
| LV Vol d, mL                    | 94.32 ± 12.96 | 95.6 ± 17.62 | 81.50 ± 33.73 | 78.81 ± 17.24 ‡‡ | 73.37 ± 14.25 ‡‡ | 79.06 ± 17.47 ‡‡ | 60.13 ± 19.8 | 71.46 ± 19.8 |
| RVD, mm                         | 1.59 ± 0.53 | 1.33 ± 0.48 | 1.49 ± 0.55 | 1.55 ± 0.35 | 1.95 ± 0.40‡ | 1.81 ± 0.37 † | 2.13 ± 0.36 | 2.14 ± 0.37 |
| LA diameter, mm                 | 2.01 ± 0.14 | 1.98 ± 0.32 | 2.41 ± 0.52 | 2.01 ± 0.37 | 2.13 ± 0.36 | 2.14 ± 0.37 | 2.13 ± 0.36 | 2.14 ± 0.37 |

Values are means ± SD; n, no. of subjects. NTg, nontransgenic; Tg-DSPWT, mice overexpressing human wild-type desmoplakin (DSP); Tg-DSPR2834H, mice overexpressing human mutant DSP-R2834H protein; EF, ejection fraction; FS, fractional shortening; IVS, interventricular septum; d, diastole; s, systole; LV, left ventricle; ID, internal diameter; PW, posterior wall; Vol, volume; RVD, right ventricular dimension; LA, left atrium. P ≤ 0.05, significant change from sedentary nontransgenic (NTg) littermates (*), from exercised NTg littermates (†), and from sedentary Tg-DSPWT (‡).
MA) was used. Proteins were quantified using the Bio-Rad protein assay (Bio-Rad, Hercules CA), and 20 μg of total protein were applied on 4–12% BT gels (Invitrogen). Blotting on a polyvinylidene difluoride membrane was done overnight at 15 volts while cooled on ice. Immobilized proteins were detected using the specific antibodies, including anti-DSP/II and anti-β-actin (Santa Cruz Biotechnology, Dallas, TX), anti-FLAG (Sigma Aldrich), anti-plakoglobin/JUP and anti-desmin (Abcam, Cambridge, MA). β-Actin was used as a reference for cytoplasmic proteins. TATA-box binding protein was used as a loading control for nuclear proteins.

Histology and ultrastructural analysis. Mice were anesthetized with pentobarbital. Hearts were perfused in vivo with a cardioplegic solution containing potassium chloride and nifedipine. Hearts were then excised, frozen, and stored at −80°C. Frozen sections were made

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**Fig. 2. Phenotype characterization of sedentary and exercised mice.**

- **A:** comparison of echocardiographic results of left ventricular (LV) mass (in mg), volume (in μl), and right ventricular (RV) dimensions (in mm) in sedentary and exercised NTg (black bars), Tg-DSPWT (white bars), and Tg-DSPR2834H (gray bars) mice. *Significant (P ≤ 0.05) change from sedentary NTg littermates; ‡significant change from exercised NTg littermates; †significant change from sedentary Tg-DSPWT.**

- **B:** representative echocardiographic images demonstrate RV, LV, and left atrial (LA) chamber sizes outlined in red in mice after exercise. C: heart wt-to-body wt ratio in sedentary NTg (black bars), Tg-DSPWT (white bars), and Tg-DSPR2834H (gray bars) mice. Significant difference: *P ≤ 0.05 and **P ≤ 0.01.

- **D:** gross morphology and 4-chamber coronal heart sections of 16-wk-old mice from sedentary and exercise groups. Scale bar = 2 mm (top) and 1 mm (bottom). Arrow indicates dilated RV and thinning of the apical portion of the RV wall in Tg-DSPR2834H mice.
To determine whether endurance exercise changes cardiac morphology and function. Expression of a reference protein was slightly increased in mutants compared with other groups. No changes in cardiac morphology were noted either (data not shown). Heart tissue from 4-wk-old mice fractioned into soluble and insoluble components was analyzed to determine the levels of overexpressed DSP protein and its effects on interacting proteins (Fig. 1B). Flag-tagged DSP was detected in soluble and insoluble fractions in both wild-type (WT) and mutant transgenic mice. In the insoluble fractions, DSP was expressed sevenfold higher in both transgenic compared with the endogenous DSP levels in nontransgenic (NTg) control hearts. Although considerably higher DSP levels were seen in transgenic mice compared with NTg animals, the levels of the DSP transgene protein were similar in these two transgenic lines (Fig. 1Ba).

Notably, overexpression of both WT and mutant DSP led to alterations in expression of the partner proteins desmin and JUP (Fig. 1B, b and c). Desmin expression was equally increased in both soluble and insoluble fractions in both transgenic lines. Levels of JUP expression increased by 1.8- and 2.5-fold in the soluble phase only, while the insoluble fractions contained no JUP. This suggested that overexpression of both WT and mutant DSP may cause translocation of JUP from intercalated disks at baseline. The level of soluble α-tubulin was slightly increased in mutants compared with other groups. Expression of a reference protein β-actin was similar for all groups.

**Results**

**DSP overexpression results in cytoplasmic translocation of soluble JUP from intercalated disk to an insoluble form at baseline.** Analogous to previously reported DSP-Tg lines (37), no cardiac dysfunction or rhythm disturbance was observed in 4-wk-old Tg-DSPWT and Tg-DSPR2834H mice. No changes in cardiac morphology were noted either (data not shown). Heart tissue from 4-wk-old mice fractioned into soluble and insoluble components was analyzed to determine the levels of overexpressed DSP protein and its effects on interacting proteins (Fig. 1B). Flag-tagged DSP was detected in soluble and insoluble fractions in both wild-type (WT) and mutant transgenic mice. In the insoluble fractions, DSP was expressed sevenfold higher in both transgenic compared with the endogenous DSP levels in nontransgenic (NTg) control hearts. Although considerably higher DSP levels were seen in transgenic mice compared with NTg animals, the levels of the DSP transgene protein were similar in these two transgenic lines (Fig. 1Ba).

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**Effect of endurance exercise on cardiac morphology and function.** To determine whether exercise changes the onset and severity of the phenotype of AVC in vivo, 4-wk-old mice were subjected to an involuntary 12-wk running regimen as shown in Fig. 1A. Serial echocardiography was performed to compare cardiac function in mice from exercised and control sedentary groups. At 16 wk of age, no evidence of impairment in cardiac function (decreased ejection fraction or FS) was found in any of the sedentary mice (Table 1). Both sedentary transgenic, Tg-DSPWT and Tg-DSPR2834H, mice showed cardiac hypertrophy compared with NTg littermates at 16 wk of age, evidenced by a significant increase in interventricular septum (IVS) dimensions. Notably, Tg-DSPR2834H mutants displayed a significantly reduced LV volume and elevated RV diameter compared with NTg and Tg-DSPWT.

Upon exercise, cardiac function remained unaffected in all groups (Table 1). Increase in IVS dimension was seen in NTg mice (0.77 ± 0.14 mm), indicating compensatory hypertrophy as a response to endurance exercise. Reduced LV volume was recorded in both Tg-DSPWT and Tg-DSPR2834H mice compared with exercised NTg. However, the RV remained larger in the mutant Tg-DSPR2834H mice than in the other two groups, with a significant difference seen between Tg-DSPR2834H and NTg littermates (Fig. 2, A and B). Taken together, echocardiography demonstrated RV enlargement and reduced LV volumes in Tg-DSPR2834H mutant mice after exercise, and this feature is seen before LV function is overtly impaired. These differential echocardiographic effects on the RV and LV could potentially be attributed to specific R2834H mutation-induced effects compared with that seen in the other control, NTg, and Tg-DSPWT lines.

**Electrocardiographic changes in Tg-DSPR2834H mice.** Because arrhythmias are commonly seen in AVC patients during exercise, ECGs from mice were compared after 16 ± 15 days of exercise to determine whether this finding is recapitulated in this animal model (Table 2). There was no significant difference in RR intervals between NTg (n = 6), Tg-DSPWT (n = 4), and Tg-DSPR2834H (n = 8) mice (P = 0.68) at sedentary baseline. Interestingly, Tg-DSPWT and Tg-DSPR2834H mice had significantly increased P wave amplitude and decreased R wave amplitude (P = 0.02 and 0.001, respectively) compared with NTg mice, correlating with echocardiographic evidence of LA enlargement (Fig. 2B). These differences were maintained even after adjusting for age and number of days exercised. No evidence of arrhythmias was recorded in any of the mice.

**Cardiac remodeling in response to exercise.** In the sedentary group, 16-wk-old Tg-DSPR2834H mutants showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the sedentary group, 16-wk-old Tg-DSPR2834H mice showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the sedentary group, 16-wk-old Tg-DSPR2834H mice showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the sedentary group, 16-wk-old Tg-DSPR2834H mice showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the sedentary group, 16-wk-old Tg-DSPR2834H mice showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the sedentary group, 16-wk-old Tg-DSPR2834H mice showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the sedentary group, 16-wk-old Tg-DSPR2834H mice showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the sedentary group, 16-wk-old Tg-DSPR2834H mice showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the sedentary group, 16-wk-old Tg-DSPR2834H mice showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the sedentary group, 16-wk-old Tg-DSPR2834H mice showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the sedentary group, 16-wk-old Tg-DSPR2834H mice showed significant increase in heart weight-to-body weight (HW/BW) ratios compared with NTg littermates (P = 0.039), suggesting hypertrophy in aging mutants at baseline (Fig. 2C). Exercised Tg-DSPR2834H mutants unlike Tg-DSPWT mice retained a significant increase in HW/BW compared with the

**Table 2. Electrocardiography of 16-wk-old mice from sedentary and exercise groups**

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<td>368 ± 19</td>
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Values are means ± SD; n, no. of subjects. Significant difference: *P ≤ 0.05 and **P ≤ 0.05; †Difference from exercised NTg littermates.

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exercised NTg mice ($P = 0.0065$), suggestive of possible pathological hypertrophy in mutants in response to exercise. Histologically, no noticeable morphologic thickening of LV or RV walls and IVS was found in any mouse from the sedentary group (Fig. 2D). Upon exercise, all mice displayed a hypertrophic LV appearance compared with sedentary littermates, including NTg mice (Fig. 2D, a–b and g–h). This likely reflects physiological endurance hypertrophy. Interestingly, only Tg-DSPR2834H mice had a markedly dilated RV with thinning of the free wall, mostly in the apical portion, the so-called “triangle of dysplasia.”

Because fibrofatty infiltration is a histological hallmark of AVC, Masson trichrome and Oil Red-O staining was performed. While no fibrotic foci were detected in the RV of any of the sedentary mice, patchy focal interstitial fibrosis appeared in the RV of exercised Tg-DSPWT and Tg-DSPR2834H in contradistinction to exercised NTg littermates (Fig. 2B). In the LV, interstitial fibrosis was seen in sedentary transgenic mice, but no fibrosis was detected in any sample after exercise (Fig. 3A). Furthermore, Oil red-O and hematoxylin and eosin staining showed lipid inclusions and interstitial infiltration along with myocyte disarray in the RV of sedentary and exercised Tg-DSPR2834H mice (Fig. 3, C and D). No abnormal fibrofatty tissues were seen in the LV of any groups. Taken together, most pathology was apparent in the RV of mutants, including thinning of the walls, myocyte disarray, and fibrofatty infiltration, while the LV demonstrated only hypertrophy, suggesting the greatest effect in exercised Tg-DSPR2834H mutant mice was early RV involvement and disease progression.

Fig. 3. Histopathology of mouse hearts at 16 wk of age. Masson’s trichrome staining of RV (A) and LV (B) at $\times 20$ magnification is shown. Left, sedentary group; right, exercise group. Focal interstitial fibrosis (in blue) is indicated by arrows in the RV of sedentary and exercised Tg-DSPR2834H as well as in LV of sedentary Tg-DSPR2834H mice. C: Oil Red-O staining of the RV for lipids. *Subepicardial lipid accumulations in the RV of sedentary and focal lipid droplets in RV of exercised Tg-DSPR12834H mice. D: hematoxylin and eosin (H&E) staining of the RV for infiltrative cells. The RV of exercised Tg-DSPR2834H mice shows interstitial fibrosis infiltration with spaces likely containing adipose cells (asterisks). Scale bars = 50 μm.
Ultrastructural changes in response to exercise. TEM revealed remarkable ultrastructural changes in the myocardium of 16-wk-old mice after exercise compared with the sedentary group (Figs. 4–5). Notable cytoplasmic protein aggregates of varying sizes, typically concentrating at the apical ends of affected cardiomyocytes close to the intercalated disks, were seen in both RV and LV from both WT and mutant Tg lines compared with NTg littersmates. Structural disarray and disruption of the intercalated disks associated with these aggregates in both LV and RV was seen in Tg-DSPR2834H mice (Figs. 4, g–i, and 5B, c and d, respectively) compared with intact intercalated disks seen in Tg-DSPWT (Figs. 4d and 5Ab, respectively). Increase in the number and changes in the shape of mitochondria was seen in both exercised WT and mutant transgenic animals compared with the NTg group, although greater abnormalities were detected in mutants DSPR2834H compared with Tg-DSPWT mice. Specifically, a notable absence of cristae, numerous electron-dense aggregates, and fat droplets dispersed throughout the mitochondrion and mitochondrial atrophy were seen in Tg-DSPR2834H hearts (Figs. 4l and 5B, c–g). T tubules were enlarged and I-A-bands were not visible in Tg-DSPR2834H cardiomyocytes with sarcomere structures still intact (Fig. 5B, d and e). Other noticeable structural changes in the RV of Tg-DSPR2834H compared with Tg-DSPWT mice included loss of desmosomes and “pulled apart” cell-cell contacts (Fig. 5Bd). Phagocytosis-like ingestion of these aggregates by the extracellular matrix (ECM) may contribute to further cellular disarray from the cell periphery to the cellular interior (Fig. 5, f and g). Numerous lipid droplets, lysosomal autophagosomes, debris, and the remains of cellular digestion were found embedded in these ECM masses. Taken together, TEM findings underscore ultrastructural changes in both transgenic lines with noticeable greater damage in Tg-DSPR2834H mutants involving both ventricles compared with both transgenic lines, DSP was expressed in desmosomes, seen as aggregates in the cell-cell contacts as expected (Fig. 6a, e and i). The normal cytoplasmic arrangement of desmin was seen in all groups; however, Tg-DSPR2834H mice showed interruption in the expected overlap of DSP and desmin at the intercalated disks (Fig. 6a, k and l) compared with NTg and Tg-DSPWT (Fig. 6a, d–h). Moreover, in exercised mutant Tg-DSPR2834H hearts, DSP aggregates were sequestered in the cytoplasm and predominantly localized to the Z-disks. These DSP aggregates appeared also to colocalize with JUP (Fig. 6bI–l) in the abnormally widened cell-cell contacts. Furthermore, colocal-

![Fig. 4](http://apjheart.physiology.org/)

Exercise

**Fig. 4.** Electron microscopy images of mouse hearts. Myocardium of LV in NTg (A–C), Tg-DSPWT (D–F), and Tg-DSPR2834H (G–I) mice. Magnification ×20,000, scale bar 1 μm. Arrow on left, intercalated disks. Insets on top depict detail of cell-cell junctions with desmosomes indicated by arrowheads. Desmosomes are severely disrupted in mutant Tg-DSPR2834H heart. Insets on bottom provide detailed view of I-bands and Z-disks, which are both diminished in tissue from transgenic animals. Electron-dense protein aggregates (asterisks, E and H) are found near the intercalated disc in both transgenic hearts. Mitochondria (M) of NTg and Tg-DSPWT (F) display normal appearance with “lacunas.” The matrix of many Tg-DSPR2834H mitochondria appears less electron dense containing no lacunas but electron-dense particles in their periphery instead (I, black arrowhead).
ization of JUP and β-catenin was detected in multiple nuclei of Tg-DSPWT mice (Fig. 5A, g–h) but not in Tg-DSPR2834H (Fig. 5A,k–l). Staining for α-tubulin, a structural component of the microtubule network, and connexin 43 (Cx43), a gap junction protein, demonstrated disarray in exercised Tg-DSPR2834H mice (Fig. 7B, k and l). This coincided with disruption of the F-actin network as demonstrated by the loss of phalloidin staining in Tg-DSPR2834H mice (data not shown). In summary, a pool of abnormally localized aggregates of desmosomal (DSP), adhesion (JUP), and gap junction (Cx43) proteins associated with endurance exercise stress was seen in Tg-DSPR2834H mice compared with NTg and Tg-DSPWT control groups. Concomitant with this, disruption of cell-cell contacts and perturbation of intermediate, microtubule, and actin networks were detected.

Exercise-induced perturbation of Wnt/β-catenin signaling. Decreased β-catenin signaling due to abnormal localization of JUP in the nucleus has been previously reported to be associated with AVC in DSP-null mice (8). To explore the effects of exercise on Wnt/β-catenin transcription signaling in our model, we further evaluated associated key proteins in nuclear and cytoplasmic protein fractions by Western blotting. As shown in Fig. 8A, DSP was detected in the nuclear fractions of all sedentary transgenic mice. After exercise, however, nuclear DSP expression was retained in Tg-DSPWT mice, whereas no nuclear DSP was detectable in the Tg-DSPR2834H mutants. High levels of JUP were found in the cytoplasmic and nuclear fractions of the sedentary transgenic animals, confirming dislocation of JUP from intercalated disks as a result of DSP overexpression (Fig. 1B). Upon exercise, cytoplasmic and nuclear JUP was retained in the Tg-DSPWT myocardium, confirming the immunohistochemical findings of nuclear JUP in Tg-DSPWT (Fig. 7Bh) control animals. However, no nuclear or cytoplasmic JUP was detectable in the mutants.

Fig. 5. Electron microscopy of 16-wk-old mouse hearts. A: myocardium of RV in NTg (a), Tg-DSPWT (b), and Tg-DSPR2834H (c) mice. B: RV of DSPR2834H (d–g) mice. Insets in top right depict detailed cell-cell junctions with desmosomes. Insets in bottom right provide detailed view of mitochondria (M). Electron-dense protein aggregates (asterisks) are found near the intercalated disk in cardiomyocytes of both transgenic lines. RV of NTg and Tg-DSPWT have preserved intercalated disks with intact desmosomes (arrows). In contrast, disruption of intercalated disks with interrupted cell-cell connections (arrowheads) is shown in DSPR2834H mice. Note the enlarged T tubules (T) while I-A-bands were not visible in the Tg-DSPR2834H heart. Mitochondria of NTg and Tg-DSPWT mice contain lacunas while these are absent in Tg-DSPR2834H hearts. The matrix of many Tg-DSPR2834H mitochondria appears less electron dense due to swollen cristae and contain numerous mitochondrial inclusions, lipid droplets (F), and autophagic vacuoles (A). Some mitochondrial membranes are disrupted, leading to mitochondrial death in the RV of Tg-DSPR2834H mice. The phagocytosome-like digestion by the extracellular matrix (ECM) of aggregated cellular organelles and debris is seen in the RV; later these cellular digestions are embedded in the ECM masses (asterisks, f and g). Magnification ×20,000, scale bar = 500 nm. C: hypothetical schema of disruption of intercalated disks in DSP-overexpressed mouse heart in response to exercise. Intercalated disk proteins are indicated as follows: desmin (DES), desmoglein (DSG), and N-cadherin (N-CAD).
Within the Wnt-β-catenin pathway, β-catenin was upregulated in the cytoplasm and nucleus in both sedentary transgenic lines compared with NTg at baseline. In the exercised group, NTg mice showed increased levels of cytoplasmic and nuclear β-catenin. Exercised Tg-DSP WT mice showed decrease in levels of cytoplasmic β-catenin, but the levels of β-catenin remained similar in nuclei. In contrast, levels of both cytoplasmic and nuclear β-catenin decreased in mutants. Upstream of β-catenin, GSK3-β, which regulates the turnover of β-catenin, was also evaluated. Total or active GSK3-β was significantly reduced with depletion of its p-Ser9 phosphorylated form (p-GSK3-βSer9) in nuclear fractions of exercised Tg-DSP R2843H mice, unlike all other groups. Further upstream, levels of total cytoplasmic AKT1 with its depleted phosphorylation at p-Ser308 and p-Thr308 (p-AKT1Ser308 and p-AKT1Thr308, respectively) was seen in exercised Tg-DSP WT mutants only. In contrast, increased levels of p-AKT1Ser473 and p-AKT1Thr308 were seen in Tg-DSP WT after exercise, suggesting that Tg-DSP WT mice have activation of AKT signaling that recruits phosphorylation and inactivation of GSK-3β, thereby leading to downstream β-catenin upregulation (Fig. 8B, left). These data suggest that DSP may have a direct (or through JUP) regulatory role on AKT1 expression and phosphorylation, whereas the DSP-R2843H mutation perturbs this function, likely by sequestering desmosomal JUP in the aggregates (Fig. 8B, left).
right), possibly blunting activation of AKT1 signaling with consequential reduction of total GSK3-β (5).

Gene expression analysis. To further analyze downstream alterations, gene expression profiling was performed for the RV since the most predominant changes were in the RV of Tg-DSPR2834H mutants. In contrast to Tg-DSPWT, exercised Tg-DSPR2834H showed increases in natriuretic peptide type A, natriuretic peptide type B, and myosin heavy chain 7, corresponding to hypertrophy and dilated RV myocardium (Fig. 9, A–D). Increased levels of Col3a1 (Fig. 9E) were detected in sedentary Tg-DSPR2834H transgenic animals only, indicative for fibrotic remodeling at sedentary baseline. Interestingly, no increase in Col3a1 was detected in any animals after exercise. Analysis of genes associated with lipid metabolism showed a significant increase in peroxisome proliferator-activated receptor-γ (Pparγ) expression in Tg-DSPR2834 mice compared with Tg-DSPWT (Fig. 9F). Expression of carnitine palmitoyltransferase (Cpt) 1b (Fig. 9G), a key enzyme in fatty acid metabolism, was significantly increased in the exercised Tg-DSPR2834H group compared with both Tg-DSPWT and NTg (P < 0.05), suggesting persistence of activated fatty acid metabolism in exercised mutants. Unlike the Cpt1b profile, the levels of peroxisome proliferator-activated receptor-γ coactivator-1α (Fig. 9H), a marker of mitochondrial biosynthesis, were similar between the exercised groups, suggesting a disconnect between fatty acid metabolism and mitochondrial biosynthesis.

Fig. 7. Immunofluorescence microscopy of RV from 16-wk-old mice subjected to exercise. Rows on top represent NTg, rows in middle represent Tg-DSPWT, and rows on bottom represent Tg-DSPR2834H. Scale bars = 10 μm. A: costaining of JUP and β-catenin. Nuclear JUP and β-catenin was only detected in Tg-DSPWT (arrowheads). B: costaining of α-tubulin (ATUB) and connexin 43 (Cx43). Presence of cytoplasmic Cx43 aggregates coincides with the disruption of α-tubulin disarray in Tg-DSPR2834H mice (arrow).
biosynthesis in mutants only. Finally, c-Myc (Fig. 9I), a direct target of β-catenin/TCF signaling, was significantly increased upon exercise in mutant Tg-DSPR2834H animals. No significant differences were seen in connective tissue growth factor, osteopontin, periostin, and peroxisome proliferator receptor-α (Ppara) genes (data not shown). Taken together, variable signaling, including fibrosis, heart failure, fatty acid metabolism, and β-catenin/TCF signaling, were perturbed in our mouse model in association with chronic endurance exercise even before echocardiographic detection of any impairment in cardiac function.

**DISCUSSION**

Endurance or acute exercise has been recognized as an environmental factor that may promote onset and progression of AVC in desmosome-encoding gene mutation-positive patients and carriers who are clinically phenotype negative. However, the management of an exercise prescription in these patients remains controversial. The goal of the present study was to unravel the mechanistic relationship between phenotypic onset and pathogenesis of AVC due to overexpressed mutant DSP and endurance exercise in vivo, potentially facilitating a consistent approach to exercise management in the clinical setting of patients with AVC. We previously reported the advanced AVC phenotype seen in 6-mo-old mutant Tg-DSPR2834H mice (37). In this study, 4-wk-old NTg, Tg-DSP R2834H, and Tg-DSP R2834H mice were reconstituted and subjected to endurance exercise for 12 wk. Serial echocardiography detected alterations in cardiac morphology, with the most profound changes observed in the RV even before cardiac function started to decline, similar to that seen in humans with AVC (23). Data showed that exercise resulted in physiological hypertrophy in NTg mice compared with sedentary NTg animals, as expected. Decrease in the LV end-diastolic volume was significant in both exercised Tg-DSP WT and mutant Tg-DSP R2834H groups, suggesting unfavorable effects of exercise on diastolic performance in both transgenic lines. Notably, significant RV dilation and wall thinning in the apical part of the RV was seen in Tg-DSP R2834H compared with Tg-DSP WT controls, similar to that reported by Kirchhof et al. in plakoglobin-deficient mice (13).
The mechanistic basis of the susceptibility to early phenotypic involvement of the RV compared with the LV has been studied and reviewed by many groups (1, 20, 33). Persistence of some embryologic differences between LV and RV in an adult heart, altered Wnt/β-catenin signaling, differential RV and LV geometry and anatomy, pressure, volumes, and cardiac pre- and afterload have been reported to contribute to the somewhat chamber-specific response of the RV (although primary LV disease can occur) in desmosomal mutation-positive subjects. In addition, chamber-specific cellular pathologies such as cell atrophy, apoptosis, and/or necrosis with or without fibrofatty replacement have been reported to be correlated in many in vivo models with specific genetic defects. For instance, necrotic cell death and subsequent myocardial inflammation with later-onset fibrous tissue replacement was observed in DSG2-N266S transgenic mice (29) while an increase in adipogenesis with myocyte apoptosis and ventricular arrhythmias was reported in JUP transgenic and DSP-deficient mice (8, 19). Heterozygous JUP-deficient mice displayed no signs of fibrofatty tissue replacement or myocyte hypertrophy but still exhibited tachycardia and reduced RV conduction (13). Our Tg-DSPR2834H model displayed specific features as well. Cardiomyocytes of Tg-DSPR2834H mice contained protein aggregates that consist of DSP, JUP, and Cx43, similar to desmin-related myopathies (9). We believe this is a direct result of a “final common pathway” disturbance of the interplay between DSP-R2834H and desmin, with the final phenotype consistent with a desminopathy. Possibly the disruption in

Fig. 9. Comparative QPCR analysis of RV myocardium in 16-wk-old mice expressed in fold changes compared with NTg. β-Actin was used as the reference gene. A: natriuretic peptide type A (Nppa); B: natriuretic peptide type B (Nppb); C: myosin heavy chain 6 (Myh6); D: myosin heavy chain 7 (Myh7); E: collagen type 1 α1 (Col1a1); F: peroxisome proliferator-activated receptor-γ (Pparγ); G: carnitine palmitoyltransferase (Cpt) 1A; H: peroxisome proliferator-activated receptor-γ coactivator-1α (Pgc1α); and I: myelocytomatosis oncogene (c-Myc). At least 4 animals per NTg (black bars), Tg-DSP (white bars), and Tg-DSPR2834H (gray bars) group were used. *P < 0.05, **P < 0.01, and ***P < 0.001.
Cx43 contributed to the perturbation in electrophysiology as observed from lower R and P wave amplitudes. Moreover, only in Tg-DSPR2834H we observed Cx43 aggregates in the cytoplasm. Because exercise endurance stress gradually stretches and disrupts desmosomal complexes in the RV of mutants, cell-cell connections and integrity are lost, probably leading to subsequent regional digestion and fibrous replacement (Figs. 3F and 6). Thus, studies in models with DSP-R2834H at levels more resembling those of the endogenous protein might provide further insights in the mechanisms involved.

One of the proposed consequences of altered DSP expression involves release of JUP from intercalated disks and its translocation to the nucleus (19). Moreover, diffuse loss of JUP from the intercalated disk appears to occur in the majority of cases (4, 25). Our study indeed demonstrated that DSP-R2834H mutation with ability to alter DSP phosphorylation (2) affects the trafficking of JUP into the nucleus in response to endurance exercise. Upon exercise, cytoplasmic JUP translocated to the nucleus in Tg-DSPWT hearts with concomitant increase of β-catenin levels triggering up- and downstream modifications in the canonical Wnt-β-catenin pathway. However, JUP was not detectable in the cytoplasm and nuclei of Tg-DSPR2834H hearts. Instead, we detected JUP in the cell apaxes clustered with aggregates of mutant DSP-R2834H, suggestive of a possible sequestration of JUP by mutant DSP-R2834H, consistent with the potential that the salvage of DSP-R2834H and JUP in the cell apaxes may interfere with activation of Wnt/β-catenin and AKT1 signaling in response to exercise in mutants (15).

Wnt/β-catenin signaling is known to enhance myogenesis and inhibit adipogenic transcription through CCAAT/enhancer-binding protein α and PPARγ coactivated with PPARα (12, 26). Cytoplasmic β-catenin is degraded in the absence of a Wnt signal as a result of phosphorylation of GSK3-β, where downstream signaling depends on fully phosphorylated forms of substrate (or β-catenin in this case) (14). Moreover, reduced AKT1 and its downstream signaling through phospho-AKT1Ser473 inactivate GSK3-β and β-catenin, providing the additional possibility that reduced GSK3-β and β-catenin in the TgDSPR2834H mutants could have an impact on adipogenic signaling and disease acceleration. In contrast, exercised Tg-DSPWT displayed a strong increase in the cytoprotective levels of phospho-AKT1Ser473 and AKT1Thr308 high nuclear p-GSK3βSer9 levels, and a subsequent β-catenin increase (28, 35). Interestingly, we did not find clear evidence of existing adipocytes in any groups after exercise; instead, focal subepicardial neutral lipid accumulation in the RV was evident. In the healthy adult heart, phosphocreatine is the main reserve source of ATP and require about three times more ATP than glucose but require increased stretch, mechanical stress, and energy requirements associated with exercise place more strain on the already functionally impaired intercalated disks due to mutant DSP-R2834H, with subsequent alterations in JUP, AKT1, and Wnt/β-catenin signaling as well as perturbations in cardiomyocyte mitochondria and the structural components α-tubulin, desmin, and Z-I-bands (34).

Study limitations. In this study, we used reconstituted transgenic mice with overexpression of human WT and mutant R2834H DSP, where overexpression of cytoplasmic DSP protein may provoke LV hypertrophy at its sedentary baseline. This fact is likely to arise from a higher level of expression of the DSP transgene in the reconstituted model used here. Because levels of overexpressed DSP protein in both WT and mutant transgenic lines are similar, it is possible that any differential effect observed between WT and mutant transgenic lines is to be attributed to the specific R2834H mutation.

Another limitation of this study is that no arrhythmias were recorded during intense exercise in mice. The limiting factor could be that ECGs were performed in sedated mice.

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