Regular Exercise Training Prevents Aortic Valve Disease in Low-Density Lipoprotein–Receptor–Deficient Mice

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Background—Regular exercise training (ET) slows the progression of atherosclerotic lesions, reduces oxidative stress, and increases nitric oxide bioavailability, all of which may be expected to improve degenerative aortic valve disease.

Methods and Results—Four-week-old low-density lipoprotein–receptor–deficient mice (n = 94) were randomly divided into 4 groups: Group 1 (control group), normal diet plus sedentary activity; group 2 (cholesterol group), cholesterol diet plus sedentary activity; group 3 (regular ET group), cholesterol diet plus regular ET (60 min/day, 5 days/week) for 16 weeks; and group 4 (occasional exercise group), cholesterol diet plus occasional ET (1 day/week) for 16 weeks. At 20 weeks of age, histological analysis was performed. A significant increase in aortic valve thickness was evident in the cholesterol group compared with the control group. Importantly, regular but not occasional ET significantly reduced aortic valve thickness compared with the cholesterol group (control 31.3 ± 3.0 μm, cholesterol 50.1 ± 3.4 μm, regular exercise 30.4 ± 1.2 μm, and occasional exercise 48.9 ± 3.2 μm). Immunohistochemistry revealed that a cholesterol diet disrupted and regular ET preserved endothelial integrity on the aortic valve surface. Furthermore, serum myeloperoxidase, accumulation of macrophages and oxidized low-density lipoprotein, in situ superoxide, activated myofibroblasts/osteoblast phenotypes, and mineralization were increased in the cholesterol group but were decreased by regular ET. Polymerase chain reaction revealed increased messenger RNA expression for α-smooth muscle actin, bone morphogenetic protein-2, runt-related transcription factor-2, and alkaline phosphatase in the cholesterol group, whereas these were diminished by regular ET. Moreover, regular ET significantly increased circulating levels of fetuin-A compared with the cholesterol group.

Conclusions—In the low-density lipoprotein–receptor–deficient mouse, regular ET prevents aortic valve sclerosis by numerous mechanisms, including preservation of endothelial integrity, reduction in inflammation and oxidative stress, and inhibition of the osteogenic pathway. (Circulation. 2010;121:759-767.)

Key Words: valves ■ exercise ■ prevention ■ inflammation ■ calcification

C urrently, no effective therapy to prevent calcified aortic valve (AV) disease exists; hence, novel therapies and their optimum timing are active areas of investigation.1 Calcified AV disease confers significant morbidity and mortality as the severity of disease progresses.2 Thus, as suggested by recent reports,3–6 adverse events can be avoided or delayed if it becomes possible to prevent the progression of AV disease at an earlier time point, before the presentation of severe valve calcification (eg, AV sclerosis).

Clinical Perspective on p 767

Accumulated evidence suggests that degenerative calcified AV disease and atherosclerosis share similar mechanisms such as clinical risk factors (eg, physical inactivity) and histopathological features (eg, valvular/vascular endothelial disruption, oxidative stress, calcification).6–10 We and others demonstrated that regular physical exercise training (ET) prevents the progression of atherosclerotic cardiovascular disease by modulating important mechanisms such as endothelial function or oxidative stress,10–13 all of which may be expected to improve degenerative AV disease. At present, it remains unknown whether ET modulates valvular heart disease, particularly AV disease. Therefore, the aim of the present study was to investigate whether ET prevents the development of AV sclerosis, and if so, what pathophysiological mechanisms are involved, including valvular endothelial integrity, inflammation, oxidative stress, and the osteogenic signaling pathway.

Methods

Animals

A total of 94 low-density lipoprotein (LDL)–receptor–deficient (LDLR−/−) mice on the C57BL/6J background were randomly divided into 4 groups at 4 weeks of age (Figure 1): group 1 (control...
group), normal diet plus sedentary lifestyle; group 2 (cholesterol group), cholesterol diet plus sedentary lifestyle; group 3 (regular ET group), cholesterol diet plus regular ET (1 h/day, 5 days/week); and group 4 (occasional ET group), cholesterol diet plus occasional ET (1 day/week). The cholesterol-rich diet (Western-type diet) contained 0.15% cholesterol and was obtained from Altromin GmbH (Lage, Germany).

The animals were housed at the Animal Research Center of the University of Leipzig in a specific pathogen-free environment in rooms with a 7 AM to 7 PM light/dark cycle. All procedures were approved by the Regierungspräsidium Leipzig (TVV 26/07).

### Training Protocol

To assess the effects of a 16-week ET program on AV thickening, LDLR−/− mice were randomly assigned to a regular ET group (5 days/week), an occasional ET group (1 day/week), or a sedentary group. Mice assigned to the ET groups were taught to run on a motorized rodent treadmill with a shock-plate incentive. The slope of the treadmill was kept constant at 5°. Mice were trained at a speed of 15 m/min for 60 min/day with 2-minute rest intervals every 15 minutes. Choosing a power of 90% and 5% type I error, we calculated that a sample size of at least 8 in each subgroup was required to detect significant differences in AV thickness by ANOVA (PASS 2008, NCSS Inc, Kaysville, Utah).

### Blood Biochemical Analysis, Histology, Immunohistochemistry, Oxidative Stress, Polymerase Chain Reaction, and Echocardiography

For a detailed description, see the online-only Data Supplement.

### Statistical Analysis

SPSS version 16.0 (SPSS Inc, Chicago, Ill) was used for all of the analyses. Data are expressed as mean±SEM. Comparisons among groups were tested with ANOVA. When data were not normally distributed or the variance was not equal, the Kruskal-Wallis nonparametric test was used. A correlation between AV thickness and AV flow velocity was assessed by Pearson coefficient. A value of \( P<0.05 \) was considered statistically significant. All of the measurements were made by investigators blinded to the treatment group.

### Sample-Size Calculation

The primary end point of the study was AV thickness at 20 weeks of age. To calculate the sample size, it was hypothesized on the basis of a recent study\(^ {15} \) that AV thickness (mean±SD) of the control, cholesterol, regular ET, and occasional ET groups was 25±15, 55±15, 30±15, and 45±15 \( \mu \)m, respectively. Choosing a power of 90% and 5% type I error, we calculated that a sample size of at least 8 in each subgroup was required to detect significant differences in AV thickness by ANOVA (PASS 2008, NCSS Inc, Kaysville, Utah).

### Results

#### Regular, Not Occasional, Exercise Reduces AV Thickening and Flow Velocity

Quantitative analysis revealed that LDLR−/− mice fed a cholesterol diet had significantly \( (\approx 60\% \) thicker AV leaflets than those fed a normal diet (control group; Figure 2; control group 31.27±2.96 \( \mu \)m, cholesterol group 50.14±3.35 \( \mu \)m, \( P<0.001 \)). In contrast, regular ET abolished the cholesterol diet–induced increase in AV thickness (Figure 2; regular ET 30.36±1.22 \( \mu \)m; \( P<0.001 \), regular ET versus cholesterol group), whereas occasional ET did not (occasional ET group 48.85±3.18 \( \mu \)m).

Although left ventricular dimensions were not different between control and cholesterol-fed animals (left ventricular dimension at diastole, control group 0.32±0.01 cm versus cholesterol group 0.30±0.01 cm, \( P=0.21 \); at systole, control group 0.20±0.01 cm versus cholesterol group 0.21±0.01 cm, \( P=0.73 \)), a cholesterol diet led to a significant \( (\approx 40\% \) increase in AV flow velocity compared with control (Figure 3; control group 0.96±0.03 m/s, cholesterol group 1.36±0.06 m/s, \( P<0.0001 \)). Regular ET was potent enough to circumvent the cholesterol-induced increase in AV flow velocity, whereas occasional ET did not (Figure 3; regular ET 0.99±0.04 m/s, occasional ET 1.23±0.04 m/s; \( P<0.0001 \), cholesterol versus regular ET group). There was a positive correlation between AV thickness and AV flow velocity \( (r=0.69, P<0.0001) \).

#### Regular Exercise Preserves Integrity of Endothelial Cell Layer on the AV

In the control animals, 95.7±1.0% of the AV surface was covered with endothelial cells (Figure 4). A cholesterol diet
for a period of 16 weeks led to a significant reduction in endothelial cell coverage (cholesterol group 73.7±2.2%; P<0.0001, control versus cholesterol group). Regular ET completely preserved the cholesterol diet–induced disruption of valvular endothelial integrity (regular exercise 95.8±2.3%; P<0.0001, cholesterol versus regular-exercise group).

Regular Exercise Reduces Macrophage Accumulation and Attenuates Valvular Fibrosis and Proosteogenesis

AV leaflets of LDLR−/− mice fed a normal diet were thin, with little if any macrophage infiltration (Figure 5). In the normal-diet (control) group, α-smooth muscle actin–positive myofibroblasts or alkaline phosphatase–positive osteoblast phenotypes were sparse at the AVs (Figure 5). A cholesterol diet significantly increased α-smooth muscle actin–positive myofibroblasts and the alkaline phosphatase–positive osteoblast phenotype at the AV leaflets, whereas regular ET reduced levels of those phenotypes significantly. To confirm these immunohistological results, quantitative reverse-transcription polymerase chain reaction analyses were also performed. A cholesterol diet significantly increased α-smooth muscle actin, bone morphogenetic protein-2, runt-related transcription factor-2, and alkaline phosphatase compared with the normal-diet (control) group. In contrast,
regular ET significantly reduced messenger RNA expression of α-smooth muscle actin, runt-related transcription factor-2, and alkaline phosphatase compared with the cholesterol-diet group (Figure 6). We also noted marked increases in valvular fibrosis and mineralization associated with thickened AVs in mice fed a cholesterol diet (Figures I and II in the online-only Data Supplement). Regular ET substantially reduced both valvular fibrosis and mineralization.

**Regular Exercise Increases Inhibitors of Calcification**

Extremely low levels of osteopontin at the AVs were comparable among 3 groups (the control, cholesterol-diet, and regular ET groups; online-only Data Supplement Figure IIIA). In contrast, positive staining of osteoprotegerin was clearly seen at the AVs in the control group, whereas its expression at the AVs in the cholesterol-diet group was extremely repressed. Interestingly, regular ET appeared to increase the levels of osteoprotegerin at the AVs compared with the cholesterol group. Moreover, a cholesterol diet significantly reduced circulating levels of fetuin-A compared with control (online-only Data Supplement Figure IIIB; control group 71.2 ± 10.5 g/mL, cholesterol group 50.2 ± 6.5 g/mL, P<0.05), but this effect was ameliorated by regular ET (regular exercise 73.9 ± 10.5 g/mL; P<0.05, regular exercise versus cholesterol).

**Regular Exercise Reduces Circulating Levels of Myeloperoxidase and Oxidative Stress of AVs**

We measured myeloperoxidase (MPO) as an enzymatic source of oxidant products. A cholesterol diet significantly increased serum levels of MPO at 10 weeks compared with the normal-diet group (Figure 7A; control group 63.1 ± 7.6 ng/mL, cholesterol group 279.0 ± 90.5 ng/mL; P<0.05, control versus cholesterol). In contrast, regular ET completely suppressed MPO levels compared with the cholesterol group (regular ET 44.8 ± 23.2 ng/mL; P<0.05, cholesterol versus regular ET). Furthermore, we assessed oxidized LDL expression and superoxide production at the AV leaflets. Both immunohistochemistry for oxidized LDL and dihydroethidium staining showed that a cholesterol diet clearly increased oxidized LDL expression and enhanced dihydroethidium fluorescence at the AV leaflets, whereas regular ET markedly reduced both parameters (Figure 7B).

**ET Did Not Alter Lipid Metabolism**

Regular or occasional ET for a period of 16 weeks did not significantly affect serum cholesterol levels in LDLR−/− mice fed a cholesterol diet (control group 859.8 ± 76.6 mg/dL, cholesterol group 1957.8 ± 235.15 mg/dL, regular ET 1978.3 ± 298.8 mg/dL, and occasional ET 2147.9 ± 341.5 mg/dL).

**Discussion**

In the present study, several findings were made as to the novel effects of ET on heart valves, particularly AVs. First, regular ET but not occasional ET prevents the development of AV sclerosis in LDLR−/− mice. Second, regular ET prevents disruption of the endothelial cell layer on the AV surface; inhibits accumulation of macrophages and oxidized LDL and reduces oxidative stress; suppresses the proosteogenic signaling pathway in the AVs; and increases inhibitors of calcification. These findings suggest that regular ET is a therapeutic option to prevent calcified AV disease.

**Effect of Exercise on AV Sclerosis**

The major finding of the present study was that regular, not occasional, ET prevents the development of AV sclerosis in LDLR−/− mice. Several previous studies have confirmed that LDLR−/− mice or apolipoprotein E–deficient mice fed an atherogenic diet reveal significant AV abnormalities.15,16 The ideal end point for measuring the effect of therapy would be direct evaluation of tissue changes in the AV leaflets.1 By using an LDLR−/− mouse model and analyzing AV tissue,
we demonstrated for the first time that regular ET but not occasional ET prevents pathological AV changes and AV dysfunction in the early stage of disease (Figures 2 and 3). AV sclerosis is an important stage at which to intervene for disease management17 because recent reports have suggested that lipid-lowering treatment should be initiated at an early stage of calcified AV disease to slow or halt disease progression.4,18

The American Heart Association recommends regular physical exercise for the prevention and treatment of atherosclerotic cardiovascular disease.19 Moreover, physical inactivity itself predicts poor outcome in patients with calcified AV disease.9 Thus, regular ET may be suggested to reduce the incidence and mortality of atherosclerotic vascular disease, as well as degenerative calcified AV disease, particularly in an early stage of disease (eg, AV sclerosis).

Effect of Regular Exercise on Endothelial Integrity at the AV Surface
We further assessed the pathophysiological mechanisms with regard to how regular ET prevents the development of AV sclerosis. The surface of AV leaflets is covered with endothelial cells, which are important for proper function.20 Evidence of valvular endothelial cell layer disruption in the pathogenesis of calcified AV disease was provided by immunohistochemistry for the endothelial marker endothelial nitric oxide synthase in a study of hypercholesterolemic rabbits.21,22 Using the same approach, we demonstrated in the present study that regular ET preserved the valvular endothelial integrity of LDLR−/− mice, even those that were fed a cholesterol diet.

Recently, valvular endothelium has been found to exert its important effects of modulating AV relaxation/contraction and regulating the changes in valve stiffness by modification of the valvular endothelial release of NO.23 In addition, valve integrity and stiffness are responsible for valve function and calcification.24,25 These findings suggest that the absence of endothelial integrity in a damaged valve could result in structural and functional damage, leading to disease progression.20 In the present study, regular ET preserved valvular endothelial integrity and thus may prevent the development of pathological valve remodeling, as demonstrated by thickened AVs (Figure 2) and accelerated AV flow velocity (Figure 3).

Effect of Regular Exercise on Inflammation, Oxidized Lipid Deposition, and Oxidative Stress at the AVs
Valvular endothelial cell layer disruption appears to initiate the recruitment of inflammatory monocytes/macrophages and the infiltration of inflammatory monocytes/macrophages and valvular myofibroblast-like cells (α-SMA), and a functional phenotypic marker of osteoblasts (alkaline phosphatase [ALP]). Semiquantitative analysis of infiltration of macrophages (B), valvular myofibroblast-like cells (C), and osteoblast-type cells (D) in 8 animals from each group. Arrows denote areas of aortic valve tissue where immunostaining is faint, whereas positive immunostaining is evident at the vascular smooth muscle cell layer (α-SMA) and the atherosclerotic areas of the aortic sinus (Mac3 and ALP), as indicated by arrowheads. Bar=100 μm. Chol indicates cholesterol diet.

Figure 6. A, Immunostaining for macrophages (Mac3), vascular smooth muscle cells and valvular myofibroblast-like cells (α-smooth muscle actin [α-SMA]), and a functional phenotypic marker of osteoblasts (alkaline phosphatase [ALP]). Semiquantitative analysis of infiltration of macrophages (B), valvular myofibroblast-like cells (C), and osteoblast-type cells (D) in 8 animals from each group. Arrows denote areas of aortic valve tissue where immunostaining is faint, whereas positive immunostaining is evident at the vascular smooth muscle cell layer (α-SMA) and the atherosclerotic areas of the aortic sinus (Mac3 and ALP), as indicated by arrowheads. Bar=100 μm. Chol indicates cholesterol diet.
phages. Notably, oxidative stress plays a crucial role in the pathogenesis of AV stenosis, including the formation of lipid-laden macrophages and the development of inflammation. In accordance with an observation by Aikawa et al., we confirmed that a high-cholesterol diet induced AV thickening in 20-week-old LDLR\(^{-/-}\) mice, which was associated with the presence of macrophage-rich lesions (Figure 5). In addition, we and others observed remarkable increases in oxidized LDL, a putative initiator of tissue calcification, in thickened mice AVs (Figure 7B) and in human calcified AVs associated with infiltrated macrophages. In contrast, regular ET markedly reduced the accumulation of macrophages (Figure 5B) and oxidized LDL, as well as levels of superoxide, in the AVs (Figure 7B). One may speculate that oxidized LDLs in the AVs are closely linked to inflammation and oxidant stress as an initiator of valve calcification. MPO can be mentioned as a possible source of valvular oxidative stress because of its potential capacity for lipid oxidation. Of note, we demonstrated that regular ET completely suppressed serum levels of MPO in mice (Figure 7A). Regular ETters to the development of valve calcification in LDLR\(^{-/-}\) mice fed a cholesterol diet.}

Figure 6. Quantitative analysis of messenger RNA expression for (A) \(\alpha\)-smooth muscle actin; B, a mediator of calcification, bone morphogenetic protein-2 (BMP-2); C, a key proosteogenic transcription factor, runt-related transcription factor-2 (Runx-2); and D, alkaline phosphatase from aortic valve cusps of 8 animals from each group. Chol indicates cholesterol diet; arb. units, arbitrary units.

Figure 7. A, Serum levels of myeloperoxidase in 7 LDLR\(^{-/-}\) mice after 6 weeks of being fed a cholesterol diet (Chol). B, Immunostaining for oxidized low-density lipoprotein (ox-LDL) and in situ detection of superoxide evaluated by staining with the \(\mathrm{O}_2^-\)-sensitive dye dihydroethidium (DHE; red fluorescence). Images for all 3 groups were acquired with identical procedures and settings. Each image is representative of results from 3 different animals. Arrowheads indicate sites of aortic valve. Bar=100 \(\mu\)m.
ET may be a tool to reduce oxidative stress by reducing MPO levels, thereby contributing to the prevention of AV disease.33,34

We lack therapies to prevent the progression of AV stenosis and thus need to discover novel potential targets for calcified AV disease.1 As suggested by recent reports,27,29,35 it is possible that treatment that targets oxidative stress during earlier stages of disease (eg, AV sclerosis) may slow the progression of AV disease.

Effect of Regular Exercise on the Osteogenic Pathway at the AVs

Recent studies reported the inhibitory effect of exercise on the extent of vascular calcification in animals and humans,36,37; however, little is known about the molecular mechanisms of how ET regulates cardiovascular calcification. In the present study, we demonstrated for the first time that regular ET not only attenuates the proosteogenic signaling pathway in the AVs but also increases inhibitors of calcification.

The primary stimulus for valvular calcification appears to be inflammation in response to endothelial dysfunction.26 In particular, oxidative stress plays an important role in the amplification of procalcific gene expression, such as bone morphogenetic protein-2 or runt-related transcription factor-2, leading to AV calcification.38 Most important, the osteoblast phenotype, through the upregulation of osteogenic transcription factors, is the final common pathway for AV calcification.21,36,39 Osteoblast phenotype is often measured by an increased expression of alkaline phosphatase and runt-related transcription factor-2, which are characteristic of various stages of bone development.39 Valvular myofibroblasts also could have the potential to differentiate into an osteoblast phenotype.25 In the present study, we observed significant increases in myofibroblast and osteoblast phenotypes, as assessed by quantitative polymerase chain reaction and immunohistochemistry, in the AVs of LDLR−/− mice fed a cholesterol diet. Notably, regular ET attenuated the levels of myofibroblast and osteoblast phenotypes through downregulation of a key osteogenic transcription factor, runt-related transcription factor-2, which probably resulted from a reduction in oxidative stress, as mentioned above.

Another potential factor regulating AV calcification is circulating fetuin-A, an inhibitor of calcification.40,41 In the present study, somewhat surprisingly, regular ET significantly increased circulating levels of fetuin-A compared with the cholesterol group (online-only Data Supplement Figure IIIIB), which may represent 1 of the mechanisms for inhibition of AV mineralization by regular ET.

A landmark echocardiographic study demonstrated that moderate or severe AV calcification has a faster disease progression and worse prognosis in patients with AV stenosis than in patients with no or mild calcification,5 which suggests the importance of intervention at an earlier stage of disease, before advanced AV calcification has occurred. Therefore, regular ET aimed at prevention of an early stage of calcified AV disease before it develops into overt AV calcification can be an effective strategy (Figure 8).

Study Limitations

Several limitations of the present study should be mentioned. First, the AV flow velocities observed in this study were within normal limits, and reproducible apical views with an acceptable intercept angle for accurate estimates are rather difficult to obtain. In that regard, potential errors may have occurred. Nevertheless, the interobserver and intraobserver variability of aortic velocity in the present study were both <5%, which represents an acceptable reproducibility and validates the results of Doppler velocity. Second, we could not perform Western blot analysis for quantitative protein measurement because of limited tissue availability in mouse AVs.4 Third, the present findings cannot be applied to the
clinical setting directly. It remains to be examined whether ET will halt or reverse early valve changes, as well as what duration and intensity of ET should be recommended. Fourth, there are some differences in the pathogenesis of AV disease between the present model and human patients, including atherogenic factors (e.g., lipids, smoking) and genetic factors (e.g., LDLR, Notch1)\textsuperscript{42,43}; however, the use of this animal model with hyperlipidemia would aid in the discovery of new therapeutic options and in our understanding of its mechanisms. Future clinical trials will need to include several atherosclerotic risk factors in addition to the potential genetic factors that affect disease development.

Conclusions

The present study reports for the first time that regular ET prevents AV sclerosis in mice by exerting numerous favorable effects, including preservation of valvular endothelial integrity, causing subsequent decreases in the recruitment of inflammatory cells, oxidative stress, and the proosteogenic pathway. On the basis of our new insights, regular physical activity may be useful to prevent the development of degenerative AV disease.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

Calcified aortic valve (AV) stenosis is a large and growing health problem with no alternatives to costly invasive treatment in Western countries. Thus, the optimum timing of novel therapy for the prevention of calcified AV disease is an active area of investigation. AV sclerosis can develop into AV stenosis in many patients. In addition, severe valve calcification indicates a rapid disease progression and poor prognosis in patients with AV stenosis. Thus, an effective intervention at an earlier stage of disease before the development of severe valve calcification (eg, AV sclerosis) would have major clinical benefits. The present study demonstrated that regular exercise training but not occasional exercise training prevented AV sclerosis in mice via numerous favorable mechanisms, including preservation of valvular endothelial integrity and a subsequent decrease in recruitment of inflammatory cells, oxidative stress, and the osteogenic process. These novel findings indicate that regular physical activity may be recommended for prevention of the early stage of calcified AV disease. Accumulated evidence suggests that several cardiovascular risk factors are associated with AV sclerosis/stenosis and that the metabolic syndrome is associated with an increased prevalence of AV calcium or progression of AV stenosis. The metabolic syndrome is a potentially preventable and modifiable condition that results primarily from a sedentary lifestyle. Many of the features of the metabolic syndrome are not reversed by the pharmacological treatment of traditional risk factors (eg, statins). Therefore, regular exercise training is warranted and may represent a promising intervention to reduce the incidence and mortality of calcified AV stenosis, particularly at an early stage of disease.
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SUPPLEMENTARY MATERIAL

Supplemental Methods

**Blood Biochemical Analysis**

Blood was collected from deeply anesthetized mice by cutting right atrial appendage and spun in a centrifuge to obtain serum, which was stored at -80°C. Serum levels of total cholesterol were determined enzymatically using a calorimetric assay kit (EnzyChrom Cholesterol Assay Kit, ECCH-100, Bioassay Systems, Hayward, USA) as recommended by the manufacturer. The serum concentration of mouse myeloperoxidase (MPO) and fetuin-A was measured by a commercial available ELISA kit (MPO: Hycult Biotechnology, Uden, The Netherlands; fetuin-A: R&D Systems, Wiesbaden, Germany). The samples were assayed in duplicate.

**Morphometric and Immunohistochemical Analysis**

The hearts were fixed in 4% formalin for 24 hours. After fixation, the hearts were embedded into paraffin, and 4-µm serial sections were cut through the AVs. Sections showing all 3 cusps were stained with hematoxylin and eosin for general morphology. Overall thickness of the leaflets was averaged over 5 equally distributed length measurements throughout the valve and
quantified with an imaging software (analysis 3.0, Olympus Soft Imaging Solutions GmbH, Münster, Germany) as previously described.\(^1\)

Sirius red staining for collagen was performed to evaluate fibrosis of the aortic valves. To avoid color balance variation, sirius red staining of all sections was performed at the same time. All sections from the different groups of mice were photographed with the same light intensity by digital image capture as previously described.\(^2\) Von Kossa staining was performed to evaluate valvular mineralization.\(^4\) A negative control for von Kossa was done by immersion of the slide in 10% formic acid for 30 minutes before von Kossa staining.\(^5\)

Immunohistochemistry for endothelial nitric oxide synthase (eNOS) (rabbit polyclonal anti-mouse NOS-3 antibody, Santa Cruz Biotechnology, Inc. Santa Cruz, USA), macrophages (rat monoclonal antibody against mouse Mac3, BD Biosciences, San Jose, USA), myofibroblast (\(\alpha\)-smooth muscle actin (\(\alpha\)SMA), 1A4, Sigma-Aldrich, Deisenhofen, Germany) osteoblast phenotype marker (rabbit polyclonal antibody against alkaline phosphatase (ALP), Abcam, Cambridge, UK) oxidized Low Density Lipoprotein (ox-LDL) (rabbit polyclonal anti-HOCl-ox-LDL, Calbiochem, Darmstadt, Germany), osteopontin (rabbit polyclonal anti-
osteopontin, Acris Antibodies, Herford, Germany) and osteoprotegerin (rabbit polyclonal anti-osteoprotegerin, Abbiotec, San Diego, USA) was performed as previously described.\textsuperscript{2, 6}

To quantify the integrity of the endothelial cell layer on the AVs, the eNOS-positive length on the AV surface was measured and calculated as ratio (%) of the total circumference of the AV surface as previously described.\textsuperscript{7} Evidence of infiltrated cells or differentiated cells was evaluated by two investigators who used light microscopy, according to a 6-tier scoring system as previously mentioned: \textsuperscript{8} grade 0, no positive-cells; grade 1, positive-cells in up to 5% of the valvular sections; grade 2, 6% to 10%; grade 3, 11% to 30%; grade 4, 31% to 50%; and grade 5, >50%.

**Superoxide Detection**

To examine the involvement of oxidative stress in the AVs, additional studies were performed with in situ dihydroethidium (DHE) fluorescence as described previously.\textsuperscript{9} Frozen sections of aortic valves (30 µm) from the different groups were incubated at the same time with DHE (10 µmol/L) in PBS for 30 minutes at 37°C in a humidified chamber protected from light. DHE is oxidized on reaction with $\text{O}_2^-$ to ethidium bromide, which binds to DNA in the nucleus and
fluoresces red. Tissue sections were then visualized with an Axioplan-2 fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a Axio Cam MRC5 (Zeiss, Oberkochen, Germany). Frozen sections from 3 groups (Con, Chol and Reg) were processed in parallel, and images were acquired with identical acquisition parameters.

RNA-Isolation and Quantitative Assessment of mRNA Expression

AV cusps were scratched off from frozen sections on glass slides under microscopic control.

Total RNA was isolated (RNeasy, Qiagen, Hilden, Germany) and reverse transcribed into cDNA using random hexamers and Sensiscript reverse transcriptase (Qiagen, Hilden, Germany). An aliquot of the cDNA was used for quantitative RT-PCR using the Syber green system on an IQ5 cycler (BioRad, Munich, Germany). The expression of specific genes was normalized to the expression of 18S-rRNA. The following primers and conditions were used; 18S rRNA:

5´-ATACAGGACTCTTTTCGAGGCC-3´ and 5´-CGGGACACTCGCTAAGAGCAT-3´, annealing at 61°C; a-smooth muscle actin: 5´-CTGACAGGGCACCAGTGAAGAAC-3´ and 5´-ATCTCACGCCTCGGCAGTAGT-3´ at 56°C annealing; bone morphogenetic protein-2 (BMP-2) primer: 5´-CTCGTCACTGGGGACAGAACTT-3´ and
5′-ACCCGCTGTCTTCTAGTGTTGC-3′; annealing at 60°C; runt-related transcription factor 2 (runx-2) primer: 5′-CTTCACAATCCTCCCCAAGTG -3′ and

5′-TCAGAGGTCAGGTCATCATC-3′; annealing at 67°C; ALP primer:

5′-GCCCTCTCCAAGACATATA-3′ and 5′-CCATGATCAGTCGATATCC-3′; annealing at 58°C.

**Functional Assessment of the Aortic Valves by Echocardiography**

To evaluate the AV function, echocardiographic assessment of AV flow velocity was performed at the day of sacrifice as previously demonstrated. Transthoracic echocardiography was performed with the Sonos 5500 echocardiogram (Agilent Technologies, Santa Clara, CA USA) equipped with a 12-Mhz phased-array transducer. The anterior thorax was shaved to optimize the acoustic interface. Warmed gel was applied, and the animal was gently cradled in the left lateral recumbent position. AV flow velocity was evaluated by continuous waves recorded through a near apical approach, and 5 beats were averaged. In 10 randomly selected mice, the inter-observer and intra-observer variability for the measurement of AV flow velocity were 3.7±1.1% and 1.9±0.3% respectively.
**Figure S1.** Inhibitory effect of regular exercise on collagen deposition at the mice aortic valve.

Sirius red staining without (upper) and with (lower) polarized light show thickened aortic valve and abundant collagen deposition in the cholesterol group (middle) as compared to control (left).

Regular exercise produced marked reduction in collagen deposition at the aortic valve (right).

Arrowheads indicated the sites of aortic valve. Images of all three groups were acquired at identical procedures and settings. Bar = 100 μm.
Figure S2. Inhibitory effect of regular exercise on mineralization at the mice aortic valve assessed with Von Kossa staining. A: Normal diet + sedentary (Control), B: cholesterol diet + sedentary, C: cholesterol diet + regular exercise training. D: A serial section of B that was decalcified by immersion in 10% formic acid for 30min and then stained with Von Kossa served as negative control. Fine speckled granules of mineralization were observed in association with thickening of aortic valve in the cholesterol group (B), while few mineralization was seen in the control (A) and regular exercise (C) group. Bar = 100 μm.
Figure S3. A, Immunostaining for osteopontin (OPN) and osteoprotegerin (OPG). The OPN expression was very faint at the aortic valves (AVs) in the control (left), cholesterol (chol) diet + sedentary (middle), and chol- diet + regular exercise training (ET) (right). Arrows denote areas of AV tissue where immunostaining was weak while arrowheads indicate atherosclerotic area where positive staining is evident. In contrast, OPG expression was clearly positive at the AV in the control, whereas it was almost negative at the AV in the chol-group. Regular ET preserved decreased expression of OPG at the AV. Bar = 100 μm. B, Serum levels of fetuin-A in LDLR<sup>−/−</sup> mice 16 weeks after feeding with cholesterol diet (8 animals in each group).
Supplementary references


