Free Radical Research, May 2011; 45(5): 550–558

informa
healthcare

RESEARCH ARTICLE

Effect of exercise on bone and articular cartilage in heterozygous manganese superoxide dismutase (SOD2) deficient mice

ALEXANDER BAUR[1,*], JAN HENKEL[1,*], WILHELM BLOCH[1], NICOLAI TREIBER[2], KARIN SCHARFFETTER-KOCHANEK[2], GERT-PETER BRÜGGEMANN[3] & ANJA NIEHOFF[3]

[1]*Department of Molecular and Cellular Sport Medicine, Institute of Cardiology and Sport Medicine, German Sport University Cologne, Cologne, Germany,* [2]*Department of Dermatology and Allergic Diseases, University of Ulm, Ulm, Germany, and* [3]*Institute of Biomechanics and Orthopaedics, German Sport University Cologne, Cologne, Germany*

(Received date: 25 October 2010; Accepted date: 5 January 2011)

Abstract

Reactive oxygen species (ROS) are involved in both bone and cartilage physiology and play an important role in the pathogenesis of osteoporosis and osteoarthritis. The present study investigated the effect of running exercise on bone and cartilage in heterozygous manganese superoxide dismutase (SOD2)-deficient mice. It was hypothesized that exercise might induce an increased production of ROS in these tissues. Heterozygous SOD2-deficient mice should exhibit an impaired capability to compensate, resulting in an increased oxidative stress in cartilage and bone. Thirteen female wild type and 20 $SOD2^{+/-}$ mice (aged 16 weeks) were randomly assigned to a non-active wild type ($SOD2^{+/+}$Con, $n = 7$), a trained wild type ($SOD2^{+/+}$Run, $n = 6$), a non-active $SOD2^{+/-}$ ($SOD2^{+/-}$Con, $n = 9$) and a trained $SOD2^{+/-}$ ($SOD2^{+/-}$Run, $n = 11$) group. Training groups underwent running exercise on a treadmill for 8 weeks. In $SOD2^{+/-}$ mice elevated levels of 15-F_{2t}-isoprostane and nitrotyrosine were detected in bone and articular cartilage compared to wild type littermates. In osteocytes the elevated levels of these molecules were found to be reduced after exercise while in chondrocytes they were increased by aerobic running exercise. The observed changes in oxidative and nitrosative stress did neither affect morphological, structural nor mechanical properties of both tissues. These results demonstrate that exercise might protect bone against oxidative stress in heterozygous SOD2-deficient mice.

Keywords: *Manganese superoxide dismutase, exercise, bone, cartilage, reactive oxygen species.*

Introduction

The over-production of reactive oxygen species (ROS) induces an imbalance between pro-oxidants and antioxidants in cells and tissues resulting in oxidative stress, which has been related to ageing, tissue inflammation and degeneration [1]. Furthermore, oxidative stress might play a role in the pathogenesis of osteoporosis [2] and osteoarthritis (OA) [3]. ROS have been shown to influence the formation, differentiation and activity of both osteoclasts and osteoblasts [4,5]. An over-production of ROS by osteoclasts leads to an imbalance between osteoblast and osteoclast activity in bone remodelling resulting in bone destruction [6]. In addition, ROS affect chondrocyte metabolism by inhibiting matrix formation, directly inducing oxidative extracellular matrix degeneration or stimulating the expression of matrix-degrading enzymes [3].

Cells and tissues possess a system of enzymatic and non-enzymatic antioxidants to protect themselves against oxidative stress and to re-establish redox homeostasis [7] including the enzyme superoxide dismutase (SOD). There are three SOD isoenzymes and the manganese superoxide dismutase (SOD2) is the major antioxidant enzyme in mitochondria. The important role of SOD2 in maintaining normal cellular function was demonstrated by the deletion of the SOD2 gene. Homozygous SOD2-deficient mice

*Baur and Henkel contributed equally.

Correspondence: Anja Niehoff, PhD, Institute of Biomechanics and Orthopaedics, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany. Tel: ++49 221 49825620. Fax: ++49 221 4971598. Email: niehoff@dshs-koeln.de

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715762.2011.555483

($SOD2^{-/-}$) elicit pathologies including dilative cardiomyopathy, steatosis hepatis, neurodegeneration and impairment of mitochondrial function and die within 5–21 days post-natally, depending on the genetic background [8,9]. Because life span is very limited in $SOD2^{-/-}$ mice, animals lacking only one allele of SOD2 are considered a much better model to study the effects of chronic exposure to oxidative stress. Depending on the tissue, heterozygous SOD2 ($SOD2^{+/-}$) mice show a reduced SOD2 activity, ranging from 30–80% of wild type mice [10].

Previous studies have demonstrated an influence of exercise on the cellular redox homeostasis [11,12]. It was suggested that intensive exercise can induce oxidative stress [13] and also has effects on the activity of cellular antioxidant enzymes [14]. Some *in vitro* studies showed that extreme mechanical loading increased the oxidative stress in chondrocytes [15–17] and osteoblast-like cells [18]. Exercise can also lead to a substantial increase of SOD activity in skeletal muscle [19,20] and cartilage [21]. However, information about the effect of physical activity on SOD in bone as well as cartilage are sparse. Therefore, the purpose of this study was to examine the effect of running exercise on bone and cartilage in heterozygous SOD2-deficient and wild type mice. We used heterozygous SOD2 deficient mice on a mixed genetic background (C57BL/6 × Sv129/Ola) with a heterozygous deficiency for SOD2 ($SOD2^{+/-}$) [22], which showed a decreased SOD2 activity [22] and an increased mitochondrial oxidative stress [22,23]. It was hypothesized that running exercise induces an increased production of ROS in the cells of bone and cartilage tissue. Heterozygous SOD2-deficient mice should exhibit an impaired capability to counteract and adapt to these conditions resulting in increased oxidative stress in bone and cartilage.

Methods

Animals

Heterozygous SOD2 mice on a mixed genetic background (C57BL/6 × Sv129/Ola) were obtained after breeding a female keratin 14 Cre transgenic mice to a floxed SOD2 male mice due to keratin 14 promoter-driven Cre expression in the oocytes [22]. C57BL/6 × Sv129/Ola mice were used as wild type controls. All animals were fed *ad libitum* and housed in standard cages. All experiments were carried out in compliance with the principles of laboratory animal care and the local animal care guidelines.

Thirteen female wild type and 20 $SOD2^{+/-}$ mice (aged 16 weeks) were randomly assigned to non-active and training groups resulting in a non-active wild type ($SOD2^{+/+}$Con, $n = 7$), a trained wild type ($SOD2^{+/+}$Run, $n = 6$), a non-active $SOD2^{+/-}$ ($SOD2^{+/-}$Con, $n = 9$) and a trained $SOD2^{+/-}$ ($SOD2^{+/-}$Run, $n = 11$) group. Mice in training groups underwent running exercise using a treadmill (EXER-3/6 Treadmill, Serial 04001-1, Columbus Instruments, Columbo, Ohio, USA) for 30 min/day, 5 days/week. Belt speed of the treadmill was 15 m/min with an incline of 5°. After 8 weeks mice were sacrificed by cervical dislocation and hindlimbs were dissected. The right hindlimbs were immediately fixed in fresh 4% paraformaldehyde (PFA) in 1 M phosphate-buffered saline solution (PBS, pH 7.4). The left hindlimbs were wrapped in gauze soaked with 0.9% saline solution (NaCl) and stored at –20°C.

Peripheral quantitative computed tomography (pQCT) and mechanical testing

Left femora and tibiae were scanned by pQCT using the XCT Research M scanner with software 5.50 (Stratec Medizintechnik GmbH, Pforzheim, Germany) as previously described [24]. In brief, transverse sections were recorded at metaphysis (femur: 15%, 17.5% and 20% bone length measured from the distal joint line, tibia: 5%, 7.5% and 10% bone length measured from the proximal joint line) and one section was taken at the midshaft (50% bone length). The voxel-size was 500 μm × 70 μm × 70 μm. At the metaphysis (mean of the three slices) total and trabecular parameters (area, bone mineral content and density) and at the midshaft cortical parameters (area, bone mineral content and density, thickness, periosteal and endosteal circumference) were determined.

In order to analyse mechanical properties of the bones, three point bending tests were performed using a materials testing machine (Z2.5/TN1S, Zwick GmbH & CoKG, Ulm, Germany). The bones were loaded at mid-diaphysis using a 100 N load cell. The distance between the lower support points was 5 mm for the femur and 10 mm for the tibia. The diameter of support points were 1.5 mm. After pre-loading at 0.1 N and a loading rate of 0.05 mm/s the bones were loaded to failure in anterior–posterior direction with a crosshead speed of 1 mm/min. Ultimate load, ultimate deformation, stiffness and energy to failure were measured from the load-deformation curve. Ultimate stress, ultimate strain and elastic modulus were calculated based on cross-sectional moment of inertia obtained from the pQCT scans.

Histology and immunohistochemistry

For immunhistochemical staining the following antibodies were used: A polyclonal rabbit antibody against SOD2 (1:100, Abcam Inc., Cambridge, UK), a polyclonal rabbit antibody directed against multi-species nitrotyrosine (1:400; Millipore GmbH, Schwalbach, Ts., Germany), a polyclonal goat antibody against 15-F_{2t}-isoprostane (Anti-8-epi-$PGF_{2\alpha}$,

1:1500, Oxford Biomedical Research, Michigan, Detroit, USA) and a monoclonal mouse antibody against human collagen II (1:1000; Calbiochem, Darmstadt, Germany). As secondary antibodies, polyclonal goat anti-rabbit immunoglobulins/biotinylated and polyclonal rabbit anti-mouse immunoglobulins/HRP (Dako Deutschland GmbH, Hamburg, Germany) were applied.

Right hindlimbs were decalcified in 10% EDTA and processed for paraffin embedding. Sections (8 μm) were routinely stained with Haematoxylin-Eosin, Safranin O/Fast Green, Alcian blue/nuclear fast red and Masson Goldner's trichrome to analyse the general tissue morphology.

For immunohistological stainings, sections were incubated with 1% H_2O_2 in methanol in order to block endogenous peroxidase activity. Sections were permeabilized with 0.25% Triton X-100 (Serva Electrophoresis GmbH, Heidelberg, Germany) and 0.5 M NH_4CL dissolved within 0.05 M TBS and blocked with 5% BSA in TBS for 1 h at room temperature. Sections were incubated with the primary antibody overnight at 4°C. Secondary antibody was added for 1 h followed by an avidin-biotin-peroxidase-complex (dilution 1:150) in 0.05 M TBS for 1 h. Finally, the sections were stained with DAB in 0.1 M PBS, containing NH_4Cl, $NiSO_4$, 10% glucose and glucose oxidase for 10–20 min depending on antibody. For collagen II staining sections were enzymatically pre-treated with 40 mU/ml chondroitinase (Sigma-Aldrich Chemie GmbH, Germany) in 0.01% BSA in TBS at 37°C for 1 h. Finally, the sections were stained using 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) as a substrate.

Immunohistochemical stainings of nitrotyrosine and 15-F_{2t}-isoprostane in cartilage (femur and tibia) were evaluated semi-quantitatively [25]. The staining of 40 randomly selected chondrocytes from the central load bearing cartilage was analysed. The cells were judged by an individual, semi-quantitative score from 0–3 points with 0 being no detectable staining, 1 being mild staining, 2 being moderate staining and 3 being intense staining. We calculated the mean staining index for cartilage of both femur and tibia by multiplying the score grade with the number of cells classified in the equivalent score. The sum was divided by the number of analysed chondrocytes ($n = 40$). Investigation was performed with a 400-fold magnification using a microscope (KS 300, Carl-Zeiss, Jena, Germany). In addition, osteocytes in the cortical bone of the distal femora were analysed semi-quantitatively in samples stained for SOD2, nitrotyrosine and 15-F_{2t}-isoprostane. A number of 50 osteocytes were randomly selected in each sample (25 in the dorsal and 25 in the ventral cortical bone) and analysed. Photos of the cortical bone were taken with a 400-fold magnification. Osteocytes were manually defined on these images and evaluated using a computer-based densitometry system (ImageJ 1.38x, National Institutes of Health, Bethesda, Maryland, USA). The measured data of all 50 osteocytes were averaged to create values representative for the cortical bone of each specimen.

The osteoclast activity was analysed in the femur using a commercial acid phosphatase leukocyte kit (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Histomorphometric measurements were performed at the secondary spongiosa of the distal femoral metaphysis (200 μm proximal to the growth plate) in an area of 0.32 mm^2 with a 200-fold magnification using a microscope (Nikon Eclipse 80i, Nikon GmbH, Düsseldorf, Germany) connected to a computer-based histomorphometry system (OsteoMeasure 4.10, OsteoMetrics Inc., Decatur, GA, USA). Tartrate-resistance acid phosphatase (TRAP) positive cells attached to the bone trabeculae were counted (N.Oc), and the ratio of osteoclasts and trabecular circumference (N.Oc/B.Pm, mm^{-1}) and trabecular cross-sectional area (N.Oc/T.Ar, mm^{-2}) were calculated. In addition, bone static histomorphometry was performed in the secondary spongiosa of the distal femoral metaphysis (200 μm proximal to the growth plate) in an area of 1.92 mm^2 with a 200-fold magnification. The trabecular bone volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, mm^{-1}) and trabecular separation (Tb.Sp, μm) was determined.

In order to measure the thickness of femoral articular cartilage three serial sections of collagen II staining were analysed. From each section three images were obtained using a computer-coupled microscope (Nikon Eclipse 80i, Nikon GmbH, Düsseldorf, Germany) with a 400-fold magnification. Measurements were accomplished by the software EclipseNet 1.20.0 (Nikon GmbH, Düsseldorf, Germany). The height of the cartilage was determined at six sites in each of the three images from the section. The values of 54 individual measurements were averaged to obtain the mean cartilage thickness.

Statistical analyses

Results are presented as means ± standard deviation. Statistical analysis was performed using STATISTICA software (Version 7.1, StatSoft Inc., Tulsa, OK, USA). A two-way analysis of variance (ANOVA) was used to detect differences between the four experimental groups followed by a DUNCAN post-hoc test. Differences were considered to be significant at $p < 0.05$.

Results

$SOD2^{+/-}$ mice conducted running exercise normally and body mass did not show significant differences among the groups ($SOD2^{+/+}$Con: 32 ± 7 g, $SOD2^{+/+}$Run: 30 ± 2 g, $SOD2^{+/-}$Con: 30 ± 4 g,

SOD2$^{+/-}$Run: 30 ± 2 g) at the end of the study. Furthermore, mass and length of tibia and femur were not significantly different between the groups.

In cortical bone both nitrotyrosine and 15-F_{2t}-isoprostane were found in osteocytes (Figure 1A). Interestingly, semi-quantitative analysis indicated a significantly ($p < 0.05$) higher level of nitrotyrosine in the SOD2$^{+/-}$Con mice compared to all other three groups (3.5 ± 1.7 in SOD2$^{+/+}$Con, 5.0 ± 1.9 in SOD2$^{+/+}$Run, 7.4 ± 2.5 in SOD2$^{+/-}$Con and 4.5 ± 1.5 in SOD2$^{+/-}$Run). The arbitrary grey values of cortical osteocytes immunohistochemically stained for

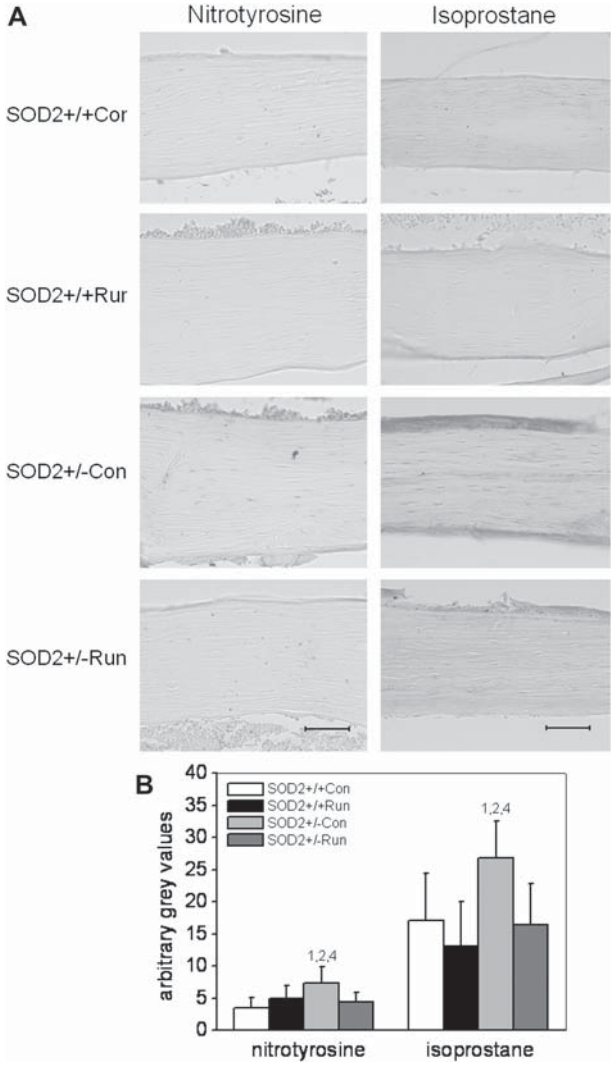

Figure 1. Comparison of anti-nitrotyrosine and anti-15-F_{2t}-isoprostane staining in cortical bone from the femur of SOD2$^{+/+}$Con, SOD2$^{+/+}$Run, SOD2$^{+/-}$Con and SOD2$^{+/-}$Run mice. (A) Representative photomicrographs of anti-nitrotyrosine and anti-15-F_{2t}-isoprostane staining from femoral cortical bone of each group. Bar: 100 µm. (B) Semi-quantitative analysis of anti-nitrotyrosine and anti-15-F_{2t}-isoprostane immunohistochemical staining. Values are presented as mean ± SD, 2-way ANOVA with DUNCAN post-hoc, Femur: SOD2$^{+/+}$Con: $n = 4$, SOD2$^{+/+}$ Run: $n = 5$, SOD2$^{+/-}$Con: $n = 8$, SOD2$^{+/-}$Run: n = 8. [1]Significantly ($p < 0.05$) different to SOD2$^{+/+}$Con, [2]Significantly ($p < 0.05$) different to SOD2$^{+/+}$Run, [3]Significantly ($p < 0.05$) different to SOD2$^{+/-}$Con. [4]Significantly ($p < 0.05$) different to SOD2$^{+/-}$Run.

15-F_{2t}-isoprostane were also significantly ($p < 0.05$) higher in the SOD2$^{+/-}$Con group compared to all other three groups (17.1 ± 7.4 in SOD2$^{+/+}$Con, 13.1 ± 7.0 in SOD2$^{+/+}$Run, 26.8 ± 5.8 in SOD2$^{+/-}$Con and 16.5 ± 6.3 in SOD2$^{+/-}$Run) (Figure 1B). The mean SOD2 expression seems to be more intensive in wild type mice (22.6 ± 9.3 in SOD2$^{+/+}$Con, 18.4 ± 9.1 in SOD2$^{+/+}$Run) compared to heterozygous SOD2-deficient mice (14.9 ± 2.9 in SOD2$^{+/-}$Con, and 17.1 ± 4.5 in SOD2$^{+/-}$Run). However, the staining showed a strong inter-individual variability and failed to reach statistically significant difference between the groups.

The pQCT measurements and mechanical testing of bones revealed no significant differences between any of the groups (Supplementary Tables 1–3). Neither physical activity nor genotype resulted in any differences of histomorphometric parameters (Supplementary Table 4). Osteoclasts could be identified in samples of all four groups due to the red staining (Supplementary Figure 1). The ratio of osteoclast-to-trabecular circumference (N.Oc/B.Pm) was found to be 1.97 ± 0.79 mm^{-1} for SOD2$^{+/+}$Con, 1.76 ± 1.02 mm^{-1} for SOD2$^{+/+}$Run, 1.39 ± 0.65 mm^{-1} for SOD2$^{+/-}$Con and 1.58 ± 1.20 mm^{-1} SOD2$^{+/-}$Run. The ratio of osteoclasts to trabecular cross-sectional area (N.Oc/T.Ar) was 13.1 ± 6.3 mm^{-2} for SOD2$^{+/+}$Con, 9.4 ± 3.4 mm^{-2} for SOD2$^{+/+}$Run, 9.4 ± 6.1 mm^{-2} for SOD2$^{+/-}$Con and 14.3 ± 11.7 mm^{-2} for SOD2$^{+/-}$Run. There was no significant difference between the groups.

Within chondrocytes an intracellular staining for nitrotyrosine was found in all four zones of articular cartilage of both femur and tibia. The ratio of cells stained positive for nitrotyrosine did obviously not differ between the different cartilage zones. The extracellular matrix of the articular cartilage did not show any nitrotyrosine staining. Mean chondrocyte grading scores were in general significantly ($p < 0.05$) higher in the heterozygous SOD2-deficient animals compared to the wild type mice (SOD2$^{+/+}$Con: 1.3 ± 0.2 and 1.1 ± 0.1, SOD2$^{+/+}$Run: 1.7 ± 0.1 and 1.5 ± 0.1, SOD2$^{+/-}$Con: 1.6 ± 0.2 and 1.4 ± 0.1, SOD2$^{+/-}$Run: 2.1 ± 0.1 and 1.8 ± 0.1 for femur and tibia, respectively) (Figures 2 and 3). In addition, the trained groups had higher levels of nitrotyrosine compared to the non-trained control groups. Staining of anti-15-F_{2t}-isoprostane was also detected in chondrocytes of all cartilage zones. The extracellular matrix displayed low staining intensity. Trained mice exhibited significantly ($p < 0.05$) higher grading scores independent of their genetic background (Figures 2 and 3). Mean chondrocyte grading scores for femur and tibia were 1.1 ± 0.3 and 1.2 ± 0.3 in SOD2$^{+/+}$ Con, 1.6 ± 0.1 and 1.6 ± 0.2 in SOD2$^{+/+}$Run, 1.3 ± 0.1 and 1.4 ± 0.1 in SOD2$^{+/-}$ Con and 1.7 ± 0.3 and 1.8 ± 0.3 in SOD2$^{+/-}$Run.

Histological analysis did not show obvious changes in the morphology of the examined cartilage between the four different groups (Figure 4). Cartilage thickness

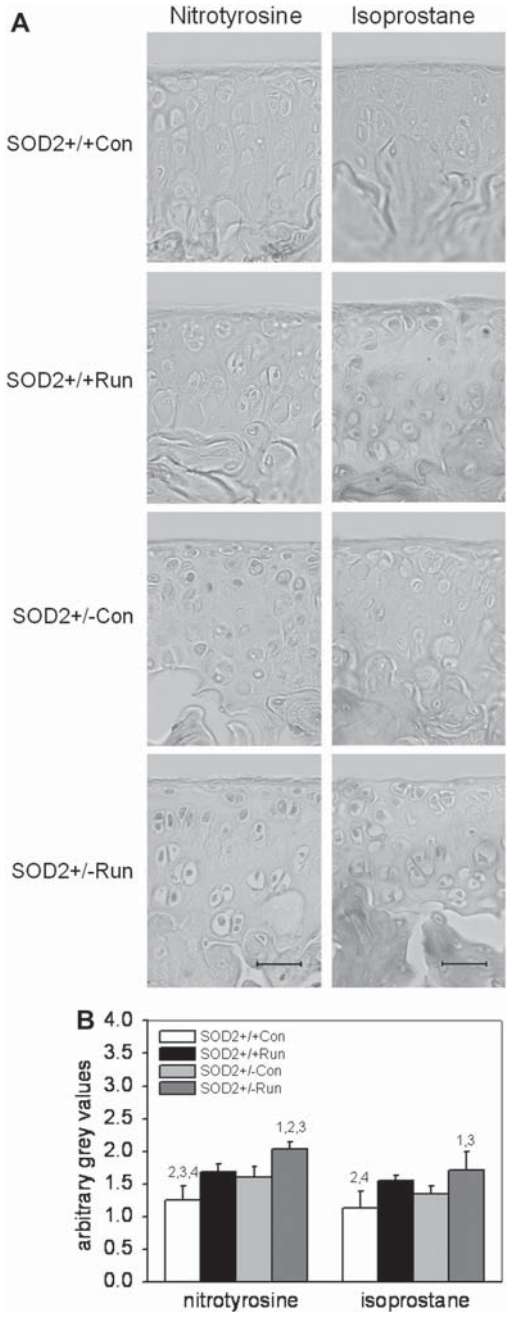

Figure 2. Comparison of anti-nitrotyrosine and anti-15-F_{2t}-isoprostane staining in articular cartilage from the femur of $SOD2^{+/+}$Con, $SOD2^{+/+}$Run, $SOD2^{+/-}$Con and $SOD2^{+/-}$Run mice. (A) Representative photomicrographs of anti-nitrotyrosine and anti-15-F_{2t}-isoprostane staining from femoral articular cartilage of each group. Bar: 50 µm. (B) Semi-quantitative analysis of anti-nitrotyrosine ($SOD2^{+/+}$Con: $n = 4$, $SOD2^{+/+}$Run: $n = 4$, $SOD2^{+/-}$Con: $n = 8$, $SOD2^{+/-}$Run: $n = 9$) and anti-15-F_{2t}-isoprostane ($SOD2^{+/+}$Con: $n = 6$, $SOD2^{+/+}$Run: $n = 6$, $SOD2^{+/-}$Con: $n = 8$, $SOD2^{+/-}$Run: $n = 10$) immunohistochemical staining. Values are presented as mean ± SD, 2-way ANOVA with DUNCAN post hoc, [1]Significantly ($p < 0.05$) different to $SOD2^{+/+}$Con, [2]Significantly ($p < 0.05$) different to $SOD2^{+/+}$Run, [3]Significantly ($p < 0.05$) different to $SOD2^{+/-}$Con. [4]Significantly ($p < 0.05$) different to $SOD2^{+/-}$Run.

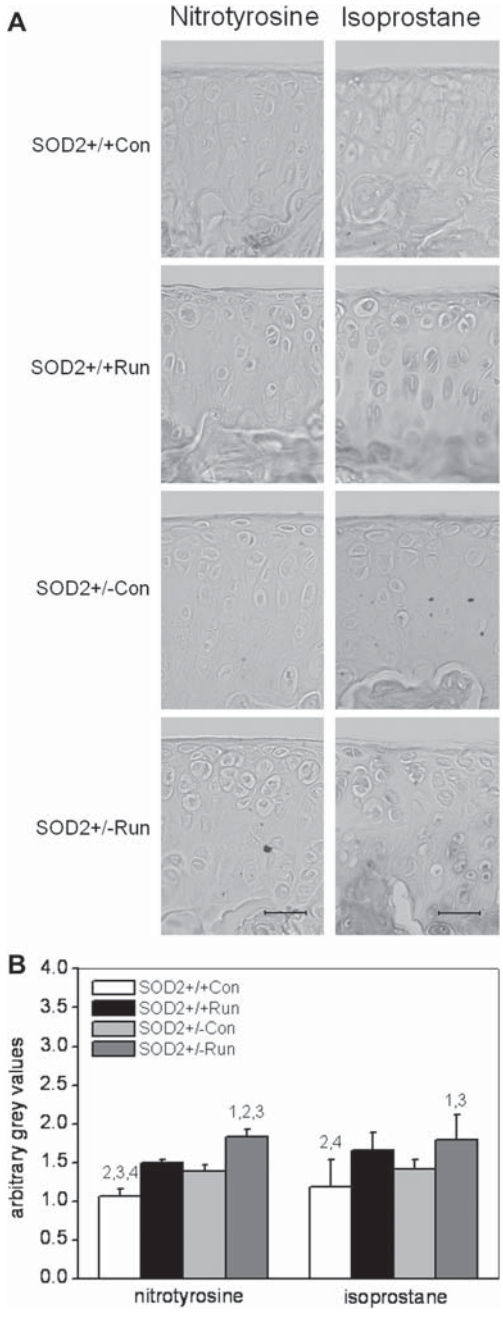

Figure 3. Comparison of anti-nitrotyrosine and anti-15-F_{2t}-isoprostane staining in articular cartilage from the tibia of $SOD2^{+/+}$Con, $SOD2^{+/+}$Run, $SOD2^{+/-}$Con and $SOD2^{+/-}$Run mice. (A) Representative photomicrographs of anti-nitrotyrosine and anti-15-F_{2t}-isoprostane staining from tibial articular cartilage of each group. Bar: 50 µm. (B) Semi-quantitative analysis of anti-nitrotyrosine ($SOD2^{+/+}$Con: $n = 2$, $SOD2^{+/+}$Run: $n = 3$, $SOD2^{+/-}$Con: $n = 8$, $SOD2^{+/-}$Run: $n = 10$) and anti-15-F_{2t}-isoprostane ($SOD2^{+/+}$Con: $n = 6$, $SOD2^{+/+}$Run: $n = 6$, $SOD2^{+/-}$Con: $n = 9$, $SOD2^{+/-}$Run: $n = 10$) immunohistochemical staining. Values are presented as mean ± SD, 2-way ANOVA with DUNCAN post hoc, [1]Significantly ($p < 0.05$) different to $SOD2^{+/+}$Con, [2]Significantly ($p < 0.05$) different to $SOD2^{+/+}$Run, [3]Significantly ($p < 0.05$) different to $SOD2^{+/-}$Con. [4]Significantly ($p < 0.05$) different to $SOD2^{+/-}$Run.

was 99 ± 6 µm for $SOD2^{+/+}$Con, 90 ± 12 µm for $SOD2^{+/+}$Run, 101 ± 12 µm for $SOD2^{+/-}$Con and 99 ± 14 µm for $SOD2^{+/-}$Run, indicating no differences between the groups.

Discussion

In this study, we used heterozygous SOD2-deficient mice ($SOD2^{+/-}$) to investigate the effect of moderate

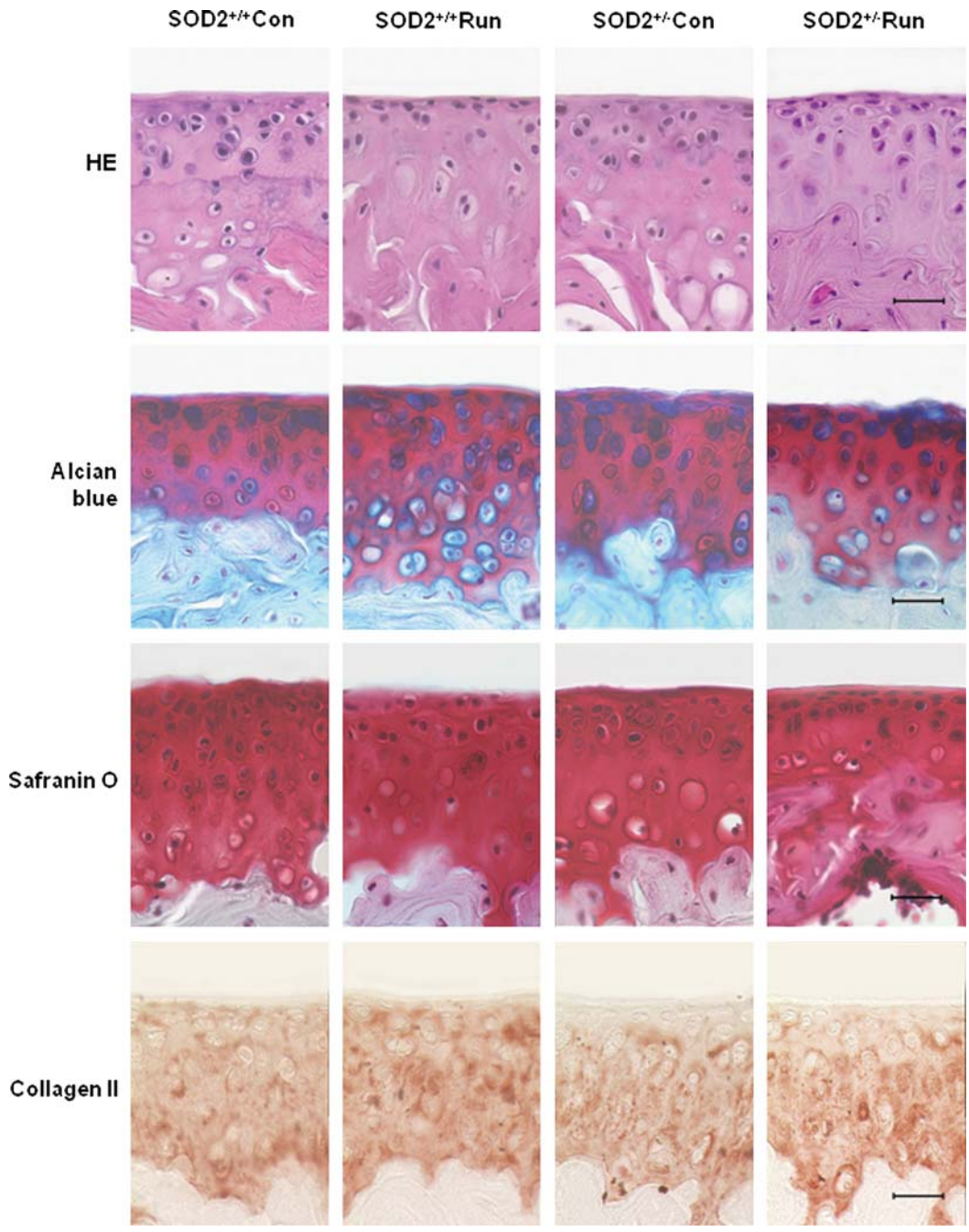

Figure 4. Cartilage morphology was not affected in $SOD2^{+/+}$Con, $SOD2^{+/+}$Run, $SOD2^{+/-}$Con and $SOD2^{+/-}$Run mice. Haematoxylin-Eosin (HE), Alcian blue/nuclear fast red and Safranin O/Fast green staining of femur paraffin histological sections. Collagen II protein was detected by immunostaining (Col II) in articular cartilage. Bar: 50 μm.

running exercise on both bone and cartilage tissue under conditions of reduced SOD2-activity. In contrast to homozygous SOD2 knockout mice ($SOD2^{-/-}$) $SOD2^{+/-}$ mice exhibit a normal life span [26] and develop no obvious phenotypic abnormalities compared to healthy littermates [8,9,22]. In $SOD2^{+/-}$ mouse models a reduction in SOD2-activity by 30–80% has been demonstrated in the mitochondria of several different tissues [9,22,27–30]. In this study the SOD2 staining of osteocytes showed a strong inter-individual variability reflecting the extreme inter-individual differences in healthy humans [31]. The expression of SOD2 seems to be more intensive in wild type mice compared to heterozygous SOD2-deficient mice, but no significant variation could be demonstrated between the groups. However, there is evidence of elevated levels of oxidative stress in examined tissues as a result of decreased SOD2-activity due to heterozygous deletion. Isoprostanes are commonly used as indirect biomarkers for oxidative stress and lipid peroxidation [32]. Nitrotyrosine, generated in the presence of ROS and NO, can be used as a marker for oxidative as well as nitrosative stress [33]. Both markers were found to be significantly elevated

in bone and cartilage tissue of untrained SOD2$^{+/-}$ mice compared to wild type littermates indicating increased levels of oxidative stress.

Previous studies have shown elevated levels of oxidative stress in different types of muscle tissue after strenuous training [11,34]. Electron leakage from the mitochondrial respiratory chain appears to be of special importance for an increased production of ROS observed in muscle tissue during aerobic exercise [35]. In accordance with this theory, we were able to show that exercise increases the oxidative stress in cartilage tissue. Nitrotyrosine and 15-F2t-isoprostane staining intensity of chondrocytes was enhanced in trained SOD$^{+/+}$ mice and both trained and untrained SOD$^{+/-}$ mice. Interestingly, our results in bone tissue were somewhat different. Training failed to cause increased oxidative stress in wild type mice. The observed increased staining for nitrotyrosine in trained SOD$^{+/+}$ mice, although not statistically significant, might be explained by an increase in the levels of NO due to exercise. A general increase in the levels of NO in several tissues and the blood plasma following exercise has been demonstrated [36]. Similar events might well take place in bone tissue.

One can speculate that the exercise was not strenuous enough to disturb the intracellular pro-oxidant–antioxidant homeostasis in osteocytes of healthy wild type mice. In SOD2-deficient mice running exercise even resulted in a reduction of oxidative stress compared to untrained SOD2-deficient animals suggesting that in osteocytes exercise somehow induces mechanisms that are able to counteract increased oxidative stress. Consistent with the observation at hand, it was shown that aerobic, anaerobic or mixed training can induce the activation of antioxidant mechanisms and provoke decreased levels of oxidative stress in several tissues [12,14,37]. Similar mechanisms have been suggested for chondrocytes and osteoblast-like cells *in vitro*, where an increase in SOD2-activity was found in response to mechanical strain beyond physiological levels [18]. Exercise might protect bone against oxidative stress. So far, a limitation of our study is the analysis of only one antioxidative enzyme. Thus, further studies are required to investigate more antioxidative enzymes to explain the underlying mechanism how exercise can protect bone against oxidative stress. Irrespective of the underlying mechanism, we have shown that exercise applied in a common used training protocol in mice studies can reduce oxidative stress in osteocytes.

Our findings of increased oxidative stress in bone and cartilage tissue of SOD2-deficient mice might also be of clinical significance. Several studies have revealed a possible link between oxidative stress and the development or progression of OA by contributing to an imbalance between anabolic and catabolic mechanisms, leading to breakdown in cartilage homeostasis by altering the first and favouring the latter [3]. Increased levels of oxidative stress have been found in osteoarthritic cartilage [38]. Recently, several studies demonstrated a decrease in SOD2 in OA cartilage [39,40]. In addition to degenerative damage to articular cartilage changes in subchondral bone including sclerosis are another common finding in OA [41]. These alterations in bone are associated with an altered metabolic activity of osteoblasts [41,42] and have also been linked to increased levels of oxidative stress [43]. However, while we found clear evidence for increased oxidative and nitrosative stress in bone and cartilage due to heterozygous SOD2-deficiency with aerobic exercise further increasing the levels in cartilage, no significant changes in morphological, structural and mechanical properties were detected. These results might indicate that the amount of oxidative stress is insufficient to induce gross changes that may alter the parameters investigated in this study at the observed age of mice. Further investigations will be necessary to clarify the effect and mechanism of increased levels in oxidative stress in articular cartilage and bone.

In conclusion, we demonstrated elevated levels of 15-F_{2t}-isoprostane and nitrotyrosine in bone and articular cartilage of SOD2$^{+/-}$ mice compared to their wild type littermates. In osteocytes the elevated levels of these molecules were found to be reduced after training while in chondrocytes they were further increased by aerobic running exercise. However, morphological, structural and mechanical properties of both bone and cartilage were not affected.

Acknowledgements

K.S-K. was supported by the Clinical Research Group KFO142: Molecular and Cellular Aging (DFG) and by the European Union through the PROTEOMAGE project (FP6-518230).

Declaration of interest

The authors report no conflicts of interest. There have been no financial, consulting, and personal relationships with other people or organizations that could inappropriately have influenced our work.

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This paper was first published online on Early Online on 11 February 2011.

Supplementary material available online

Supplementary Tables S1 to S4.
Supplementary Figure S1.