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ARTICLE

Exercise restores endothelial function independently of weight loss or hyperglycaemic status in *db/db* mice

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Abstract
Aims/hypothesis Exercise ameliorates oxidative stress-mediated diabetic vascular endothelial dysfunction through poorly defined mechanisms. We hypothesised that, in addition to improving metabolic parameters, upregulation of antioxidants such as superoxide dismutase (SOD) mediates exercise-induced reductions of oxidative stress and increased nitric oxide (NO) bioavailability, and also restores vasodilatation.
Methods Type 2 diabetic *db/db* and normoglycaemic wild-type mice were exercised at moderate intensity for 1 h a day for 7 weeks, leading to a 10% body weight loss. Sedentary animals or those undergoing a low-intensity exercise regimen causing non-significant weight loss were also used. We examined aortic endothelial cell function, NO bioavailability and various biomarkers of oxidative stress.
Results Moderate-intensity exercise lowered body weight, increased mitochondrial manganese SOD (MnSOD) and both total and phosphorylated (Ser1177) endothelial nitric oxide synthase (eNOS) protein production; it also reduced whole-body (plasma 8-isoprostane) and tissue oxidative stress (nitrotyrosine immunostaining or protein carbonyl levels in the aorta). Low-intensity exercise did not alter body weight; however, it upregulated cytosolic Cu/Zn-SOD instead of MnSOD, and still demonstrated all the above benefits in the *db/db* aorta. Importantly, both exercise protocols improved endothelial-dependent vasodilatation and NO bioavailability without altering hyperglycaemic status in *db/db* mice.
Conclusions/interpretation Exercise reverses diabetic vascular endothelial dysfunction independently of improvements in body weight or hyperglycaemia. Our data suggest that upregulation of eNOS and specific SOD isoforms could play important roles in improving NO bioavailability, as well as in reversing endothelial dysfunction in type 2 diabetes patients through lifestyle modifications in the management of diabetes.

Keywords *db/db* · Endothelial function · Endothelial nitric oxide synthase · eNOS · Exercise · Oxidative stress · Superoxide dismutase

Abbreviations

ACh	acetylcholine
L-Arg	L-arginine
BH_4	tetrahydrobiopterin
EC_{50}	half-effective concentration
E_{max}	maximum effective concentration
eNOS	endothelial nitric oxide synthase

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MnSOD	manganese superoxide dismutase
NMR	nuclear magnetic resonance
NO	nitric oxide
PSS	physiological salt solution
SNP	sodium nitroprusside
SOD	superoxide dismutase
T_2	time 2
TBS-T	Tris-buffered saline containing 0.1% Tween-20
WT	wild-type

Introduction

Cardiovascular disease is the leading cause of mortality in patients with diabetes [1]. Vascular abnormalities in these patients may be partly due to endothelial dysfunction, leading to atherosclerosis, hypertension and hypercoagulability [2]. Although the exact molecular mechanisms are unclear, changes in lifestyle are routinely advocated for the management of type 2 diabetes [3]. In diabetes, exercise-induced improvements in vascular function are believed to be secondary to lowering of metabolic risk factors such as plasma lipids, blood glucose [4] or oxidative stress biomarkers [5].

Regarding oxidative stress, reactive oxygen species such as superoxide react with nitric oxide (NO) causing a loss of NO bioavailability [6]. Such reactions can lead to the formation of peroxynitrite, a potent oxidant that causes irreversible oxidative modifications to the endothelium and ultimately cell death [7]. Superoxide dismutases (SODs) are endogenous antioxidants, which compete with NO for superoxides and neutralise them. Since NO is a relatively stable, highly diffusible molecule, relative distribution of cellular SOD isoforms is important in determining the extent and location of peroxynitrite-induced oxidative damage in blood vessels [8, 9]. In addition to SODs, catalase is another endogenous antioxidant able to protect blood vessels from hydrogen peroxide-mediated oxidative stress in diabetes [10]. However, the relative importance of different intracellular SOD isoforms or catalase during diabetes remains unclear.

Whereas mitochondrial free radicals and mitochondrial manganese SOD (MnSOD) may be important regulators of oxidative stress in the diabetic heart and endothelial cells [11–13], Cu/Zn-SOD, the cytosolic isoform, accounts for approximately 50% to 80% of total SOD activity in blood vessels [14, 15]. Based on previous findings in humans and animals, we hypothesised that exercise decreases oxidative stress by reducing body weight, improving metabolic parameters and increasing the expression of antioxidant enzymes in the aorta of *db/db* mice, a routinely used murine model of obesity and type 2 diabetes.

Methods

Animals and exercise regimen Protocols were designed in accordance with the University of British Columbia Animal Care Committee Guidelines. We randomly grouped 5-week-old male *db/db* (BKS.cg-m +/+*Lepr*^{db}/J) and wild-type (WT) mice (Jackson Laboratory, Bar Harbor, ME, USA) in sedentary (no-exercise), low-intensity exercise and moderate-exercise groups (n=8–10 per group). Body weights were recorded weekly. Mice assigned to exercise protocols were trained to run on a motorised exercise wheel system (Lafayette Instruments, Lafayette, IN, USA). To allow for animal acclimatisation, exercise intensity was gradually increased over the first 2 weeks till a target of 1 h of daily exercise at a speed of 3.6 m/min in the low-intensity or 5.2 m/min in the moderate-intensity group was achieved. For the duration of the 7-week training period mice were exercised daily at a set time each day for 5 days a week. Non-exercised *db/db* or WT mice were placed in non-rotating wheels for the same duration. The experimental protocol was terminated when the mice reached 12 weeks of age.

Estimation of body fat using nuclear magnetic resonance At 12 weeks of age, whole-body fat measurements were made using an animal magnetic resonance imaging scanner (Bruker, Karlsruhe, Germany). The nuclear magnetic resonance (NMR) signal from the entire body was acquired with a quadrature volume radio-frequency coil tuned to 300 MHz. A standard Carr Purcell Meiboom Gill (CPMG) sequence (TE= 2.377 ms, TR=10 s) was used to acquire 256 echoes, from which the time 2 (T_2) decay curve was extracted. The decay curves were fitted to a double exponential function using a software procedure developed in house with Igor (WaveMetrics, Portland, OR, USA). The component corresponding to T_2≈40 ms was identified as water in lean tissue and the T_2≈200 ms component was identified as body fat [16]. The diffusion coefficient (dc) shift of the double exponential function was identified as a ‘free’ water component corresponding to body fluids, e.g. urine and cerebrospinal fluid, with typical amounts of less than 5% of the total signal. The ratio of lean tissue: body fat expressed as weight: weight was calculated from the NMR data [17].

Collection of blood and tissue samples Animals were anaesthetised with pentobarbital (50 mg/kg, i.p.) combined with heparin (50 U/kg) and blood samples were taken. A portion of each blood sample was separated for use in the 8-isoprostane enzyme immunoassay (see below). The remaining blood was immediately centrifuged (10 min at 4°C, 1,000 *g*) to separate plasma, which was stored at −76°C for later analysis. The animals were killed after collection of blood samples. Thoracic aorta were removed, placed in

ice-cold physiological salt solution (PSS) and then dissected and cleaned of connective tissue. Part of the aorta was immediately assayed for nitrite levels, whereas other pieces were snap-frozen in liquid nitrogen and stored at −76°C for western blotting analyses and protein carbonyl analyses under basal conditions.

Measurement of plasma parameters Plasma glucose levels were measured by Trinder Assay using a commercial kit (Diagnostic Chemicals, Oxford, CT, USA). Insulin levels were determined in plasma using an assay kit (Mercodia Ultrasensitive Mouse Insulin Assay; Alpco, Salem, NH, USA). Photometric measurements were performed by Dimension Clinical Chemistry System (GMI, Ramsey, MN, USA) to obtain plasma triacylglycerol, total cholesterol and HDL-cholesterol levels. LDL-cholesterol was then calculated by the following formula:
$\mathrm{LDL-cholesterol} = \mathrm{total\,cholesterol} - (\mathrm{HDL-cholesterol} + \mathrm{triacylglycerol}/2.2)$.

Oral glucose tolerance test Mice underwent an OGTT when they reached 10 weeks of age (i.e. after 5 weeks of exercise). After 6 h of fasting, mice were glucose loaded (1.5 g/kg) with a 40% glucose solution by oral gavage. Blood samples were taken at times 0, 10, 20, 60 and 120 min. Plasma was separated by centrifugation (1,000 *g*) and stored at −76°C for later analysis of glucose and insulin. To reduce short-term treatment effects, animals were not exercised for 24 h prior to the OGTT.

Isometric force measurement Ring segments of aorta were threaded with stainless steel wire (0.02 mm diameter) and attached to the tissue holders of a four-channel wire myograph (JP Trading, Aarhus, Denmark). Tissues were allowed to equilibrate for 60 min at 37°C, during which time the PSS was replaced at 20-min intervals. During equilibration, the resting tension was gradually increased to 5 mN and remained at this resting tension for 20 to 30 min. Each tissue was maximally activated with a solution of KCl (80 mmol/l) that was prepared by equimolar substitution of NaCl in PSS. Following washout with fresh PSS and return of tension to basal preload, phenylephrine (1 μmol/l) was added to establish a stable contraction. Thereafter, cumulative additions of acetylcholine (ACh) (1 nmol/l to 10 μmol/l) were made. After washout, the ACh concentration–response curve was repeated in the presence of SOD (150 U/ml) or L-arginine (L-Arg) (10^3 μmol/l) plus tetrahydrobiopterin (BH_4) (10 μmol/l). The same protocol was repeated for sodium nitroprusside (SNP) (1 nmol/l to 10 μmol/l) after washout. Vasodilatory responses were recorded on a computer using MyoDaq Acquisition software (version 2.01; Danish MyoTechnology, Aarhus, Denmark) and expressed as per cent dilation of phenylephrine-induced constriction [18].

8-Isoprostane enzyme immunoassay Just before the animals were killed, blood samples were collected in ice-cooled heparinised Eppendorff tubes, to which 10 mg butylated hydroxy toluene was added. The tubes were immediately centrifuged (1,000 *g*) and the plasma fraction separated and stored at −76°C until further use. Plasma levels of free 8-isoprostane were determined using a commercially available enzyme immunoassay kit (8-isoprostane EIA; Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's protocol.

Spectrophotometric quantification of tissue nitrite Pieces of aorta were incubated for 30 min in 0.5 ml Krebs–Henseleit solution at 37°C. Following the equilibration period, the solution was changed to 0.5 ml Krebs–Henseleit (37°C) containing ACh (10 μmol/l). After 5 min, 100 ml of the perfusate was mixed with 100 ml Griess reagent from an assay kit (Calbiochem, San Diego, CA, USA). To convert all nitrate in the sample to nitrite, samples were treated with nitrate reductase and NADPH. Standards were prepared with sodium nitrite at concentrations ranging from 1 to 35 mmol/l. Spectrophotometric measurements were made using a microplate reader set at 550 nm. The values were expressed per mg dry weight of the tissues.

Western blot Thoracic aortae were initially cut into small pieces and homogenised in ice-cold homogenisation buffer. The protein contents of the homogenates were then quantified using a Bradford protein assay. The homogenates were diluted and boiled with sample loading dye. Samples of this, corresponding to 50 μg protein, were used in SDS-polyacrylamide gel electrophoresis. After transfer, nitrocellulose membranes were blocked overnight in 5% skimmed milk in Tris-buffered saline containing 0.1% (vol./vol.) Tween-20 (TBS-T). Membranes were incubated for 2 h at room temperature with antibodies raised in (1) rabbit (endothelial nitric oxide synthase [eNOS], phospho-eNOS [#SC654; #SC12972R; Santa Cruz Biotechnology, Santa Cruz, CA, USA] or catalase [#219010; Calbiochem, San Diego, CA, USA]), sheep (Cu/Zn-SOD or MnSOD; #574597, #574596; Calbiochem) or (2) mouse (nitrotyrosine; #189542; Cayman Chemicals). Following triple washes in TBS-T, membranes were incubated for 2 h at room temperature with secondary goat anti-rabbit and donkey anti-sheep or goat anti-mouse horseradish peroxidase-conjugated antibodies and visualised using an electrochemiluminescence detection kit. Values were expressed as arbitrary units per mg protein.

Protein carbonyl levels Protein carbonyls were assayed as an index of oxidative modification of proteins. Briefly, 50 μl of the plasma fractions were added to an equal volume of 10% (vol./vol.) trichloroacetic acid, centrifuged for 5 min at 6,000 *g* and 4°C, and the supernatant fraction

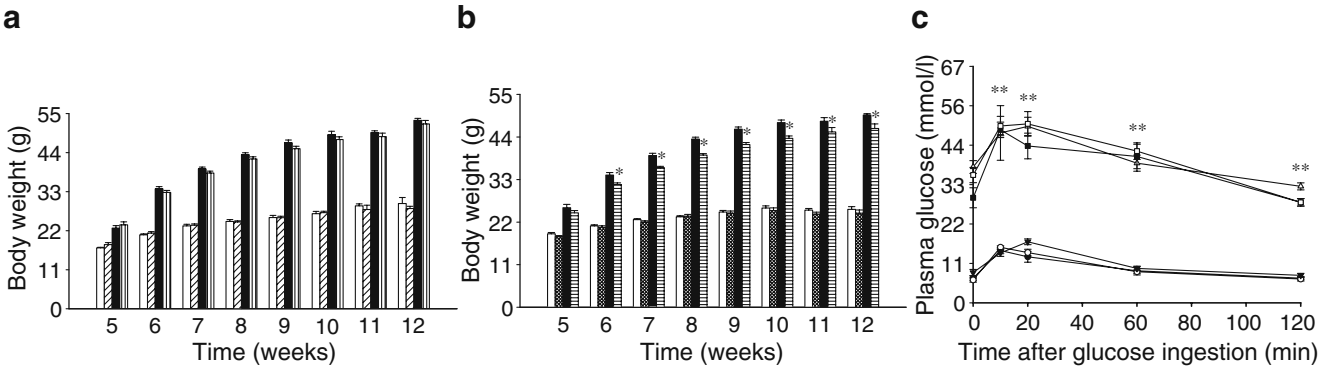

Fig. 1 Age- and exercise-related changes in body weight of mice and OGTT values. **a** Low-intensity exercise did not significantly change body weight in *db/db* mice over 7 weeks (white columns, WT; hatched columns, WT low-intensity exercise; black columns, *db/db*; vertically striped columns, *db/db* low-intensity exercise), while **b** *db/db* mice exercised at moderate intensity gained less weight than control littermates (white columns, WT; cross-hatched columns, WT moderate-intensity exercise; black columns, *db/db*; horizontally striped columns, *db/db* moderate-intensity exercise). This difference was apparent from 6 weeks of age onward. *$p<0.05$ (repeated measures ANOVA), $n=8$–10 per group. Weight gain in WT mice was not affected by either low- or moderate-intensity exercise. **c** OGTT showing glucose changes after oral ingestion of 1.5 g/kg glucose at the tenth week of age in fasted mice (black circles; WT; white circles, WT low-intensity exercise; black triangles, WT moderate intensity exercise; white triangles, *db/db*; black squares, *db/db* low-intensity exercise; white squares, *db/db* moderate-intensity exercise). **$p<0.01$ for difference between *db/db* and WT groups (repeated measures ANOVA), $n=9$–10 per group. Results are means±SEM

discarded. The precipitated proteins were resuspended in 0.2% 2,4-dinitrophenyl hydrazine and incubated for 1 h at 37°C. Subsequently, proteins were precipitated again with trichloroacetic acid, centrifuged at 4,000 *g*, washed with ethanol/ethyl acetate (1:1), dissolved in 6 mmol/l guanidine hydrochloride, and the absorbance measured spectrophotometrically at 370 nm [11, 19].

Statistical analysis and calculations Results are expressed as mean±SEM. Data were analysed using NCSS-2000 (Kaysville, UT, USA) computer software. Repeated measures ANOVA with multiple comparisons using Bonferroni's test or one-way ANOVA was performed where appropriate. GraphPad Prism (version 3.02–2000; San Diego, CA, USA) was used for linear regression, curve fitting and dose–response analysis. The results of statistical tests were considered significant if p values were less than 0.05.

Results

Influence of exercise on body weight and body fat The effects of exercise on body weight are shown in Fig. 1a,b. Body weight in *db/db* mice was initially higher than in WT mice at 5 weeks of age and continued to increase to nearly twice that of WT mice at 12 weeks (Fig. 1a). Low-intensity exercise did not influence body weight in *db/db* mice

Table 1 Plasma parameters of diabetic (*db/db*) and WT mice

Parameters	WT mice exercise groups			*db/db* mice exercise groups		
	None	Low intensity	Moderate intensity	None	Low intensity	Moderate intensity
Lipid profile						
TG (mmol/l)	0.5±0.07	0.71±0.12	0.61±0.14	1.32±0.14	0.62±0.07[a]	0.50±0.08[b]
Chol (mmol/l)	2.7±.0.20	2.65±0.08	3.04±0.04	3.97±0.20	4.1±0.10	2.9±0.16[b]
LDL-cholesterol (mmol/l)	0.91±0.07	0.88±0.05	0.99±0.08	1.48±0.16	1.55±0.06	0.830±0.23[b]
HDL-cholesterol (mmol/l)	1.44±0.13	1.48±0.05	1.65±0.15	1.66±0.27	2.26±0.13[a]	1.74±0.12[b]
Glycaemic status						
Glucose (mmol/l)	6.44±0.29	6.46±0.18	5.72±0.26	47.56±3.83	43.96±1.16	48.24±4.00
Insulin (pmol/l)	257.8±25	283.7±56	199.0±17	628.0±89	622.8±69	643.5±96

Values are means±SE for six to eight mice in each group. Plasma parameters were measured at the time of killing (12 weeks old)
Chol, cholesterol; TG, triacylglycerol
[a] $p<0.05$ for difference from sedentary *db/db* mice
[b] $p<0.05$ for difference from low-intensity exercised *db/db* mice

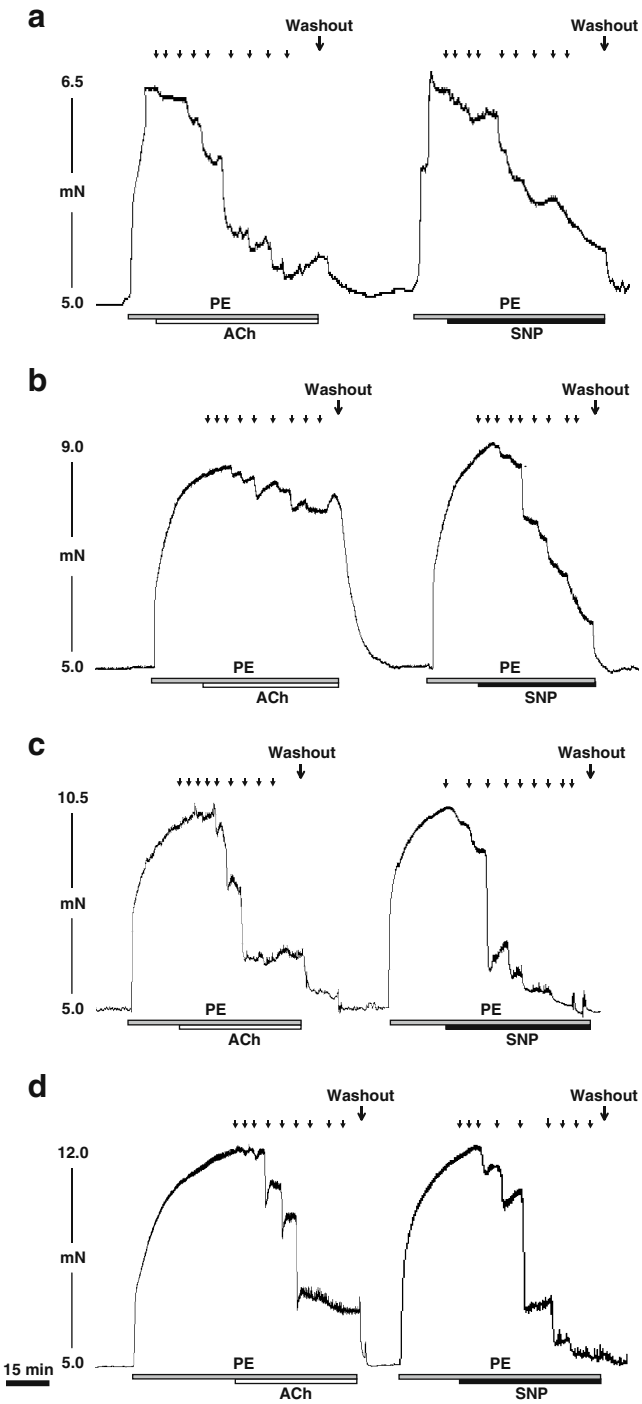

Fig. 2 Traces illustrating tension (mN) of mouse aortic rings. **a** WT, **b** *db/db*, **c** *db/db* exercised at low intensity and **d** *db/db* exercised at moderate intensity. Arteries were preconstricted with phenylephrine (PE) (1 μmol/l) and challenged with cumulative concentrations of ACh (10^{-3} to 10 μmol/l) and SNP (10^{-3} to 10 μmol/l). In WT mice (**a**) ACh and SNP caused vasodilation of aortic rings by ~80%. A marked attenuation of ACh-induced vasodilation was seen in *db/db* mice (**b**) (~25% dilation), while SNP-mediated vasodilation was unchanged. Both low- (**c**) and moderate-intensity exercise (**d**) improved ACh-induced aortic dilation in *db/db* mice

compared with non-exercised groups (Fig. 1a). In contrast, even after 1 week of moderate-intensity exercise, the weights of *db/db* mice were significantly (~10%) lower than those of non-exercised *db/db* mice (Fig. 1b). Neither exercise protocol affected body weight of WT mice (Fig. 1a,b). When the mice were 12 weeks old, the lean to fat ratio was significantly higher in WT than in *db/db* mice; neither of the exercise protocols significantly affected the lean to fat ratio in the *db/db* mice (WT, 1.75±0.05 vs *db/db*, 0.58±0.04, *db/db* low-intensity exercise, 0.6±0.03, *db/db* moderate-intensity exercise, 0.6±0.02, $p<0.05$).

Influence of exercise on plasma lipid profile Plasma cholesterol, triacylglycerol, LDL-cholesterol and HDL-cholesterol were measured as indicators of whole-body lipid profile (Table 1). Triacylglycerol, cholesterol and LDL-cholesterol were elevated in non-exercised *db/db* mice compared with non-exercised WT mice. Neither low nor moderate-intensity exercise changed plasma lipids in the WT mice. However, exercise with both protocols decreased plasma triacylglycerol in *db/db* mice. Moderate-intensity exercise also lowered total cholesterol and LDL-cholesterol, whereas low-intensity exercise increased HDL-cholesterol levels in *db/db* mice plasma (Table 1).

Influence of exercise on glycaemic status Both plasma insulin and glucose values were elevated in *db/db* compared with WT mice. Neither form of exercise altered these parameters in either *db/db* or WT mice (Table 1). As exercise has often been linked to improved whole-body insulin resistance, an OGTT was performed after 5 weeks of exercise in *db/db* and WT mice (Fig. 1c). Neither exercise regimen (low or moderate intensity) altered plasma glucose levels in *db/db* or WT mice within 120 min after an oral glucose load (Fig. 1c).

Endothelium-dependent and -independent vasodilation following exercise To evaluate endothelial function, endothelium-dependent and -independent vasodilation was evaluated using ACh and SNP respectively. The tracings illustrating tension (mN) of aortic rings from different mouse groups are depicted in Fig. 2, while Fig. 3 depicts ACh and SNP concentration–response curves in aortic rings preconstricted with phenylephrine with their E_{max} (maximum effective concentration) and EC_{50} (half-effective concentration) values given in Table 2. The endothelium-dependent vasodilation produced by ACh was impaired in aortic rings from *db/db* mice compared with WT counterparts (Figs 2a,b and 3a,c; Table 2). Exercising *db/db* mice with either low or moderate intensity restored endothelium-dependent vasodilation (Figs 2c,d and 3a,c; Table 2). The EC_{50} for ACh-induced vasodilation was similar in all groups (Table 2). Finally, endothelium-independent vasodi-

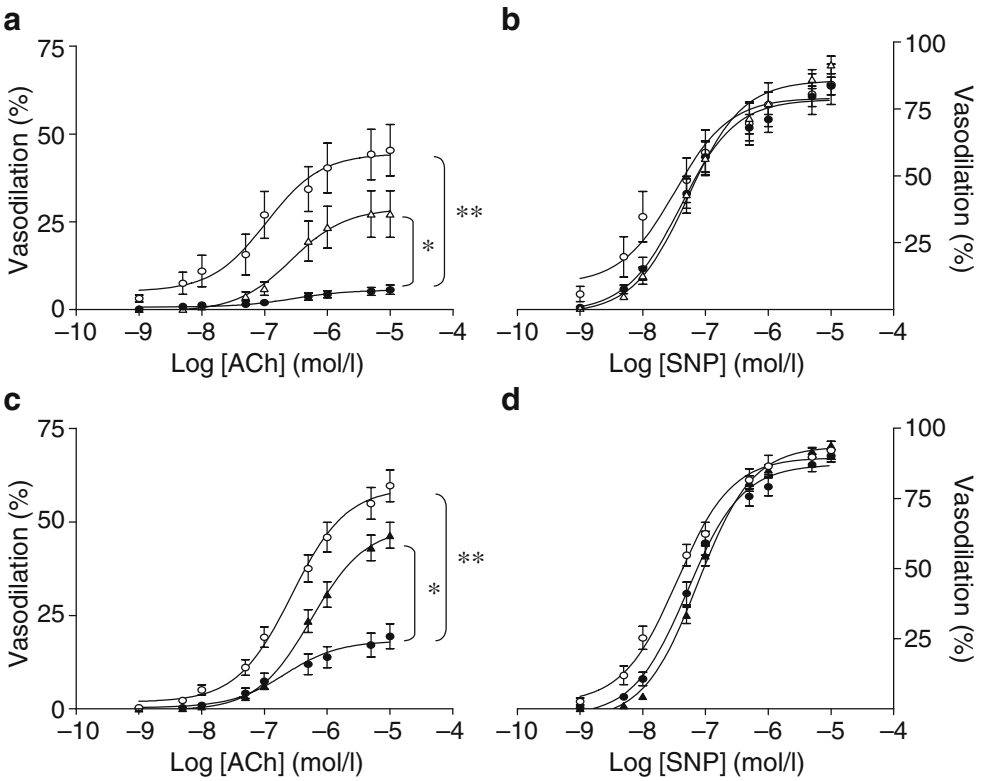

Fig. 3 ACh and SNP concentration–response curves from aortic rings preconstricted with phenylephrine (1 μmol/l). Endothelium-dependent vasodilation in response to ACh was significantly impaired in aortas of *db/db* mice (black symbols) compared with WT littermates (white symbols). Both low- (white triangles) and moderate-intensity (black triangles) exercise improved endothelium-dependent vasodilation in *db/db* mice (**a**, **c**). Endothelium-independent vasodilation of aortic rings induced by SNP was not statistically different in any of the groups (**b**, **d**). *$p<0.05$ and **$p<0.01$, repeated measures ANOVA; n=9–10 per group

lation induced by SNP was similar in *db/db* and WT mice and exercise did not alter this response in any of the experimental groups (Fig. 3b,d).

Aortic nitric oxide bioavailability following exercise We used two strategies to investigate endothelial-dependent vasodilation in *db/db* mice. First, we incubated aortic rings with SOD (150 U/ml), to improve NO availability by removing superoxide. Second, we incubated arteries with the nitric oxide synthase precursor, L-Arg (10^{-3} mol/l), combined with an eNOS cofactor, BH_4 (10^{-5} mol/l), to augment NO production [20]. Vasodilation in response to ACh in sedentary *db/db* mice improved significantly with both SOD (Fig. 4a) and L-Arg/BH_4 supplementation (Fig. 4b), implying the presence of increased superoxide and/or impaired NO production/availability in *db/db* mice. In contrast, neither SOD incubation nor L-Arg/BH_4 altered ACh responses in exercised *db/db* mice (Fig. 4c–f), suggesting that in these mice superoxide generation was decreased or endogenous SOD activity improved following exercise. Vasodilation in response to ACh in sedentary or exercised WT groups was not changed by SOD or L-Arg/BH_4 (data not shown). E_{max} for ACh responses were significantly increased in *db/db* mice after incubation with either SOD or L-Arg plus BH_4. However, such treatment had no effect in exercised *db/db* mice (Table 3); the EC_{50} values did not change in any group before and after incubations with these cofactors (Table 3).

Table 2 E_{max} and EC_{50} values for ACh and SNP concentration–response curves

	−Log EC_{50}		E_{max}	
	Ach	SNP	ACh	SNP
Figure 3a,b				
WT	7.0±0.2	7.5±0.1	44.4±3.6	79.1±3.1
db/db	6.5±0.3	7.4±0.1	5.6±0.6**	78.5±2.0
db/db low intensity	6.6±0.2	7.3±0.1	28.8±3.1*	85.6±2.3
Figure 3c,d				
WT	6.6±0.05	7.4±0.1	59.0±1.3	89.6±1.7
db/db	6.7±0.1	7.3±0.05	18.2±0.8**	87.1±1.6
db/db moderate intensity	6.2±0.02	7.1±0.04	48.5±0.6*	93.6±1.4

Results are the means±SE
*$p<0.05$ for difference between control *db/db* and *db/db* mice exercised at low and moderate intensity (one-way ANOVA), n=9–10 per group
**$p<0.01$ for difference between *db/db* and WT mice (one-way ANOVA), n=9–10 per group

Changes in eNOS production and tissue nitrite levels following exercise We evaluated protein production of eNOS and SOD isoforms under basal conditions (without treatment) in *db/db* aorta. Total eNOS protein was unchanged in non-exercised *db/db* mice (compared with WT mice), but was upregulated to an equal extent by the exercise protocols

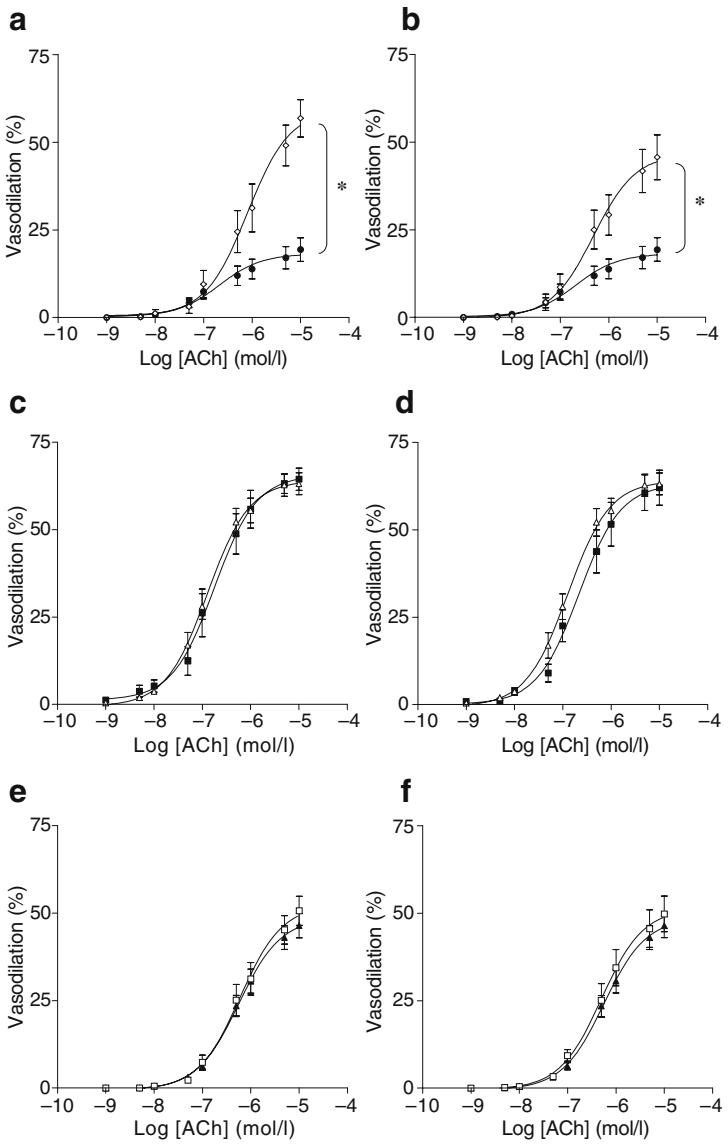

Fig. 4 Effect of SOD and L-Arg plus BH_4 incubation on ACh-induced vasodilation. **a** SOD and **b** L-Arg plus BH_4 incubation of aortic rings improved endothelium-dependent vasodilation in *db/db* mice. Black symbols, no treatment; white symbols, *db/db*+SOD (**a**), *db/db*+L-Arg plus BH_4 (**b**). **c** SOD and **d** L-Arg plus BH_4 had no effect in *db/db* mice exercised at low intensity. White symbols, *db/db* low-intensity exercise; black symbols, *db/db* low intensity+SOD (**c**), *db/db* low-intensity+L-Arg plus BH_4 (**d**). **e**, **f** The same treatments also had no effect at moderate intensity. Black symbols, *db/db* moderate-intensity exercise; white symbols, *db/db* moderate-intensity+SOD (**e**), *db/db* moderate-intensity+L-Arg plus BH_4 (**f**). *$p<0.05$, repeated measures ANOVA; n=8–10 per group

(Fig. 5a). Evaluation of phosphorylated eNOS at Ser1177 [21] revealed that diabetes decreases eNOS phosphorylation at Ser1177 in the aorta of non-exercised *db/db* compared with those of WT mice (Fig. 5b); however, eNOS phosphorylation at Ser1177 was increased with both exercise intensities, with low-intensity exercise demonstrating a greater benefit. When expressed as a ratio of 'active' eNOS (phospho-eNOS) to total eNOS protein, sedentary *db/*

Table 3 E_{max} and EC_{50} values for ACh concentration–response curves before and after SOD and L-Arg plus BH_4 incubation

Group	−Log EC_{50} (mol/l)			E_{max} (% vasodilation)		
	Control	SOD	L-Arg+BH_4	Control	SOD	L-Arg+BH_4
WT	6.57±0.05	6.40±0.05	6.16±0.07	59.03±1.33	66.83±1.65	57.37±2.11
db/db	6.68±0.11	6.10±0.06	6.31±0.05	18.18±0.81	59.12±1.97**	46.58±1.13**
db/db low intensity	6.89±0.02	6.75±0.04	6.67±0.05	64.04±0.55	65.52±1.04	63.15±1.22
db/db moderate intensity	6.25±0.02	6.22±0.05	6.30±0.03	48.53±0.58	52.24±1.25	51.31±0.78

Values are means±SE for eight to ten mice in each group
*$p<0.01$ for difference in *db/db* mice after incubation with both SOD or L-Arg plus BH_4 (one-way ANOVA)

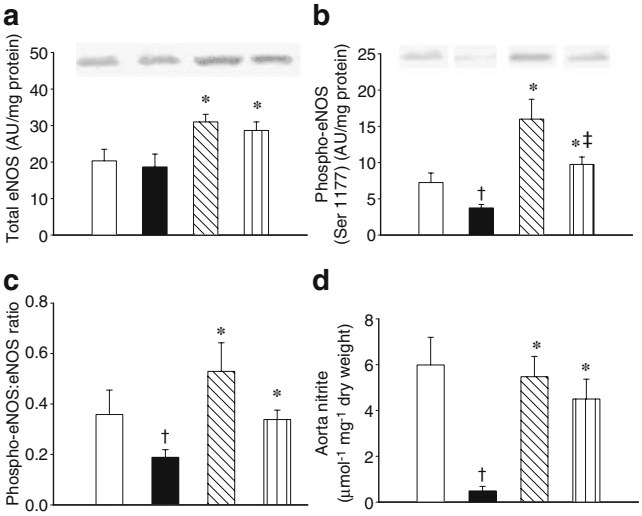

Fig. 5 Endothelial nitric oxide synthase protein production and aorta nitrite levels. **a** Total eNOS and **b** phosphorylated (Ser1177) eNOS protein production were measured by western blot followed by densitometry. Representative bands from each group are shown. **c** Ratio between phosphorylated eNOS and total eNOS protein as indication of 'active' eNOS levels. **d** Spectrophotometric quantification of nitrite (NO_2^-) in the aortas. Results are the means±SE of six rats in each group. White columns, WT; black columns, *db/db*; hatched columns, *db/db* low-intensity; vertically striped columns, *db/db* moderate-intensity. *$p<0.05$ for difference from sedentary *db/db* mice; †$p<0.05$ for difference from sedentary WT mice; ‡$p<0.05$ for difference from low-intensity exercised *db/db* mice. AU, arbitrary units; Phospho-eNOS, phosphorylated eNOS

db mice demonstrated decreased NOS activity, which was reversed by both exercise protocols (Fig. 5c). Non-exercised *db/db* mice also had lower plasma nitrite levels than WT mice. Exercise-induced tissue eNOS activation was associated with corresponding increases in aorta NO release, as measured by tissue nitrite levels, which increased by almost five- to sixfold with both forms of exercise (Fig. 5d).

Differential regulation of intracellular antioxidants following exercise Catalase levels were unchanged in all WT and *db/db* mice groups (Fig. 6a). As shown in Fig. 6b, low-intensity exercise caused an upregulation of Cu/Zn-SOD, the cytosolic SOD isoform, in aorta from diabetic mice. On the other hand, MnSOD, the mitochondrial isoform, was decreased in *db/db* mice and was selectively upregulated by a moderate-intensity exercise regimen (Fig. 6c).

Influence of exercise on whole-body and tissue-specific oxidative stress Plasma free 8-isoprostane is formed by whole-body lipid peroxidation reactions and tissue protein carbonyls are formed by protein oxidation. Plasma free 8-isoprostane (Fig. 7a) and aorta protein carbonyls (Fig. 7b) were higher in *db/db* than in WT mice. Both low- and moderate-intensity exercise lowered the levels of plasma 8-isoprostane and aorta protein carbonyls in *db/db* mice to levels similar to those in WT mice. Nitrotyrosine (biomarker for peroxynitrite-induced protein nitration) immunostaining increased following diabetes and decreased maximally with moderate-intensity exercise (Fig. 7c) [7].

Discussion

It is estimated that approximately 70% of people with diabetes will die as a result of a vascular event [22]. Endothelial dysfunction occurs early in diabetes and improving endothelial dysfunction is an important goal of therapeutic strategies to combat the disease [2]. Increased physical activity is routinely recommended in the management of human type 2 diabetes [22] and is believed to improve glycaemic control and plasma lipids, while simultaneously decreasing insulin resistance and body weight [23]. Although loss of body weight is often an important consideration in life style changes, beneficial effects of exercise, especially on cardiovascular complications, can also occur independently of body weight loss [24]. Thus, the molecular mechanisms by which exercise is beneficial in type 2 diabetes remain largely unknown.

There are several proposed mechanisms for the cardiovascular benefits of exercise in diabetes. Exercise improves

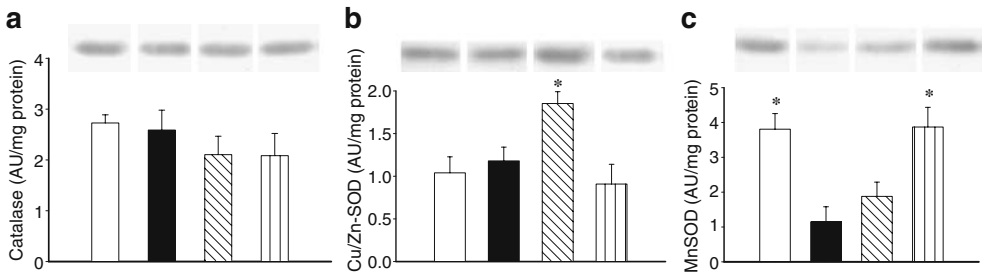

Fig. 6 Western blot analysis of antioxidant protein production. **a** Catalase, **b** Cu/Zn-SOD, **c** MnSOD protein production were measured by western blot followed by densitometry. Representative bands from each group are shown. Results are the means±SE of six rats in each group. White columns, WT; black columns, *db/db*; hatched columns, *db/db* low-intensity; vertically striped columns, *db/db* moderate-intensity. *$p<0.05$ for difference from sedentary *db/db* mice. AU, arbitrary units

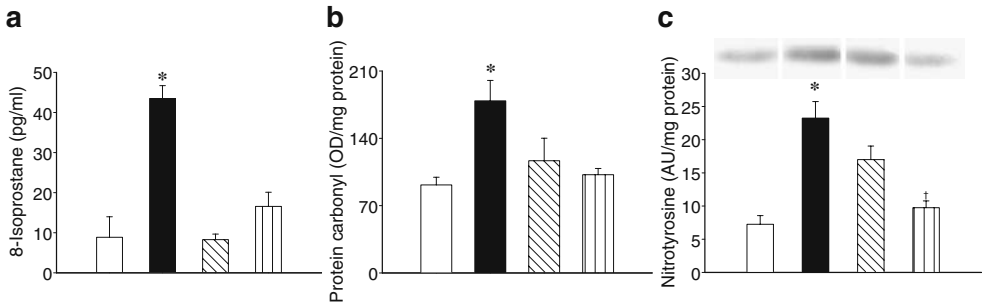

Fig. 7 Oxidative stress-related parameters. **a** Spectrophotometric determination of plasma free 8-isoprostane levels, **b** Western blot of aorta nitrotyrosine protein production and **c** spectrophotometric determination of aorta protein carbonyls. Representative bands (**c**) from each group are shown. Results are the means±SE of six rats in each group. White columns, WT; dark columns, *db/db*; hatched columns, *db/db* low-intensity; vertically striped columns, *db/db* moderate-intensity. *$p<0.05$ for difference from all other groups; †$p<0.05$ for difference from low-intensity exercised *db/db* mice. AU, arbitrary units; OD, optical density

cardiovascular function by decreasing risk factors such as plasma lipids, blood glucose or body mass index [4, 22, 23]. However, although long-term vigorous exercise decreases some of these metabolic parameters, exercise is also known to improve vascular function without significantly altering those risk factors [25]. In our study, *db/db* mice exercised at low intensity had improved vascular function without lowering of cholesterol, LDL-cholesterol, glucose or insulin levels. However, low-intensity exercise did reduce circulating triacylglycerol levels, while at the same time increasing HDL-cholesterol levels, which may be related to improved vascular endothelial function in exercised mice [26]. Exercise-induced increases in vascular shear stress may also be an important stimulus for NO release in vivo [27]. Since we were able to record improved vascular function in isolated blood vessel segments where no shear stress was present, our data suggest a more enduring effect of chronic exercise. Reductions in hyperglycaemia and insulin resistance, independently of body weight loss, have also been suggested to improve endothelial function and vasodilation [28]. In our study, following 5 weeks of exercise at different intensities, plasma insulin or glucose levels in a 2-h OGTT in *db/db* mice were not different from those in sedentary animals. The failure to improve insulin sensitivity is supported by previous studies in this model [29] and could be related to the exercise protocol used, since repeated short periods of daily exercise are more effective in reducing OGTT results than a longer period of exercise of the same duration every day [30].

Blood vessels from diabetic animals and humans exhibit attenuated endothelium-dependent relaxation in response to ACh [31]. Acetylcholine interacts with endothelial muscarinic M_3 receptors and causes NO release and vasodilatation [32]. It is possible that vascular dysfunction in diabetes is a result of impaired muscarinic receptor activity [33]. We show here that decreased endothelium-dependent vasodilation in *db/db* mice is unrelated to alterations in the receptor sensitivity to ACh. The decline, in the present study, of ACh E_{max} in the absence of changes in EC_{50} indicates that ACh receptor function remained relatively unaltered in diabetic mice. It is also unlikely that the activity of guanaylate cyclase is altered in diabetes, as indicated by similar responses to SNP, an exogenous donor of NO [34], and by the fact that in human diabetes the vasodilator response to SNP remained intact even though there was a marked decline in vasodilation in response to ACh [35].

We (in this study) and others have reported that diabetes does not alter the total protein production of eNOS [36]. However, some studies suggest a downregulation of eNOS activity during diabetes [37]. Phosphorylation of eNOS is important for post-translational regulation of eNOS activity [21]. The activation of eNOS catalytic function by Ser1177 phosphorylation inhibits the dissociation of calmodulin from eNOS and so increases the rate of eNOS electron transfer to produce NO [21]. In the present study, investigation of eNOS phosphorylation revealed a novel finding, namely that following diabetes Ser1177 phosphorylation was decreased in the aorta of *db/db* mice, and that an upregulation of phosphorylation levels occurred following exercise, suggesting greater eNOS activity without changes in body weight or glucose related parameters, which correlated well with aorta nitrite levels.

Apart from eNOS expression and phosphorylation, decreased availability of the eNOS substrate L-Arg or cofactors such as BH_4 also occurs in diabetes [38] and has been reported to be significantly lower in diabetic patients [39, 40]. In our study, although direct measurements of L-Arg and BH_4 were not performed, incubation with these agents potentiated the relaxation induced by ACh in aorta of sedentary *db/db* mice. One of the major reasons for a lack of L-arg and BH_4, is increased peroxynitrite, a free radical and strong oxidant, in the diabetic aorta. The formation of peroxynitrite occurs through a reaction between excess superoxide and NO, and takes place three times faster than the reaction between superoxide and its corresponding antioxidant, SOD [7]. BH_4 can be degraded directly by

peroxynitrite [20]. Additionally, BH_4 deficiency and peroxynitrite can uncouple eNOS [21]. Uncoupled eNOS consumes L-Arg to generate more superoxide instead of NO, forming a positive feedback loop and a progressive loss of available NO. In support of this hypothesis, we found increased levels of nitrotyrosine, a biomarker for peroxynitrite in the aorta of sedentary *db/db* mice.

Three isoforms of SOD are currently known, but their relative role in protecting against hyperglycaemia-induced vascular dysfunction is unclear. In our study, following diabetes and various intensities of exercise, we found differential regulation of these SOD isoforms. SOD-2 has manganese as cofactor, and is located mainly in the mitochondria, which it protects from oxidative damage [41]. On the other hand, cytosolic Cu/Zn-SOD may counteract NADPH oxidase and xanthine oxidase activity, which are the prime generators of cytosolic superoxides [41]. In this study, we observed a specific downregulation of aorta MnSOD following diabetes. Interestingly, although Cu/Zn-SOD did not change following diabetes as such, low-intensity exercise increased Cu/Zn-SOD protein production in *db/db* aorta. Although the exact reasons for such an upregulation are unknown, it has been reported that during mild to moderate training exercise laminar blood flow increases shear stress and upregulates Cu/Zn-SOD [42, 43]. Although whole-body 8-isoprostane, a marker of lipid peroxidation, decreased with low-intensity exercise, aorta nitrotyrosine and protein carbonyls were not affected by Cu/Zn-SOD upregulation. It may be speculated that being a cytosolic enzyme, Cu/Zn-SOD was unable to neutralise mitochondrial oxidative stress, which is the prime regulator of tissue peroxynitrite levels and protein oxidation in diabetes [44]. However, with higher intensities of exercise, we report a specific upregulation of MnSOD, the mitochondrial SOD isoform. During higher intensities of exercise, whole-body and muscle oxygen consumption increases up to 20- and 100-fold respectively [45], leading to increased release of superoxide from the mitochondrial electron transport chain. As Cu/Zn-SOD is downregulated with increased exposure to oxygen [46], upregulation of MnSOD under these conditions is common across various tissues [5, 47, 48] and may play a role in compensating a lack of Cu/Zn-SOD upregulation, thus providing greater protection by preventing formation of nitrotyrosine and protein oxidation, as shown in our study.

In conclusion, the primary defect in the diabetic aorta seems to be the prevalence of free radicals like superoxide and peroxynitrite, which lower NO bioavailability by degrading this compound and decreasing eNOS substrate (L-Arg) and cofactor (BH_4) availability. We demonstrate that a loss of body weight, body fat or improvement in glucose parameters are not obligatory in exercise-induced reversal of the above vascular defects. Additionally, exercise may upregulate aorta eNOS activity independently of its total protein production in diabetic blood vessels. Finally, in this study, we demonstrate for the first time that differential regulation of SOD isoforms occurs in the diabetic aorta, depending on exercise intensity. This, along with improved NO bioavailability may be pivotal in the reversal of diabetic endothelial dysfunction by lifestyle modification approaches such as exercise.

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