

Research Article

Effect of Moderate-Intensity Exercise on Plasma C-Reactive Protein and Aortic Endothelial Function in Type 2 Diabetic Mice

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The aim of this study was to evaluate the effects of moderate-intensity exercise on plasma levels of C-reactive protein (CRP) and tumor necrosis factor- α (TNF- α) as markers of low-grade inflammation and endothelial function in diabetic (db/db) mice. Control and db/db mice were divided into sedentary and exercised groups. Aortic endothelial function was evaluated after two- and six-week exercises using a wire myograph. Plasma CRP levels were measured at baseline, and after two and six weeks of exercise. Baseline plasma CRP levels were significantly higher in db/db mice compared to control ($P < .05$). After two weeks of exercise, aortic endothelial function was significantly improved without affecting body weight or plasma CRP levels. Six weeks of exercise not only improved endothelial function, but also significantly reduced body weight and plasma CRP levels in db/db mice. Thus short-term exercise has beneficial effect on endothelial function without reducing low-grade inflammation while more prolonged exercise periods are required to reduce inflammatory markers.

1. Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in diabetic patients [1], and it is likely that vascular abnormalities may be responsible for the higher incidence of cardiovascular diseases in diabetes. Although it is suggested that endothelial dysfunction is an important contributor to the vascular complications of diabetes [2, 3], the exact mechanisms of impaired endothelial function are unclear.

Lifestyle modification, especially exercise, is routinely recommended for the management of human type 2 diabetes [4, 5]. Exercise is thought to improve vascular function by reducing plasma lipids and blood glucose level [6], oxidative stress [7], and increasing insulin sensitivity [8]. Endothelial dysfunction is one of the earliest events in the progression of cardiovascular diseases.

Chronic low-grade inflammation, as reflected by elevated plasma levels of CRP, is an independent predictor of cardiovascular disease [9, 10] and diabetes [11]. CRP has a number

of roles in several cardiovascular diseases [12], and levels of CRP are positively correlated with obesity and insulin resistance [13]. Many studies suggest that a chronic inflammatory process promotes the progression of endothelial dysfunction [14]. In this study, we hypothesized that moderate-intensity exercise improves endothelial function by decreasing low-grade inflammation in db/db mice, a frequently used animal model of type 2 diabetes.

2. Materials and Methods

2.1. Animal Groups. Six-week-old control wild type and diabetic db/db mice (BKS.cg-m $+/+$ Lepr db/J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All experiments were performed according to the guidelines of the University of British Columbia Animal Care Committee. After one week of acclimatization, animals were randomly divided into four groups ($n = 10$ each): two groups each of control (control sedentary and control exercised) and diabetic mice (diabetic sedentary, diabetic exercised). The

animals were housed ten per cage under conditions of a 12-hour light/dark cycle, 22°C temperature, and with free access to food and water. Body weights were recorded weekly.

2.2. Exercise Program. Mice were exercised using a running wheel (Lafayette Instruments, Lafayette, IN, USA) as previously described in [2, 15]. Mice assigned to the exercise groups were placed in individual running wheels for one hour of daily exercise at a speed of 5.2 m/min (which represents a daily forced exercise of 312 m) for 6 weeks. During the training period (two weeks), mice were exercised daily at a set time each day for 5 days a week. The sedentary db/db or control groups were placed in nonrotating wheels for one hour per day.

2.3. Plasma Variables. Animals were anaesthetized with pentobarbital (50 mg/kg, *i.p.*) combined with heparin (50 U/kg). Blood samples were taken at baseline (6 weeks old), after two weeks of exercise following a two-week training period (the 10th week) and at the end of study (the 14th week). Fasting blood glucose was measured using commercially available kits. Plasma CRP and TNF- α levels were measured using ELISA kits (Alpco Diagnostic, USA).

2.4. Evaluation of Endothelial Function. Thoracic aortas were removed and placed in ice-cold physiological salt solution (PSS) and cleaned of connective tissue. Segments of aorta were threaded with stainless steel wire (0.04 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 minutes at 37°C during which time the PSS was replaced at 20-minute intervals. During the equilibration period, the resting tension was gradually increased to 5.5 mN and kept at this level for 20 to 30 minutes. Each tissue was maximally activated with a solution of KCl (80 mmol/L) that was prepared by equimolar substitution of NaCl. Following washout with fresh PSS and return of tension to basal preload, phenylephrine (1 μ mol/L) was added to establish a stable contraction. Thereafter, cumulative additions of acetylcholine (ACh) (1 nmol/L to 10 μ mol/L) were made. Vasodilatory responses were recorded on a computer using MyoDaq Acquisition software (version 2.01; Danish MyoTechnology, Aarhus, Denmark) and expressed as percent dilation of phenylephrine-induced constriction.

2.5. Citrate Synthase Assay. To document the efficacy of an endurance-trained state, citrate synthase activity levels were measured in skeletal muscle. Thigh adductor muscles were gently removed after sacrificing the animal, and citrate synthase activity was measured as previously described in [16].

2.6. Drugs and Chemicals. Acetylcholine, and phenylephrine were purchased from Sigma Chemical Co (St. Louis, MO). The composition of the PSS (mM) was NaCl (119), KCl (4.7), KH₂PO₄ (1.18), MgSO₄ (1.17), NaHCO₃ (24.9), EDTA

(0.023), CaCl₂ (1.6), and dextrose (11.1). Isotonic substitutions (replacement of Na⁺ with equimolar concentrations of K⁺) were used when using PSS solutions with increased K⁺ concentrations.

2.7. Statistical Analysis. Results are expressed as mean \pm SEM. Data analysis was done using NCSS-2000 software and GraphPad Prism (version 3.02-2000). ANOVA with multiple comparisons was performed using the Bonferroni's test. Correlation analysis using Spearman coefficient tests were performed where appropriate. $P < .05$ was considered as being statistically significant.

3. Results

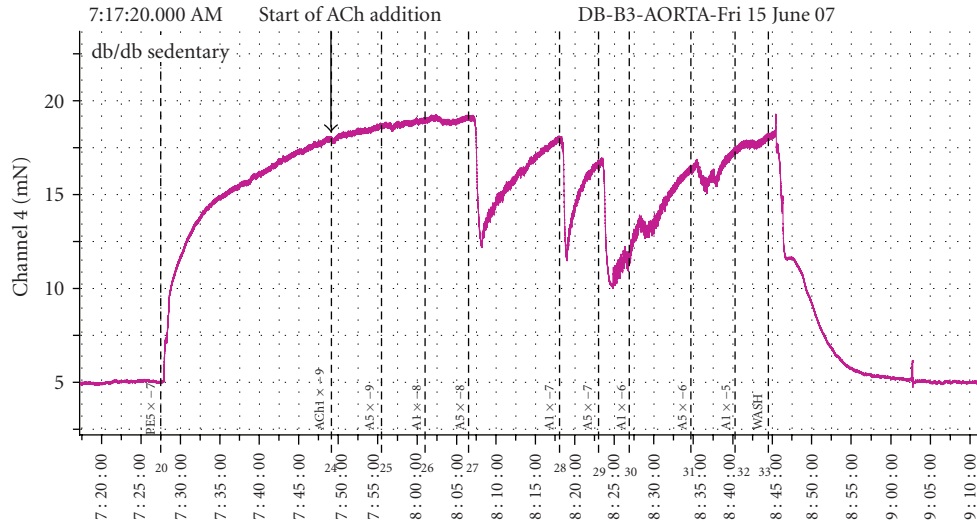
3.1. Body Weight, Blood Parameters, and Effect of Exercise. Six-week old diabetic mice had higher body weights than control mice. After six weeks of exercise, db/db exercised had lower body weights compared to the sedentary group (Table 1). Analysis of baseline blood parameters (6 weeks old), after two weeks (10 weeks old) and six weeks (14 weeks old) of exercise are shown in Table 1. Diabetic mice had higher fasting blood glucose levels at all time points, and while two weeks exercise did not alter blood glucose levels in db/db mice, six weeks of exercise reduced blood glucose levels in diabetic mice ($P < .05$).

Baseline plasma CRP levels were higher in db/db mice compared to control (3.81 ± 0.23 versus 1.83 ± 0.30) ($P < .05$). Plasma CRP levels in db/db mice were not affected by two weeks of exercise but were significantly reduced after 6 weeks exercise (at the 14th week) (3.59 ± 0.41 versus 5.12 ± 0.25) ($P < .05$). Plasma levels of CRP were significantly correlated with body weight ($r = 0.5855$, $P < .0001$) and blood glucose ($r = 0.4821$, $P = 0.0003$) when analyzed by the Pearson test.

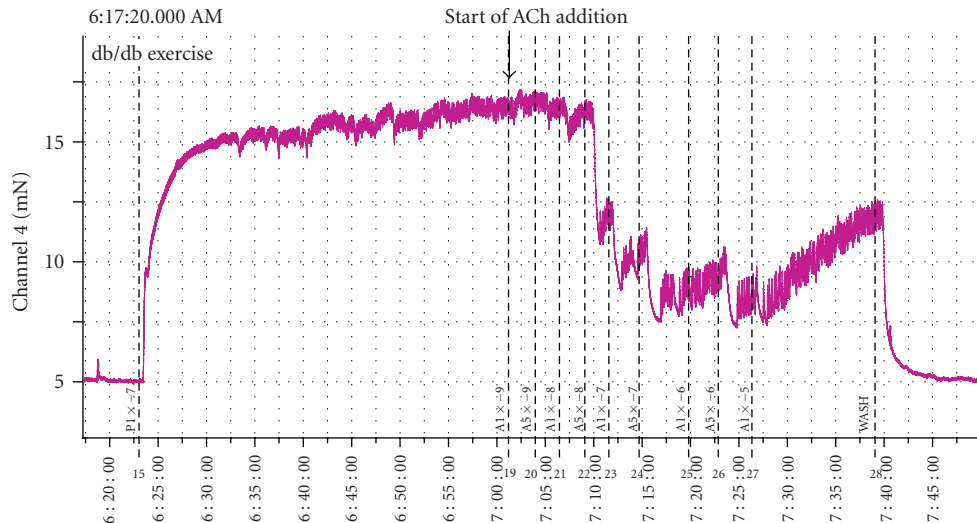
The level of plasma TNF- α in sedentary db/db mice at 14 weeks old (18.62 ± 2.11 pg/mL) tends to be higher than in control mice (14.88 ± 0.35 pg/mL); however, it does not reach statistical significance ($P > .05$).

3.2. Endothelial Function. Acetylcholine (ACh) was used to evaluate endothelial-dependent vasodilatation. Responses to ACh vasodilation were impaired in aortic rings from db/db mice compared with control counterparts (Figure 1). Moderate-intensity exercise in db/db mice for either two or six weeks restored endothelium-dependent vasodilation (Figure 1). The maximal vasodilatation (% loss of induced tone) and sensitivity (EC₅₀) is shown in Figure 2.

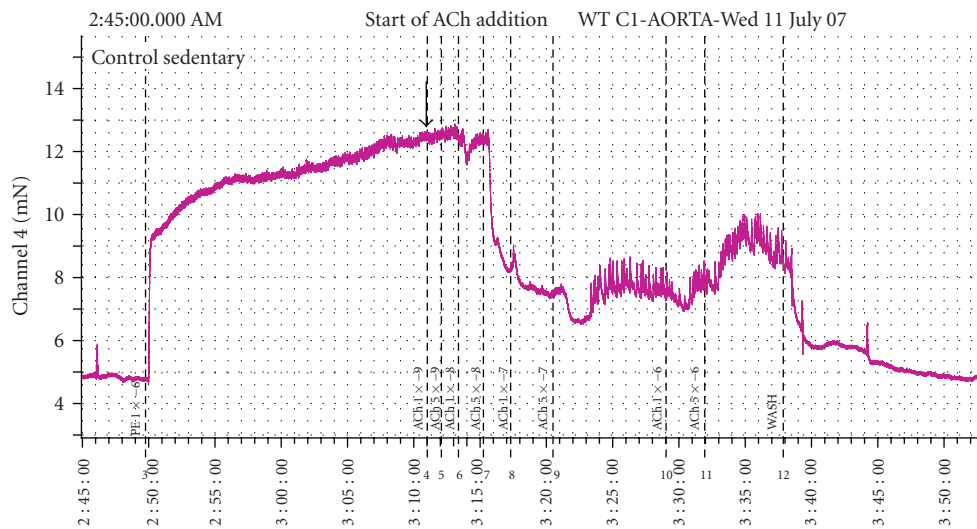
3.3. Citrate Synthase Activity. As shown in Table 2, tissue levels of citrate synthase activity were significantly increased in the thigh adductor muscles of db/db and control exercised mice compared to the sedentary groups at both time points (after two and six weeks of exercise) ($P < .01$, $n = 10$).



(a)



(b)



(c)

FIGURE 1: Continued.

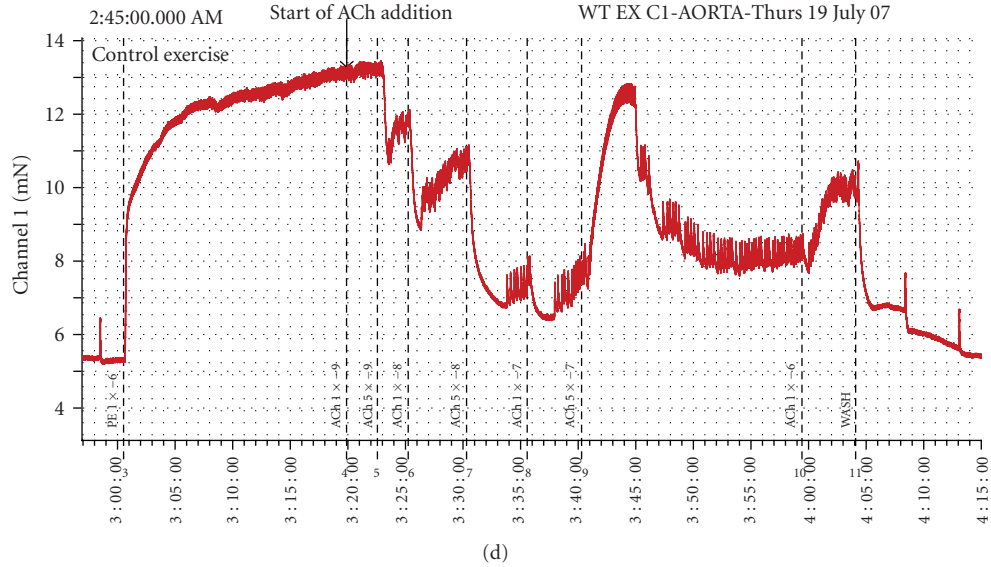


FIGURE 1: Representative traces showing ACh-induced vasodilation in aortae from diabetic (db/db) and control mice that were either sedentary or exercised.

TABLE 1: Body weights, plasma glucose CRP, and TNF- α levels in control or diabetic (db/db) mice that were either sedentary or exercised.

	Control sedentary			Control exercise			db/db Sedentary			db/db exercise	
	6 wk	10 wk	14 wk	10 wk	14 wk	6 wk	10 wk	14 wk	10 wk	14 wk	
Body weight (gm)	20.7 \pm 0.3	28.0 \pm 0.3	31.9 \pm 0.4	25.8 \pm 0.4	28.3 \pm 0.6*	30.7 \pm 0.4#	45.9 \pm 0.7#	48.9 \pm 1.2#	43.6 \pm 1.0#	44.6 \pm 1.3*#	
Fasting blood glucose (mg/dL)	2.3 \pm 0.1	5.0 \pm 0.2	5.9 \pm 0.5	5.3 \pm 0.2	5.8 \pm 0.2	5.7 \pm 0.3#	31.5 \pm 1.3#	54.7 \pm 1.5#	30.3 \pm 1.9#	44.6 \pm 1.6*#	
Plasma CRP (ng/mL)	1.8 \pm 0.3	2.6 \pm 0.3	2.5 \pm 0.4	3.8 \pm 0.2*	4.5 \pm 0.3*	3.3 \pm 0.3#	4.1 \pm 0.2#	5.1 \pm 0.3#	3.8 \pm 0.4#	3.6 \pm 0.4*	
Plasma TNF- α (pg/mL)	N/A	N/A	14.88 \pm 0.35	N/A	14.30 \pm 0.74	N/A	N/A	18.62 \pm 2.11	N/A	20.53 \pm 1.85	

* $P < .05$ compared to sedentary group at the same age.

$P < .05$ compared to control groups.

N/A: variable not measured

4. Discussion

This study examined the effects of moderate levels of exercise on vascular endothelial function and plasma CRP levels in control and type 2 diabetic (db/db) mice. We report that endothelial function (endothelium-dependent relaxation) was significantly impaired in db/db mice, as also reported in other studies [17–19]. There is much evidence to support the notion that endothelial dysfunction precedes the development of type 2 diabetes [20, 21]. Two-week and six-week of moderate-intensity exercise both significantly improved endothelium-dependent relaxation in db/db mice.

There is a strong association between endothelial dysfunction and inflammation. Endothelial dysfunction and plasma markers of inflammation are consistently increased in type 2 diabetes [22]. Our results show that diabetic mice initially have higher CRP levels compared to control

animals. An association between CRP levels and diabetes has been reported in other studies. For example, plasma levels of plasma CRP and ICAM levels are higher in diabetic subjects [23–25], and it is likely that increases in CRP levels also occur in patients with impaired glucose tolerance [26]. Thus, hyperglycemia may be one reason for endothelial dysfunction and low-grade inflammation in db/db mice [27]. Hyperglycemia is thought to activate the immune and macrophage-monocyte systems and so stimulate the production of cytokines and acute phase proteins, which are also proposed to reduce endothelial dependent vasodilation [22, 28]. Moreover, highly-glycated haemoglobin impairs NO-mediated vascular responses by a mechanism involving superoxide anions but not cyclooxygenase derivatives [7, 29]. In addition, db/db mice are obese, and there is also a close association between adiposity and CRP [13, 30]. Adipose tissue secretes inflammatory mediators (especially

TABLE 2: Citrate synthase activity (umole/mL/min) in thigh adductor muscle of all experimental groups.

	Control sedentary	Control exercised	db/db sedentary	db/db exercised
(10 Week old)	4.7 ± 0.053	5.0 ± 0.064*	3.6 ± 0.058	3.9 ± 0.042*
(14 Week old)	4.21 ± 0.32	6.61 ± 0.54*	1.67 ± 0.18	2.16 ± 0.12*

* $P < .05$ compared to sedentary group.

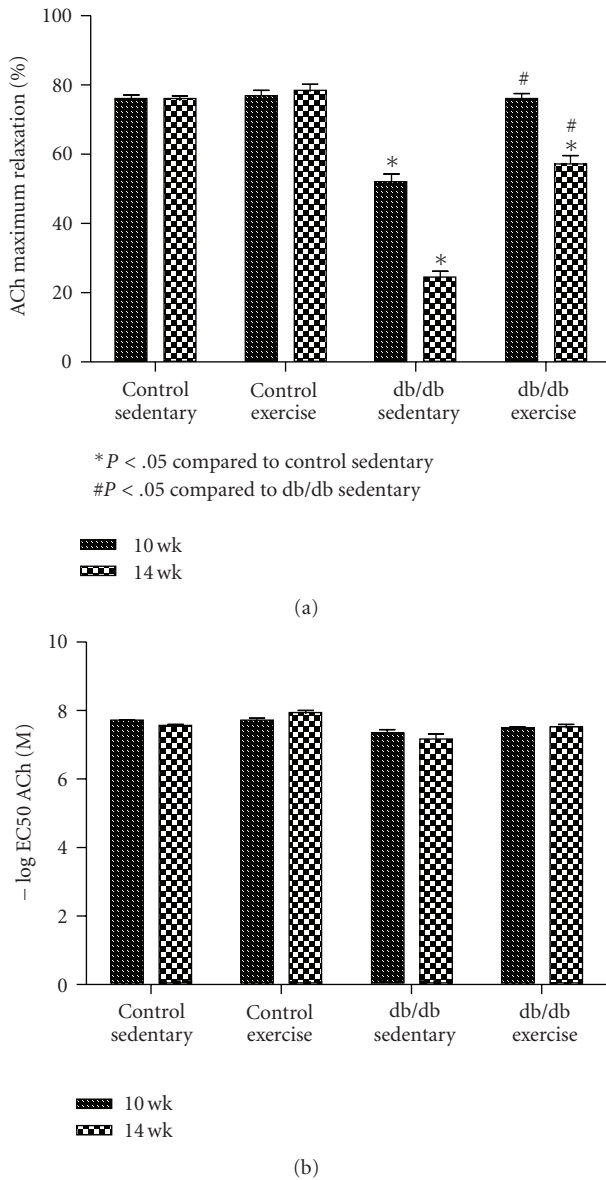


FIGURE 2: Emax (a) and EC50 (b) of ACh vasodilation after two weeks (10-week old mice) and six weeks (14-week old mice) of moderate-intensity exercise.

IL-6) which stimulates CRP synthesis in the liver [31]. CRP is related to insulin resistance and is a marker of endothelial dysfunction [32].

In our experiments, exercise improved endothelium-dependent relaxation in db/db mice after two-week exercise

independently of reductions in weight, blood glucose, or plasma CRP levels; our data shows a lack of a correlation between improved vasodilatation to ACh and decreased plasma CRP levels after two weeks of exercise as shown by the nonsignificant ($P = .1941$) Pearson correlation coefficient for the relationship between maximal ACh dilation and plasma CRP levels. However, six weeks of exercise improved ACh-mediated vasodilatation while also reducing plasma CRP levels in db/db mice; this was associated with a significant correlation between plasma CRP levels and body weight, a finding that is consistent with other reports in experimental [33] and human diabetes [34].

Our results indicate that CRP levels are increased in control mice that underwent a period of forced-exercise. This finding is in keeping with recent studies in healthy humans indicating that there were significant increases in plasma CRP and TNF alpha following a two-week bout of exercise [35]. In addition, exercise has also been shown to stimulate a marked but transient increases in inflammatory markers such as IL-6 and cortisol (which subsequently stimulate hepatocytes to generate the synthesis of acute phase proteins such as CRP), responses that may reflect muscle injury [36, 37].

Since CRP can cause a dose-dependent decrease in NO production in endothelial cells [38], it is possible that this effect is time-dependent and occurs independently of inflammation as reported by CRP levels. Other studies have reported that CRP directly inhibits the endothelium-dependent NO mediated dilation of porcine retinal arterioles [39], and down-regulates eNOS protein to decrease NO release [40].

The plasma levels of TNF- α in sedentary db/db mice tends to be higher than in control mice; however, it does not reach statistical significance. Previous reports have failed to demonstrate a parallelism between changes in plasma levels of CRP, IL-6, and TNF- α under pathological conditions [41–43]. Overweight adolescent boys had higher TNF- α , but not CRP or IL-6 levels compared to normal weight controls [42]. A systematic review demonstrated that exercise decreases CRP with no apparent effects on TNF- α [41]. However, CRP is the marker of chronic inflammation most frequently studied [44] and has been shown to predict cardiovascular diseases more than other cytokines [45].

In conclusion, we report a reciprocal association between endothelial dysfunction and CRP levels in diabetic db/db mice. Short-term exercise improves endothelial function without changing plasma CRP levels (two weeks of exercise). Longer periods of exercise (six weeks) reduce plasma CRP levels and maintain improved endothelial function in diabetic mice.

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