Effect of exercise on augmented aortic vasoconstriction in the db/db mouse model of type-II diabetes

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Summary

We evaluated the effects of exercise on the vascular constrictor responses to α-adrenergic stimulation in the db/db mice. Twenty male db/db and their age-matched wild-type (WT) mice were exercised (1 hour/day, five days a week). Mice were anaesthetized 7 weeks later and thoracic aortae mounted in wire myograph and constrictor responses to phenylephrine (PE, 1nM-10μM) obtained. Citrate synthase activity measured in the thigh adductor muscle was significantly increased in db/db mice that were exercise trained. Maximal force generated by PE was markedly greater in db/db aortae and exercise did not attenuate this augmented contractile response. Vessels were incubated with inhibitors of nitric oxide synthase (L-NAME, 200μM), endothelin receptors (bosentan, 10 μM), protein kinase C (PKC) (calphostin C, 5μM), cyclooxygenase (indomethacin, 10μM) or Rho-kinase (Y-27632, 0.1μM). Only calphostin-C normalized the augmented PE-induced constriction in db/db and db/db- exercised mice to that observed in WT ($p<0.05$). Cumulative additions of indolactam, a PKC activator, induced significantly greater constrictor responses in aortic rings of db/db compared to WT and exercise did not affect this response. Data suggest that the augmented vasoconstriction observed in the aorta of db/db mice is likely due to increased PKC activity and that exercise don't ameliorate this increased PKC-mediated vasoconstriction.

Keywords: Exercise, vasoconstriction, diabetes

Introduction

Diabetes is associated with an increased incidence of cardiovascular disease (Garcia *et al.*, 1974). It is likely that vascular smooth muscle cell responsiveness is altered in diabetes where there is an attenuated response to nitric oxide (Tesfamariam & Cohen, 1992), while at the same time the constrictor response to phenylephrine (PE) is enhanced (Agrawal and McNeill, 1987, Kamata *et al.* 1988). These abnormal vasomotor regulatory effects may in part underlie the cardiovascular morbidity and mortality that is a common complication of non-insulin dependent diabetic mellitus. Several studies revealed that contractile response to α-adrenergic stimuli in diabetic rats are enhanced (Abebe *et al.* 1990; MacLeod, 1985, Taylor *et al.* 1994), but the effect of exercise on increased contractile response of vascular smooth muscle cell is unknown.

Exercise is generally thought to provide cardioprotection (Sesso *et al.* 2000; Paffenbarger, Jr. *et al.* 1993, Myers *et al.* 2002; Jolliffe *et a.* 2001) and has several well-documented cardiovascular and systemic effects. The effects of exercise include improvements in endothelial function, left ventricular diastolic function, arterial stiffness, systematic inflammation, and reduced total and abdominal fat; it is thought that these effects of exercise may be largely responsible for improving insulin sensitivity and endothelial function in diabetes (Stewart, 2004a, Stewart, 2004b). The beneficial effects of life style modifications such as exercise in diabetic subjects is as important as controlling plasma glucose (Knowler *et al.* 2002). In this study, we used db/db mice, an animal model of non-insulin

dependent diabetic model (Guo *et al.* 2005; Kamata *et al.* 1988, Pannirselvam *et al.* 2002, Pannirselvam *et al.* 2003) to test the hypothesis that exercise decreases the constrictor response of vascular smooth muscle cells to adrenergic stimulation.

Methods

Animal groups

Twenty male db/db (BKS.cg-m +/+ *Lepr*^{db}/J) and age-matched wild-type (WT) mice (aged 5 weeks) were purchased from Jackson Laboratories (U.S.A.). All experimental protocols were approved by the Animal Care Committee of the University of British Columbia. Animals were housed in groups of 5 per cage with a 12h light/dark cycle at 26ºC and allowed access to food and drinking water *ad libitum*. Each group was randomized to exercised (n=10) and sedentary subgroups (n=10).

Exercise training program

Mice in the exercise group were trained to run on a motorized exercise wheel system (Lafayette Instrument Co, IN, USA). The initial two-week period involved a training period during which the exercise intensity was gradually increased. The initial exercise speed was 2.5 meters/min for one hour (150m) and incrementally changed to 5.2 meters/min (312 m) (table 1), which is well below the exercise tolerance level in mice (Verma-Ahuja *et al.* 2000). Mice were exercised five days per week for the duration of the experiment (8 weeks) at a set time each day (De

Angelis *et al.*, 2004; Tang & Reed, 2001). The integrated digital interface on the motorized exercise training wheel system controlled the wheel speed and duration of exercise. Sedentary animals were placed in non-rotating wheels daily for the same duration as the exercise group.

Isometric force measurement

Mice were sacrificed when aged 12 weeks: mice were anaesthetized by injection of pentobarbital sodium (Somnotol 30 mg/kg, i.p.) and heparin sodium (50 U/kg; i.p). The thoracic aortae were excised and placed in ice-cold physiologic salt solution (PSS, see solutions and chemicals) where they were carefully cleaned of fat and surrounding connective tissue. Segments of aortae were threaded with stainless steel wire (0.02mm diameter) and attached to tissue holders of a 4-channel wire myograph (JP Trading, Aarhus, Denmark) containing PSS solution aerated with 95% O_2-5% CO_2. Tissues were allowed to equilibrate for 60 min at 37°C, during which time the PSS was replaced at 20-30 minute intervals. During the equilibration, the resting tension was gradually increased to 5mN (milli newton) and kept at this level for 20-30 minutes. Each tissue was maximally activated with a solution of KCl (80mM) that was prepared by equimolar substitution of NaCl in PSS. Following washout with fresh PSS and return of tension to basal preload, phenylephrine (PE, 1 μmol/l) was added to establish a stable contraction. Thereafter, cumulative additions of Acetyl Choline (ACh) (1 nmol/l to 10 μmol/l) were made. The same protocol was repeated for sodium

nitroprusside (SNP, 1 nmol/l to 10 μmol/l). Following washout, constrictor responses to phenylephrine (PE, 1nM to10μM) were obtained using cumulative additions. After washout, the PE concentration-response curves were repeated in the presence of each of the following: nitric oxide synthase (NOS) blocker, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 200μM), endothelin dual receptor (A and B) antagonist (bosentan, 10 μM), protein kinase C (PKC) inhibitor (calphostin C, 5μM), cyclooxygenase inhibitor (indomethacin, 10μM) or the Rho-kinase inhibitor (Y-27632, 0.1μM). In other tissues, concentration-response curves were also made to indolactam, a PKC activator, (10^{-8} to 10^{-5}M). All data were recorded on a computer using MyoDaq Acquisition software (Danish Myo Technology, Aarhus, Denmark).

Measurement of plasma parameters

Animals were fasted for 12 h before sacrifice. Blood sample was collected from the inferior vena cava and immediately dispensed into tubes (Microtainer, Becton Dickinson, USA) and centrifuged at 8000g for 10 min for plasma generation. Plasma samples were then collected in separate Eppendorf tubes and stored at –70˚C for further analysis. Plasma lipid concentrations were measured using a Dimension® Clinical Chemistry System (GMI, Ramsey, Minnesota, USA). Plasma glucose and insulin levels were measured using commercially available assay kits.

Citrate synthase assay

To document the presence of an endurance-trained state, citrate synthase activity assays were performed on skeletal muscle. After sacrificing the animals, thigh adductor muscles were gently removed and frozen, and citrate synthase activity was measured as previously described (Korzick *et al.* 2004, Spier *et al.* 1999).

Drugs and Chemicals

Acetylcholine, sodium nitroprusside , phenylephrine, L-NAME, calphostin C, indomethacin and Y-27632 were purchased from Sigma Chemical Co (St. Louis, MO). The composition of the PSS (mM) was: NaCl (119), KCl (4.7), KH_2PO_4 (1.18), $MgSO_4$ (1.17), $NaHCO_3$ (24.9), EDTA (0.023), $CaCl_2$ (1.6), dextrose (11.1). Isotonic substitutions (replacement of Na^+ with equimolar concentrations of K^+) were used when using PSS solutions with increased K^+ concentrations.

Statistical analysis and calculations

Results are expressed as mean ± SEM. Data analysis and curve fitting were made with NCSS-2000 software and GraphPad Prism (version 3.02-2000), respectively. ANOVA with multiple comparisons using Bonferroni's test or student t-test was performed where appropriate. A value of $p< 0.05$ was considered as being statistically significant.

Results

Body weight

Figure 1 illustrates age related changes in the weight of mice in the experimental groups. At the beginning of the study (mice aged 5 weeks), the body weights of db/db mice were greater than WT, and this increased to an approximately two-fold difference at the end of the study. Exercised db/db mice had lower body weights compared to their sedentary counterparts ($p<0.05$).

Plasma Parameters

At the end of the study, plasma glucose and insulin concentrations in db/db mice were significantly greater than in WT mice. Exercised did not alter either plasma glucose or insulin levels ($p>0.05$) (table 2). Plasma levels of cholesterol, LDL, and TG were higher in db/db sedentary compared to WT ($p<0.05$). Exercise significantly decreased cholesterol, LDL and TG in diabetic mice ($p<0.05$) without significantly changing the HDL concentration ($p>0.05$).

Efficacy of exercise training program

The levels of citrate synthase activity were significantly higher in the thigh adductor muscles of db/db-exercised mice (69.44 ± 4.05) compared to the sedentary db/db mice group (51.42 ± 2.41) ($p<0.01$, n=5-7). In addition, there was

a significant difference between WT and WT exercised mice (52.44 ± 3.05 vs. 70.33 ± 4.10; $p<0.05$, n=6-7).

Endothelium-dependent and -independent vasodilation

The maximum response of endothelium-dependent vasodilation produced by ACh was impaired in aortic rings from *db/db* mice compared to their control counterparts (Figure 2). Exercise significantly improved endothelium-dependent vasodilation in *db/db* ($p<0.01$). Endothelium-independent vasodilation induced by SNP was similar in *db/db* and WT mice and exercise did not alter this response in any of the experimental groups (data not shown).

Aortic contractile responses

Contractility of mice aortae to 80mM KCl was not significantly different in sedentary and exercised db/db and WT mice (figure 4-B). The maximal force generated in response to the alpha-adreneregic receptor agonist PE (1nM to10μM) was markedly greater in db/db aortae. Exercise did not attenuate this augmented contractile response (Fig.3 & 4-A). Figure 4-C illustrates the E_{max} and EC_{50} of PE-response in all groups. Both sensitivity (EC_{50}) and maximum constriction (E_{max}) to PE was greater in diabetic mice compared to WT; these parameters were unaffected by exercise.

Effect of cyclooxygenase inhibitor (indomethacin)

The endothelium of the mouse aorta produces sufficient PGH_2/TXA_2 to initiate large contractions (Okon *et al.* 2002). To examine the role of PGH_2/TXA_2 in the augmented contractile response in diabetic mice aortae, we used indomethacin, a cyclooxygenase inhibtor. Incubation of aortae with indomethacin (10μM) did not attenuate either the enhanced PE-induced constriction in db/db and db/db exercised mice or the altered the sensitivity to PE (data not shown).

Effect of endothelin-1 receptor antagonist (bosentan), and Rho-kinase inhibitor (Y-27632)

To examine the possible role of endothelin-1 and Rho-kinase in the augmented PE-induced contractions in db/db aortae, PE-concentration response curves were reproduced in the presence of either bosentan or Y-27632. Pre-treatment with bosentan (10^{-5}M) or Y-27632 (10^{-7}M), did not change the maximal PE-induced constriction or EC_{50} in db/db mice. Similar results were obtained in the db/db exercised group (data not shown).

Effect of NOS blocker (L-NAME)

The role of basal NO in the PE-induced contractions in WT and db/db aortae was studied by comparing PE-concentration response curves in the absence and presence of L-NAME, a NOS inhibitor. Pre-treatment with L-NAME (200μM),

did not change the maximal PE-induced constriction or EC_{50} in db/db mice. Similar results were obtained in the exercised db/db mice (data not shown).

Effect of PKC inhibitor (Calphostin-C)

The PE concentration-response curves in the aorta from WT mice were not affected by pretreatment of vessels with calphostin-C, a PKC inhibitor (5×10^{-6}M). In contrast, calphostin-C reduced the increased PE-induced constriction in both db/db sedentary and db/db- exercised mice to levels similar to that observed in WT *(*$p<0.05$) (Figure 5).

PKC activator (indolactam) concentration-response curve

Cumulative concentrations of indolactam (10^{-8} to 10^{-5}), a PKC activator, induced significantly greater constrictor responses in aortic rings of db/db compared to WT mice. Exercise did not affect this exaggerated response to PKC activation (Figure 6).

Discussion

The aim of this study was to examine the effect of exercise on PE-constrictor responses in db/db mice. We demonstrate that the constrictor responses of smooth muscle cells to the alpha-adrenergic stimulant, PE, and the PKC activator, indolactam, are markedly enhanced in aortae of diabetic mice and that exercise did not attenuate this exaggerated constrictor response. Levels of citrate synthase

activity in the thigh adductor muscle (Korzick *et al.* 2004, Spier *et al.* 1999) were significantly increased in exercised db/db mice, confirming the systemic effects of the exercise protocol used in this study. The db /db mice had raised plasma glucose and insulin levels, likely as a result of insulin resistance in this model of type 2 diabetes. Exercise did not change plasma glucose and insulin levels, at least at the time of sacrifice of these diabetic mice. However, exercise reduced the raised plasma levels of LDL cholesterol and triglycerides in db/db mice, which may at least be partially related to the improved endothelial function in diabetic exercised mice (Bartus *et al.* 2005, Bae *et al.* 2001). However, exercise failed to improve serum HDL levels in diabetic mice.

Vascular responses to depolarization with KCl were similar in db/db and WT mice, both in the sedentary and exercised groups. Thus, it is unlikely that a generalized increase in responsiveness of arteries or changes in calcium-activated contractile mechanisms are involved in the augmented PE contractile response in diabetic mice. Alterations of vascular smooth muscle function have been implicated in the development of vascular complications and circulatory dysfunction in diabetes such as the increased aortic contractile response to PE (Zhu *et al.* 2001, Okon *et al.* 2003, Kawasaki, 1997, Guo *et al.* 2005; Abebe *et al.* 1990). While augmented responses to PE have been reported by several groups, there are also some reports showing changes or even decreased contractions of aorta (Mulhern and Docherty, 1989, Keegan *et al.* 1995). The endothelium produces vasoconstrictors such as eicosanoids and endothelin-1 (Tesfamariam *et*

al. 1989, Vanhoutte, 1994). Serum isolated from db/db mice induces COX-2 expression and increases TXA_2 production in primary cultured vascular smooth muscle cells (Guo *et al.* 2005, Xavier *et al.* 2003), suggesting that they may at least partially contribute to the vascular smooth muscle contractile hyperactivity in db/db mice (Guo *et al.* 2005). Endothelin-1 is also a powerful paracrine regulator of vascular smooth muscle tone (Yanagisawa *et al.* 1988). We speculated that the augmented constrictor response in db/db mice could be due to increased activity of endothelin-1 (Arikawa *et al.* 2001) and/or TXA_2/PGH_2 (Abebe *et al.* 1990; Tesfamariam *et al.* 1989). We excluded these possibilities by demonstrating that bosentan and indomethacin did not attenuate the enhanced PE-constrictor responses in db/db mice. Another possibility is that a decrease in basal NO production in diabetic mice may be responsible for the augmented PE response. We examined this possibility by repeating PE concentration-response curves after incubation with L-NAME. In the presence of L-NAME, PE-induced constriction remained significantly higher in db/db and db/db exercised mice compared to WT mice. This suggests that lower NO production may not underlie the enhanced contractile response to alpha-adrenergic stimulation in diabetic mice.

Contractile responses can be modulated by agonists independently of changes in intracellular Ca^{2+}, a process known as Ca^{2+} sensitization (Bradley and Morgan, 1987; Himpens *et al.* 1990, Morgan and Morgan, 1984). Rho-kinase inhibits myosin light chain phosphatase activity and has a key role in Ca^{2+} sensitization (Somlyo and Somlyo, 2000, Satoh *et al.* 1994). Therefore, it is possible that that

changes in Ca^{2+} sensitivity, for example mediated by Rho-kinase or PKC pathways (Sandu *et al.* 2000, Buus *et al.* 1998) could be responsible for the enhanced PE-constriction in diabetes. To investigate these possibilities, we inhibited Rho-kinase and PKC using Y-27632 and calphostin-C, respectively. Y-27632 did not change, while calphostin-C suppressed, the augmented contractile response to PE. Inhibition of PKC restored the contractions in db/db mice to levels observed in WT. In addition, cumulative concentrations of indolactam-V, a PKC activator, induced higher constrictor responses in db/db mice compared to WT. Therefore, increased Ca^{2+} sensitization due to increased protein kinase C activation likely mediates the enhanced alpha-adrenergic-mediated contractile response. Others have suggested that exposure of vascular smooth muscle to elevated concentrations of glucose increases protein kinase activity through activation by DAG (Abebe and MacLeod, 1991) and that this may be an important causal factor in diabetic vascular dysfunction (Haller *et al.* 1995, Inoguchi *et al.* 1992, Koya and King, 1998).

Our data suggests that the exercise improves endothelial function in db/db mice without affecting blood glucose or insulin level. We also demonstrated that augmented PE-induced constriction in db/db mice is not relieved by lifestyle medications such as exercise. It is likely that increased PKC activity may underlie the enhanced constrictor response in db/db mice. Exercise does not appear to modulate PKC activation of the db/db mouse aorta.

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Table 1 Exercise training protocol for mice.

Day	Exercise speed (m/min)
1	2.5
2	2.6
3	2.8
4	3.0
5	3.2
8	3.4
9	3.6
10	3.9
11	4.2
12	4.6
13	5.2
14	5.2
15	5.2
. . . . .	Continues same for 8 weeks

Table 2 Serum plasma parameters at the end of study in all experimental groups (n=6-8 each group).

Mouse group / Plasma parameters	WT	WT Exercised	db/db	db/db Exercised
Triglycerides	0.50±0.07	0.61±0.14	1.32±0.14*	0.50±0.08**
Cholesterol	2.7±0.20	3.04±0.04	3.97±0.20*	2.9±0.16**
LDL	0.91±0.07	0.99±0.08	1.48±0.16*	0.830±0.23**
HDL	1.44±0.13	1.65± 0.15	1.66±0.27	1.74±0.12
Glucose	6.44±0.29	5.72±0.26	47.56±3.83*	48.24±4.00
Insulin	1.41±0.53	1.51±0.36	6.48±0.52*	6.33±1.33*

** $p<0.05$ when compared to WT sedentary.*

*** $p<0.05$ when compared to db/db sedentary.*

Figure captions

Figure 1: Age- and exercise-related change in body weight of WT and db/db mice (* significant difference from db/db exercised; ANOVA; $p<0.05$).

Figure 2: The maximum response to ACh in aortic rings of WT, db/db and db/db exercised mice. Endothelium-dependent relaxation was significantly impaired in db/db aortae compared to WT and this response was significantly improved in db/db exercised animals (ANOVA; *$p<0.01$, WT & WT exercised vs. db/db; º$p<0.01$, db/db vs. db/db exercised; n = 6-7).

Figure 3: Traces illustrating tension (mN) of aortic rings from the following mouse groups: WT (**a**), *db/db* (**b**), *db/db* exercised (**c**). The maximal force generated in response to PE (10^{-9}-10^{-5}M) was markedly greater in db/db aortae. Exercise did not attenuate the augmented contractile response.

Figure 4: A- PE-induced constriction in WT, db/db, and db/db exercised mice aortae. The constrictor responses are potentiated in db/db compared to WT mice and exercise did not attenuate this augmented response (ANOVA; *$p<0.01$, n = 8-10 per group). **B-** Vascular contractile responses to depolarization with KCl were similar in db/db and WT mice. C- E_{max} and EC_{50} values for PE concentration-response curves. E_{max} and EC_{50} values were significantly higher in db/db mice compared to WT ($p<0.05$, n = 8-10).

Exercise did not alter the E_{max} and EC_{50} values in db/db mice (**p<0.05, n = 8-10 per group).

Figure 5: PE concentration-response curves in the presence and absence of calphostin-C in WT (A), db/db (B), db/db exercised mice (C). Treatment with calphostin C restored PE- induced constriction in diabetic mice to the levels that were similar to those in WT mice (D) (t-test; *p<0.01; n= in each group).

Figure 6: Indolactam concentration-response curve. The constrictor response to indolactam was markedly higher in diabetic (control and exercised) compared to WT mice (ANOVA; *p<0.05, n = 4)

Figure 1

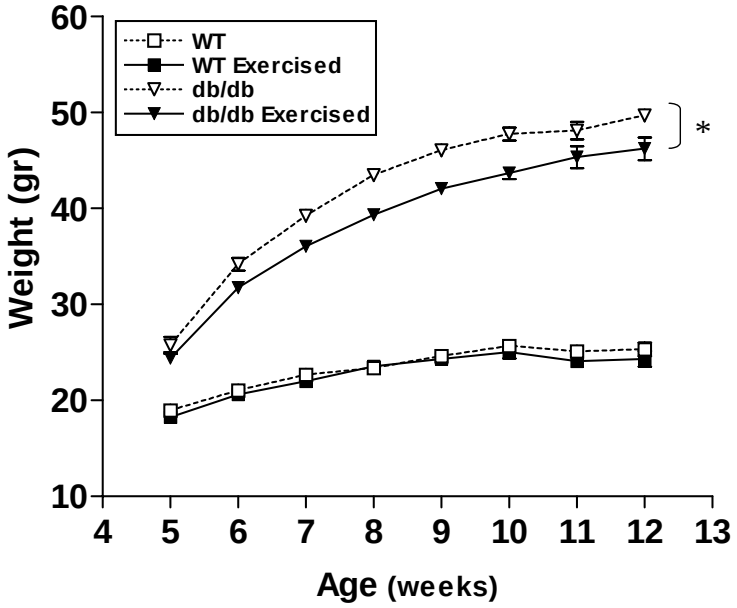

WT
WT Exercised
db/db
db/db Exercised
*
Weight (gr)
60
50
40
30
20
10
4
5
6
7
8
9
10
11
12
13
Age (weeks)

Figure 2

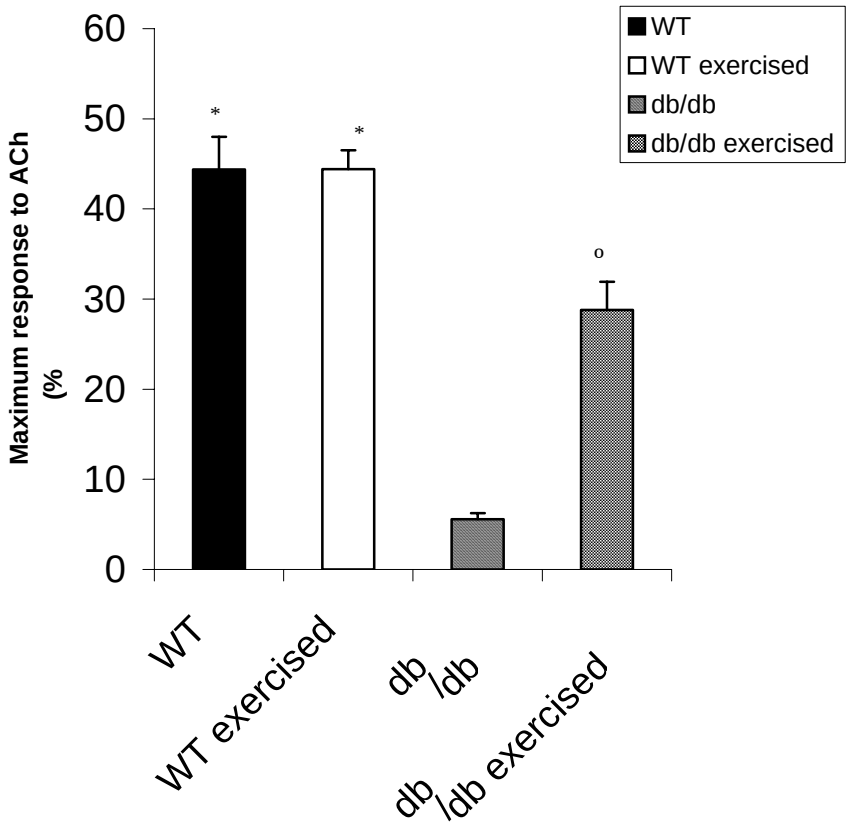

Maximum response to ACh (%
60
50
40
30
20
10
0
*
*
o
WT
WT exercised
db/db
db/db exercised
WT
WT exercised
db/db
db/db exercised

Figure 3

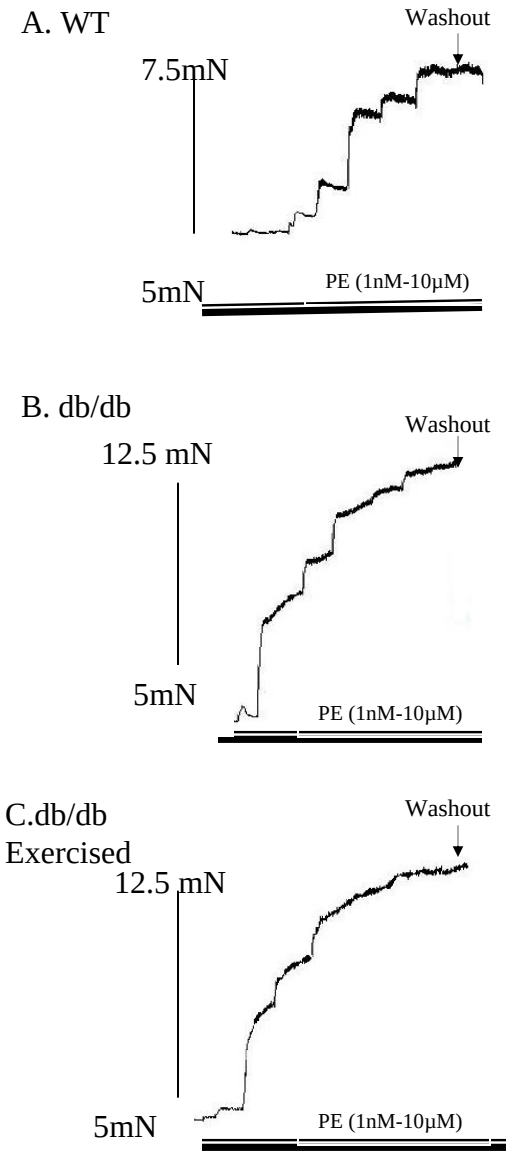

A. WT
Washout
7.5mN
5mN
PE (1nM-10µM)
B. db/db
Washout
12.5 mN
5mN
PE (1nM-10µM)
C.db/db
Exercised
Washout
12.5 mN
5mN
PE (1nM-10µM)

Figure 4

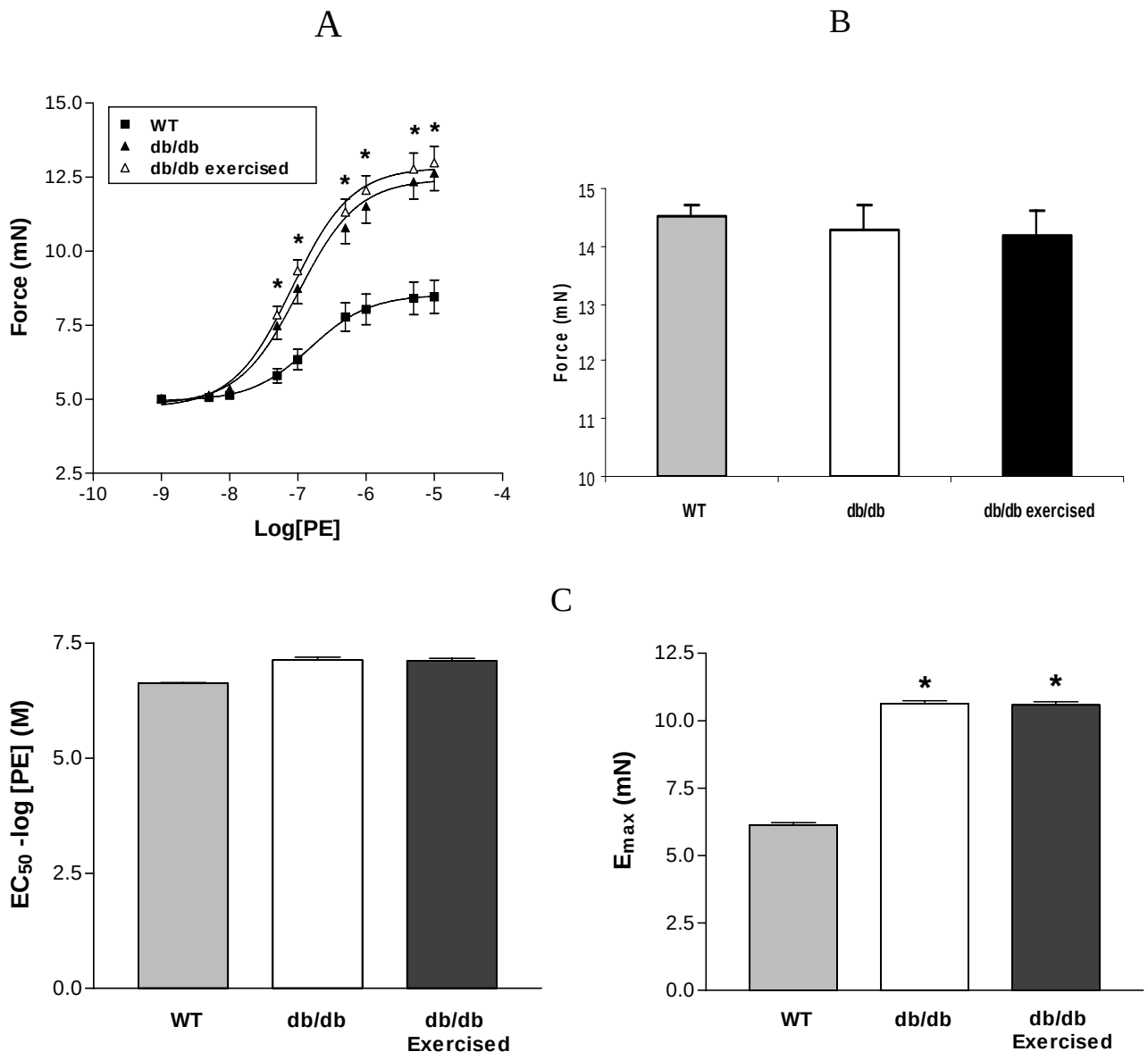

A
B
C
WT
db/db
db/db exercised
Force (mN)
Log[PE]
Force (mN)
WT
db/db
db/db exercised
EC50 -log [PE] (M)
WT
db/db
db/db Exercised
Emax (mN)
WT
db/db
db/db Exercised

Figure 5

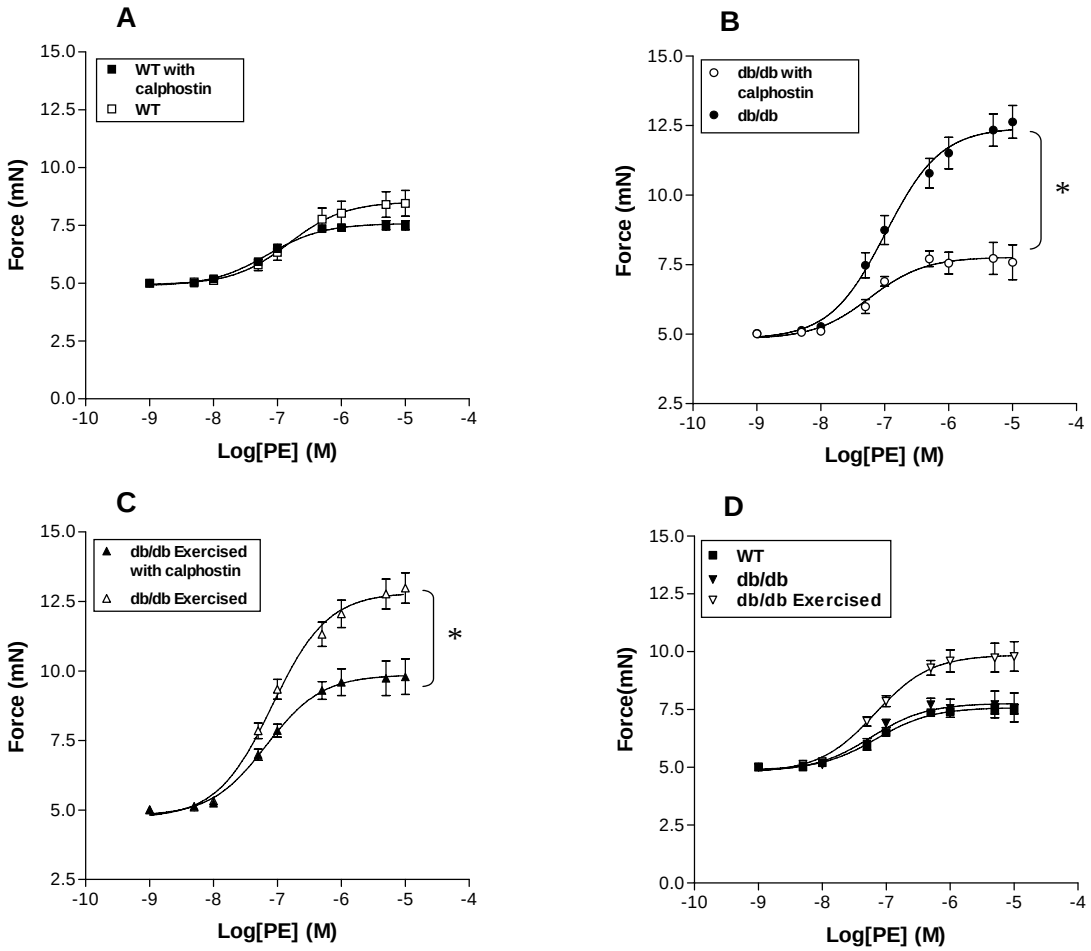
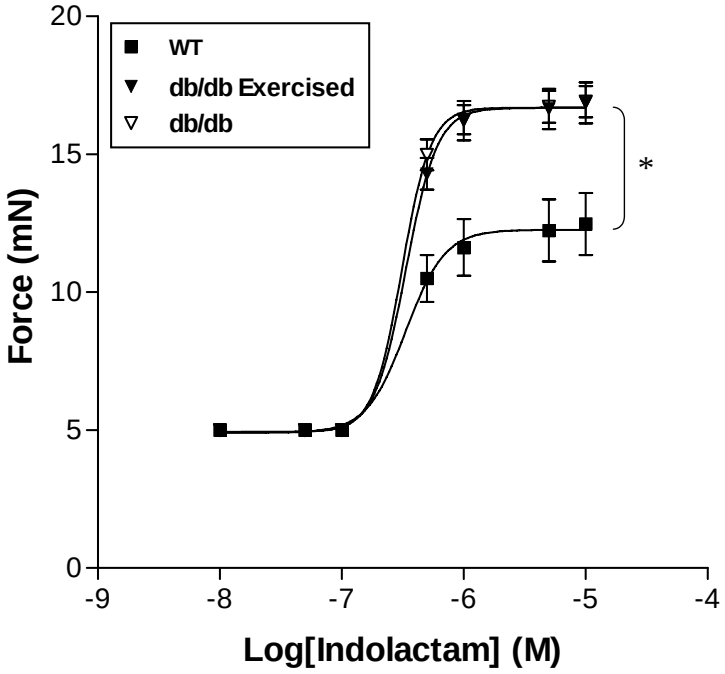

A
WT with calphostin
WT
Force (mN)
Log[PE] (M)
B
db/db with calphostin
db/db
*
Force (mN)
Log[PE] (M)
C
db/db Exercised with calphostin
db/db Exercised
*
Force (mN)
Log[PE] (M)
D
WT
db/db
db/db Exercised
Force(mN)
Log[PE] (M)

Figure 6


WT
db/db Exercised
db/db
Force (mN)
Log[Indolactam] (M)
*
20
15
10
5
0
-9
-8
-7
-6
-5
-4