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Pioglitazone ameliorates the lowered exercise capacity and impaired mitochondrial function of the skeletal muscle in type 2 diabetic mice

Running title: Exercise capacity and insulin resistance

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ABSTRACT

We have reported that exercise capacity is reduced in high fat diet (HFD)-induced diabetic mice, and that this reduction is associated with impaired mitochondrial function in skeletal muscle (SKM). However, it remains to be clarified whether the treatment of diabetes ameliorates the reduced exercise capacity. Therefore, we examined whether an insulin-sensitizing drug, pioglitazone, could improve exercise capacity in HFD mice. C57BL/6J mice were fed a normal diet (ND) or HFD, then treated with or without pioglitazone (3 mg/kg/day) to yield the following 4 groups: ND+vehicle, ND+pioglitazone, HFD+vehicle, and HFD+pioglitazone (n=10 each). After 8 weeks, body weight, plasma glucose, and insulin in the HFD+vehicle were significantly increased compared to the ND+vehicle group. Pioglitazone normalized the insulin levels in HFD-fed mice, but did not affect the body weight or plasma glucose. Exercise capacity determined by treadmill tests was significantly reduced in the HFD+vehicle, and this reduction was almost completely ameliorated in HFD+pioglitazone mice. ADP-dependent mitochondrial respiration, complex I and III activities, and citrate synthase activity were significantly decreased in the SKM of the HFD+vehicle animals, and these decreases were also attenuated by pioglitazone. NAD(P)H oxidase activity was significantly increased in the HFD+vehicle compared with the ND+vehicle, and this increase was ameliorated in HFD+pioglitazone mice. Pioglitazone improved the exercise capacity in diabetic mice, which was due to the improvement in mitochondrial function and attenuation of oxidative stress in the SKM. Our data suggest that pioglitazone may be useful as an agent for the treatment of diabetes mellitus.

Keywords: insulin resistance, diabetes, mitochondria, muscle, oxidative stress

1. Introduction

One of the pathophysiological features of patients with metabolic syndrome and type 2 diabetes is lowered exercise capacity (Regensteiner et al., 2005; Yokota et al., 2011). Indeed, this feature has been reported to be an independent predictor of mortality (Wei et al., 2000). Life style intervention, including physical exercise, is an important component of the prevention and treatment of diabetes. However, the appropriate therapy with exercise could be limited by the lowered exercise capacity in patients with type 2 diabetes. Therefore, the development of a pharmacological intervention to specifically improve the exercise capacity is an essential issue. The abnormalities in skeletal muscle energy metabolism are the most crucial factor for the lowered exercise capacity (Okita, 1998; Yokota et al., 2011; Yokota et al., 2013). Moreover, it has been reported that mitochondrial function is impaired in the skeletal muscle of patients with type 2 diabetes (Mogensen et al., 2007; Yokota et al., 2011; Yokota et al., 2013).

Houssay and Martinez for the first time reported diet-induced diabetes model in 1947 (Houssay and Martinez, 1947). Since that, diet-induced type 2 diabetes model became popular. Although agreed cut-off values for a definition of diabetes in mice have not been identified, high fat diet (HFD)-fed mice clearly demonstrated obesity, insulin resistance and glucose tolerance compared with those in ND-fed mice for 8 weeks (Suga et al., 2014; Takada et al., 2013; Yokota et al., 2009). Therefore, HFD-fed mice could be suitable as type 2 diabetes model (Islam and Loots du, 2009). In our previous study, we reported that the lowered exercise capacity and impaired mitochondrial function in the skeletal muscle in this model were due to enhanced oxidative stress via the activation of NAD(P)H oxidase (Takada et al., 2013; Yokota et al., 2009).

Furthermore, the activation of NAD(P)H oxidase was due to activation of the renin-angiotensin system (RAS) in the skeletal muscle, and angiotensin II type 1 receptor blocker (ARB) partially improved the limited exercise capacity (Takada et al., 2013). The NAD(P)H oxidase-induced enhancement of oxidative stress was also demonstrated in skeletal muscle from patients with type 2 diabetes (Roberts et al., 2006). Therefore, RAS-dependent activation of NAD(P)H oxidase plays an important role in the limited exercise capacity in HFD-induced diabetic mice.

NAD(P)H oxidase activity can be increased by high fatty acid levels and activation of RAS, as well as by high glucose, insulin and insulin resistance (Yang and Kahn, 2006). NAD(P)H oxidase activity and the expression levels of the NAD(P)H subunit have been shown to be activated in the skeletal muscle of insulin resistance-induced diabetes mice (Bonnard et al., 2008; Takada et al., 2013; Yokota et al., 2009). Therefore, insulin resistance may also play an important role in NAD(P)H oxidase and impaired mitochondrial function, leading to the lowered exercise capacity in HFD-induced type 2 diabetic mice. We thus hypothesized that the insulin-sensitizing drug, pioglitazone (Pio), could ameliorate the activated NAD(P)H oxidase and lowered exercise capacity in these mice. The purpose of the present study was to determine whether the administration of Pio to HFD-induced diabetic mice can ameliorate the impaired mitochondrial function and the lowered exercise capacity.

2. Materials and methods

2.1. Experimental animals

Male C57BL/6J mice (10-12 weeks of age) were housed in an animal room under controlled conditions on a 12-h:12-h light/dark cycle. Mice were fed either a normal diet (ND) containing 4.2% fat and 54.6% carbohydrate or an HFD (HFD32) containing 32.0% fat and 29.4% carbohydrate for 8 weeks. Mice were divided into two groups with or without addition of Pio (3 mg/kg/day; Takeda Chemical Industries, Osaka, Japan) to the ND or HFD diet. The quantities of food consumed by each mouse (2.4-2.5 g/day/mouse) and body weights were monitored every week, and the dose of Pio in the diets was adjusted. The concentration of Pio was chosen on the basis of previous study (Ishida et al., 2004). The present study was thus performed in the following 4 groups of mice: 1) ND+vehicle, 2) ND+Pio, 3) HFD+vehicle, and 4) HFD+Pio (n=10 for each group). These assignment procedures were performed using numeric codes to identify the animals. All procedures and animal care were approved by our institutional animal research committee and conformed to the Animal Care Guideline for the Care and Use of Laboratory Animals at Hokkaido University Graduate School of Medicine.

Eight weeks after treatment, exercise tests and intraperitoneal glucose or insulin tolerance tests were performed. Then blood samples were collected, and all mice were euthanized and their organ weights measured. Because the amount of hindlimb skeletal muscle samples was limited, these samples were divided into the experiments for mitochondrial oxygen consumption and biochemical assay, including NAD(P)H oxidase activity (n=6-10 for each assay).

2.2. Biochemical measurement and organ weight

After animals fasted for 6 h, blood samples were collected from the inferior vena cava before euthanization under deep anesthesia with tribromoethanol-amylen hydrate. Plasma insulin, total cholesterol, triglyceride, and nonesterified fatty acid (NEFA) levels were measured as previously described (Takada et al., 2013). Heart, epididymal fat, and unilateral hindlimb skeletal muscle were then excised and weighed. Total hindlimb skeletal muscle was used in all experiments.

2.3. Intraperitoneal glucose and insulin tolerance test

For the glucose or insulin tolerance test, mice were fasted for 6 h and were given an intraperitoneal injection of glucose (1 mg/g) or human regular insulin (0.25 mU/g) in purified water. Blood samples were repeatedly drawn from the tail vein of the same mice before and 30, 60, 90, and 120 min after the injection. Blood glucose levels were determined using a glucometer (Glutest Ace R; Sanwa Kagaku Kenkyusho, Nagoya, Japan).

2.4. Treadmill testing

Mice were treadmill tested to measure indexes defining whole body exercise capacity as previously described (Kinugawa et al., 2005; Suga et al., 2014; Takada et al., 2013; Yokota et al., 2009). The work was defined as the product of the vertical running distance to exhaustion and body weight.

2.5. Mitochondrial O_2 consumption in the skeletal muscle

Hindlimb skeletal muscle tissues were quickly harvested, and mitochondria were isolated as previously described (Takada et al., 2013; Yokota et al., 2009). The isolated

mitochondrial protein concentration and O₂ consumption by the isolated mitochondria were measured as previously described (Takada et al., 2013; Yokota et al., 2009). Mitochondrial respiration was initiated by the addition of 2.5 mmol/L L-glutamate and L-malate as substrates. ADP-stimulated (state 3) respiration was determined after adding ADP (300 µmol/L) (Mogensen et al., 2007; Takada et al., 2013; Yokota et al., 2009). Non-ADP-stimulated (state 4) respiration was measured in the absence of ADP phosphorylation and validated by oligomycin (2 mg/L), an ATPase inhibitor. An inflection point was objectively determined as previously described (Takada et al., 2013; Yokota et al., 2009). The respiratory control index (RCI) was calculated as the ratio of state 3 to state 4 respiration, and the P/O ratio was calculated as the ratio of the ATP amount to consumed O₂ during state 3. Therefore, RCI indicates overall mitochondrial respiratory activity and the P/O ratio indicates efficiency of ATP synthesis

2.6. Mitochondrial complex activities and citrate synthase activity in the skeletal muscle

The specific enzymatic activities of mitochondrial electron transport chain (ETC) complex I (rotenone-sensitive NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome c oxidoreductase), and complex IV (cytochrome c oxidase) were measured in the mitochondria isolated from skeletal muscle as previously described (Suga et al., 2014; Yokota et al., 2009).

The enzymatic activity of citrate synthase (CS, a key enzyme of tricarboxylic acid cycle) was spectrophotometrically determined in the tissue homogenate from skeletal muscle sample, as described previously (Inoue et al., 2012; Suga et al., 2014).

2.7. NAD(P)H oxidase activity in skeletal muscle

NAD(P)H oxidase activity was measured in the homogenates isolated from hindlimb skeletal muscle by the lucigenin assay after the addition of NAD(P)H (300 $\mu\text{mol/L}$) as previously described (Suga et al., 2014; Takada et al., 2013; Yokota et al., 2009).

2.8. Administration of amiloride

Previous study reported that thiazolidinediones increased body fluid volume through salt absorption in the renal collecting duct, which was blocked by amiloride (Guan et al., 2005). Therefore, to investigate the effect of Pio-associated fluid retention on exercise capacity, another set of mice (ND+vehicle, ND+Pio, HFD+vehicle, HFD+Pio; $n=4$ for each group) were treated with amiloride for 2 days before treadmill test (Hasegawa et al., 1995). Body weight was monitored before and after the treatment of amiloride, and the treadmill test was performed.

2.9. Statistical analysis

Data are expressed as means $\pm$ S. E. M. For multiple-group comparisons, two-way ANOVA followed by the Tukey's test was performed. In intraperitoneal glucose and insulin tolerance tests, differences between groups were determined with repeated-measures ANOVA. The effects of amiloride on body weight were analyzed separately using paired t -tests. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Animal characteristics

Table 1 shows the animal characteristics in the 4 groups of mice. Body weight was significantly higher in the HFD+vehicle compared with the ND+vehicle mice, and this increase was accompanied by a significant increase in the epididymal fat weight. There was no difference in the total weight of lower limb skeletal muscle between ND+vehicle and HFD+vehicle mice. Fasting blood glucose and plasma insulin levels were significantly higher in HFD+vehicle mice. Total cholesterol was also significantly higher in HFD+vehicle mice, but NEFA and triglyceride were comparable between ND+vehicle and HFD+vehicle mice. Moreover, blood glucose levels during an intraperitoneal glucose and insulin tolerance test were significantly higher in HFD+vehicle than in ND+vehicle mice (**Fig. 1**).

Pio significantly increased body weight, but did not affect the organ weight or biochemical measurements in ND mice (**Table 1**). HFD+Pio mice showed no significant differences from HFD+vehicle mice in body weight, heart weight, epididymal fat weight, total skeletal muscle weight, fasting glucose, NEFA, or triglyceride levels (**Table 1**). On the other hand, the plasma insulin levels were completely normalized in HFD+Pio mice. Moreover, blood glucose levels during an intraperitoneal glucose tolerance test were significantly lower in HFD+Pio than in HFD+vehicle mice (**Fig. 1**). These results showed that HFD+vehicle feeding for 8 weeks induced type 2 diabetes with the characteristic obesity and glucose intolerance, and Pio improved insulin resistance.

3.2. Exercise capacity

Fig. 2 shows the indices of exercise capacity. The work, run distance, and run time to exhaustion were significantly decreased in HFD+vehicle compared with ND+vehicle mice. The lowered exercise capacity was ameliorated in HFD+Pio mice. In particular, the work, which is an index used to account for the influence of body weight, was completely normalized in HFD+Pio mice. In contrast, Pio significantly decreased exercise capacity in ND mice.

3.3. Mitochondrial O₂ consumption in the skeletal muscle

Exercise capacity is largely dependent on mitochondrial O₂ consumption, which is energy production, in the skeletal muscle. Therefore, mitochondrial O₂ consumption was measured (**Fig. 3A**). State 3 respiration and RCI were significantly decreased in HFD+vehicle compared with ND+vehicle mice without any changes in state 4 respiration in the presence of glutamate-malate as substrate. The P/O ratio did not differ between groups. HFD+Pio mice had significantly improved state 3 respiration and RCI compared to the HFD+vehicle mice. In contrast, Pio did not affect mitochondrial O₂ consumption in ND mice.

3.4. Mitochondrial complex activities and citrate synthase activity in the skeletal muscle

Coincident with the impaired mitochondrial respiratory activity in the HFD+vehicle group, mitochondrial ETC complex I and III activities were significantly decreased in the HFD+vehicle compared with the ND+vehicle mice, and this decrease was normalized by Pio (**Fig. 3B**). Pio did not affect complex I and III activities in the ND+vehicle group. There were no significant differences in complex II and IV activities among the 4 groups (**Fig. 3B**).

CS activity was also significantly decreased in the HFD+vehicle compared with ND+vehicle group, and this decrease was also inhibited by Pio (**Fig. 3C**).

3.5. *NAD(P)H oxidase activity in the skeletal muscle*

NAD(P)H oxidase activity was significantly increased in the skeletal muscle from the HFD+vehicle compared with the ND+vehicle mice, and this change was completely inhibited by Pio (**Fig. 4**).

3.6. *Effects of Pio-associated fluid retention on exercise capacity*

Pio significantly increased body weight, and decreased exercise capacity in ND mice (**Table 1** and **Fig. 2**), which may have been due to Pio-associated fluid retention. Body weight was significantly lower after amiloride treatment in all groups (**Fig. 5A**). Similarly to the results in the group without amiloride treatment (**Fig. 2**), exercise capacity was decreased in the HFD+vehicle compared with ND+vehicle mice, and the decrease was ameliorated in HFD+Pio mice (**Fig. 5B**). Importantly, Pio did not affect exercise capacity in ND mice under treatment with amiloride (**Fig. 5B**).

4. Discussion

In the present study, HFD-induced type 2 diabetic mice exhibited lowered exercise capacity, impaired mitochondrial respiratory activities, decreased enzyme activities of mitochondrial complex and CS, and enhanced oxidative stress in skeletal muscle, and all these effects were significantly ameliorated by chronic treatment of HFD mice with Pio. Therefore, the lowered exercise capacity and mitochondrial dysfunction of HFD-induced type 2 diabetes may be associated with insulin resistance.

Our previous study showed that RAS-NAD(P)H oxidase system-induced reactive oxygen species played an important role in the impairment of mitochondrial dysfunction in the skeletal muscle, which led to lowered exercise capacity in HFD mice (Yokota et al., 2009) (Takada et al., 2013). In the present study, chronic administration of Pio in HFD mice almost completely ameliorated the lowered exercise capacity and the impaired mitochondrial function (**Fig. 2 and 3**), and these restorations were accompanied with the normalization of plasma insulin levels and the inhibition of NAD(P)H oxidase activation (**Table 1 and Fig. 1, 4**). These results suggested that aggravated insulin resistance could be involved in the activation of NAD(P)H oxidase and the lowered exercise capacity.

Pio completely inhibited the activation of NAD(P)H oxidase (**Fig. 4**) in a manner similar to the ARB or the reactive oxygen species inhibitor. Although the ARB and the reactive oxygen species inhibitor partially improved the exercise capacity in HFD mice (Takada et al., 2013; Yokota et al., 2009), Pio completely improved it (**Fig. 2**). These facts suggested that Pio improved the exercise capacity through mechanisms other than the inhibition of NAD(P)H oxidase activation. Previous studies have reported that various regulating factors for mitochondrial function are decreased in diabetic model

animals (de Las Heras et al., 2013; Escande et al., 2010; Lee et al., 2012; Safwat et al., 2013; Zhang et al., 2010). Adiponectin (Iwabu et al., 2010; Lee et al., 2012; Lin et al., 2013; Safwat et al., 2013) and sirtuin-1 (sirt-1) (de Las Heras et al., 2013; Escande et al., 2010; Price et al., 2012) in particular have been widely investigated. Iwabu et al. reported that adiponectin regulated exercise capacity by the increases in mitochondria content and function in the skeletal muscle via the activation of AMP kinase/sirt-1/peroxisome proliferator-activated receptor gamma (PPAR γ) coactivator 1- α (Iwabu et al., 2010). It is well known that Pio binds to and activates the ligands of PPAR γ , which regulates adiponectin or sirt-1. (Kumagai et al., 2013; Lin et al., 2013). Kumagai et al. reported that administration of Pio increased blood adiponectin levels in KKAY diabetic mice (Kumagai et al., 2013). Furthermore, Dutchak et al. reported that Rosiglitazone increased adiponectin via an increase of FGF-21 in adipocytes from stromal vascular cells (Lin et al., 2013). Therefore, Pio might improve the lowered exercise capacity and impaired mitochondrial function through an increase in adiponectin. Sirt-1 is a key factor regulating mitochondrial function, but our previous study showed that there was no difference between ND mice and HFD mice in the gene expression of sirt-1 in skeletal muscle (Takada et al., 2013). Therefore, sirt-1 might not have been associated with mitochondrial dysfunction in the HFD mice used in the present study.

It is known that Pio induces edema in patients (Guan et al., 2005). Pio activates a PPAR γ -dependent pathway within the renal collecting duct that directly stimulates epithelial Na⁺ channel γ subunit transcription and amiloride-sensitive Na⁺ absorption (Guan et al., 2005). In the present study, Pio significantly increased body weight and decreased exercise capacity in ND-feeding mice (**Table 1 and Fig. 2**). As expected,

treatment with amiloride for 2 days decreased body weight and canceled the adverse effects of Pio-associated fluid retention on exercise capacity (**Fig. 5**). In contrast, Pio did not increased body weight in HFD-feeding mice. Previous study reported that administration of Pio at 6 or 12 mg/kg/day did not increase body weight in HFD-fed B6 mice compared with HFD-fed control mice for 9 weeks (Matsui et al., 2010), which was consistent with our present study. The reason why Pio did not increase body weight in HFD mice is not clear. It has been reported that α -subunit and β -subunit, but not γ -subunit, of the epithelial sodium channel are upregulated in HFD rat model (Zhou et al., 2006). Therefore, the role of the increased epithelial sodium channel γ -subunit by Pio might be relatively small in HFD mice.

There are several limitations that should be acknowledged. First, NEFA and triglyceride were comparable between ND-fed and HFD-fed mice in the present study (**Table 1**), whereas they were increased in HFD-induced diabetes mice in the previous study (Hsu et al., 2014). Epididymal fat weight was increased in these mice (**Table 1**), which suggests that an excess energy was stored in the adipose tissue, and did not overflow into the blood. Our previous study also showed that 12 weeks feeding did increase NEFA and triglyceride (Suga et al., 2014). Therefore, the term of feeding with HFD may be important determinant of increase in their blood levels.

Second, the association between fatty acid metabolism and mitochondrial function in HFD-induced diabetes is controversial. Our and other studies reported that HFD-feeding induced insulin resistance and intramuscular lipid accumulation through the increase in gene expression of lipid transportation and the decreases in the expression of mitochondrial biogenesis related genes and CS activity (Chen et al., 2011; Suga et al., 2014; Yokota et al., 2009). Therefore, mitochondria in HFD-fed mice would prefer the

utilization of free fatty acid to other substrates, which could decrease CS activity through the enhanced oxidative stress (**Fig. 3**). In contrast, Turner et al. reported that HFD-feeding mice had higher mitochondrial oxidative enzyme capacities including CS activity than ND-feeding mice accompanied with an increase in blood level of free fatty acid (Turner et al., 2007). Furthermore, the increase in free fatty acid induced the increases in mitochondrial biogenesis and enzyme activities (Garcia-Roves et al., 2007; Hancock et al., 2008). It has been also reported that HFD increases angiogenesis in the skeletal muscle through the increase in fatty acid oxidation (Silvennoinen et al., 2013). The discrepancy between our and these data would be due to the difference in blood level of free fatty acid, which might be caused by the different HFD components (22.3% saturated, 66.5% monounsaturated, and 10.4% polyunsaturated fatty acid profile vs. 31.4%, 35.5%, and 33.1%, respectively). Although we could not clearly explain this issue, we think that the difference in the kind of feeding and the term of feeding may be associated with the difference in the results.

The incidence of type 2 diabetes has been steadily increasing, creating both medical and social challenges in industrialized countries. The lowered exercise capacity in type 2 diabetes could lead to aggravation of the disease by limiting the applicability of or compliance with exercise therapy. Our present data showed that pharmacological treatment with Pio performed to attenuate insulin resistance improved exercise capacity. Indeed, Regensteiner et al. reported that chronic administration of another thiazolidinedione, rosiglitazone, in patients with type 2 diabetes improved their exercise capacity (Regensteiner et al., 2005). In another experiment, the administration of Pio for an additional 4 weeks was performed in mice that had been fed an HFD for 8 weeks, in which exercise capacity was already lowered and mitochondrial function was impaired.

However, Pio did not improve the exercise capacity or mitochondrial dysfunction in their study (Suga et al., 2014). Given the close association between exercise capacity and prognosis, the present findings may draw further attention to the option of early and intensive treatment of type 2 diabetes using an insulin-sensitizing drug. Although clinical use of Pio might cause some adverse events, previous large-scale clinical trials showed that Pio had many protective effects to various organs including skeletal muscle (Scherthaner et al., 2013). Therefore, Pio represents an important therapeutic option in patients with type 2 diabetes.

In conclusion, Pio improved the exercise capacity in diabetic mice, which was attributed to the improvement in mitochondrial function and attenuation of oxidative stress in the skeletal muscle. Our data suggest that Pio would contribute ameliorating activities to the treatment of diabetes mellitus.

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Disclosures

No conflicts of interest.

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Figure legends

Fig. 1. (A) Blood glucose levels during intraperitoneal glucose tolerance test in the normal diet (ND)+vehicle, ND+Pioglitazone (Pio), high fat diet (HFD)+vehicle, and HFD+Pio (n = 9-10 for each group) mice. (B) Area under the curve of blood glucose levels during intraperitoneal glucose tolerance test in the ND+vehicle (white column), ND+Pio (gray column), HFD+vehicle (black column), and HFD+Pio (dark gray column) mice. (C) Blood glucose levels during intraperitoneal insulin tolerance test in the ND+vehicle, ND+ Pio, HFD+vehicle, and HFD+Pio mice (n=9-10 for each group). (D) Area under the curve of blood glucose levels during intraperitoneal insulin tolerance test in the ND+vehicle, ND+Pio, HFD+vehicle, and HFD+Pio mice. Data are expressed as means $\pm$ S. E. M. Experiments were performed after 8 weeks of feeding in all groups. * P <0.01 vs. ND; † P <0.05 vs. HFD at each time point.

Fig. 2. The summarized data of (A) the work, (B) run distance and (C) run time to exhaustion in the ND+vehicle, ND+Pio, HFD+vehicle, and HFD+Pio mice (n=10 for each group) are shown. Data are expressed as means $\pm$ S. E. M. * P <0.05 vs. ND; † P <0.05 vs. HFD.

Fig. 3. (A) The summarized data of ADP-stimulated (state 3) respiration, non-ADP-stimulated (state 4) respiration, respiratory control index (RCI) and the ratio of ATP amount to consumed O₂ during state 3 (P/O) ratio in the isolated mitochondria in glutamate and malate (n=6-7 for each group), (B) mitochondrial electron transport chain (ETC) complex I, II, III, IV enzymatic activities in the isolated mitochondria (n=10 for

each group), and (C) citrate synthase (CS) activity in the skeletal muscle from 4 groups of ND+vehicle, ND+Pio, HFD+vehicle, and HFD+Pio mice. Data are expressed as means $\pm$ S. E. M. * P <0.05 vs. ND; † P <0.05 vs. HFD.

Fig. 4. NAD(P)H oxidase activity measured by lucigenin chemiluminescence in the skeletal muscle obtained from 4 groups of ND+vehicle, ND+Pio, HFD+vehicle, and HFD+Pio mice (n=9 for each group). Data are expressed as means $\pm$ S. E. M. RLU, relative light unit. * P <0.05 vs. ND; † P <0.05 vs. HFD.

Fig. 5. The summarized data of the (A) body weight of pre- and post-treatment of amiloride. (B) The work, run distance, and run time to exhaustion in the ND+vehicle, ND+Pio, HFD+vehicle, and HFD+Pio mice (n=4 for each group) treated with amiloride are shown. Data are expressed as means $\pm$ S. E. M. * P <0.05 vs. ND; † P < 0.05 vs. HFD.

Table 1. Animal Characteristics

	ND+	ND+	HFD+	HFD+
	vehicle	Pio	vehicle	Pio
<i>Body weight and organ weight</i>				
N	10	10	10	10
Body wt (g)	29±1	31±1 ^a	39±1 ^a	38±1 ^a
Heart wt (mg)	121±3	138±9	132±5	129±3
Epididymal fat wt (mg)	771±59	827±68	2790±93 ^a	2575±79 ^a
Skeletal muscle wt (mg)	1090±38	1072±25	1131±49	1156±29
<i>Biochemical measurements</i>				
N	8	8	8	8
Fasting glucose (mg/ml)	139±8	136±11	210±8 ^a	208±8 ^a
Insulin (ng/ml)	0.24±0.09	0.25±0.12	1.07±0.38 ^a	0.15±0.03 ^b
Total cholesterol (mg/dl)	69±5	62±3	145±9 ^a	135±3 ^a
NEFA (mEq/l)	0.55±0.05	0.46±0.06	0.54±0.05	0.49±0.09
Triglyceride (mg/dl)	59±3	62±6	54±5	54±4

Data are expressed as means ± S. E. M. ND, normal diet; HFD, high-fat diet; Pio, pioglitazone; wt, weight; NEFA, non-esterified fatty acid. ^a $P < 0.05$ vs. ND+vehicle; ^b $P < 0.05$ vs. HFD+vehicle.

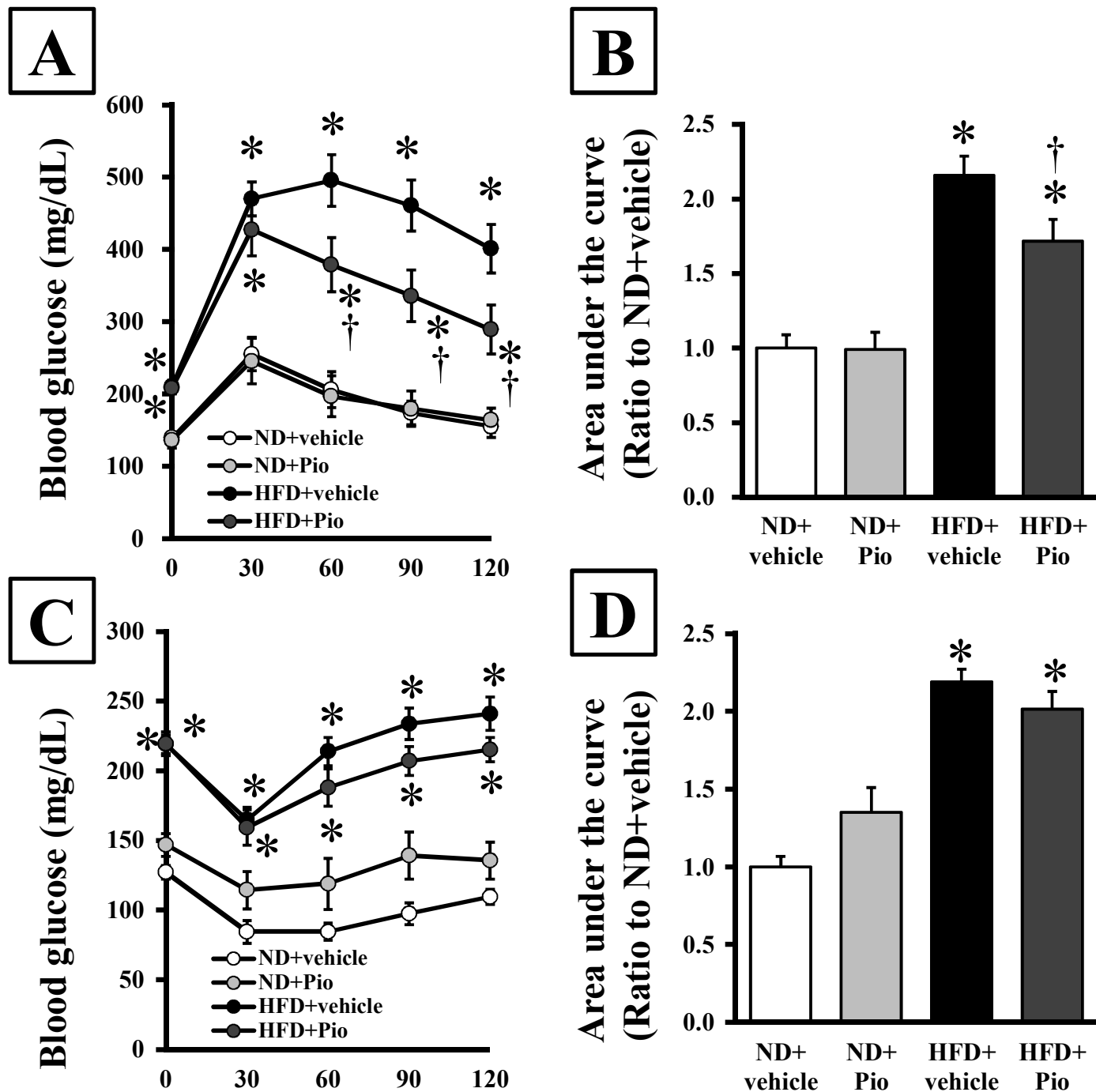


Figure 1

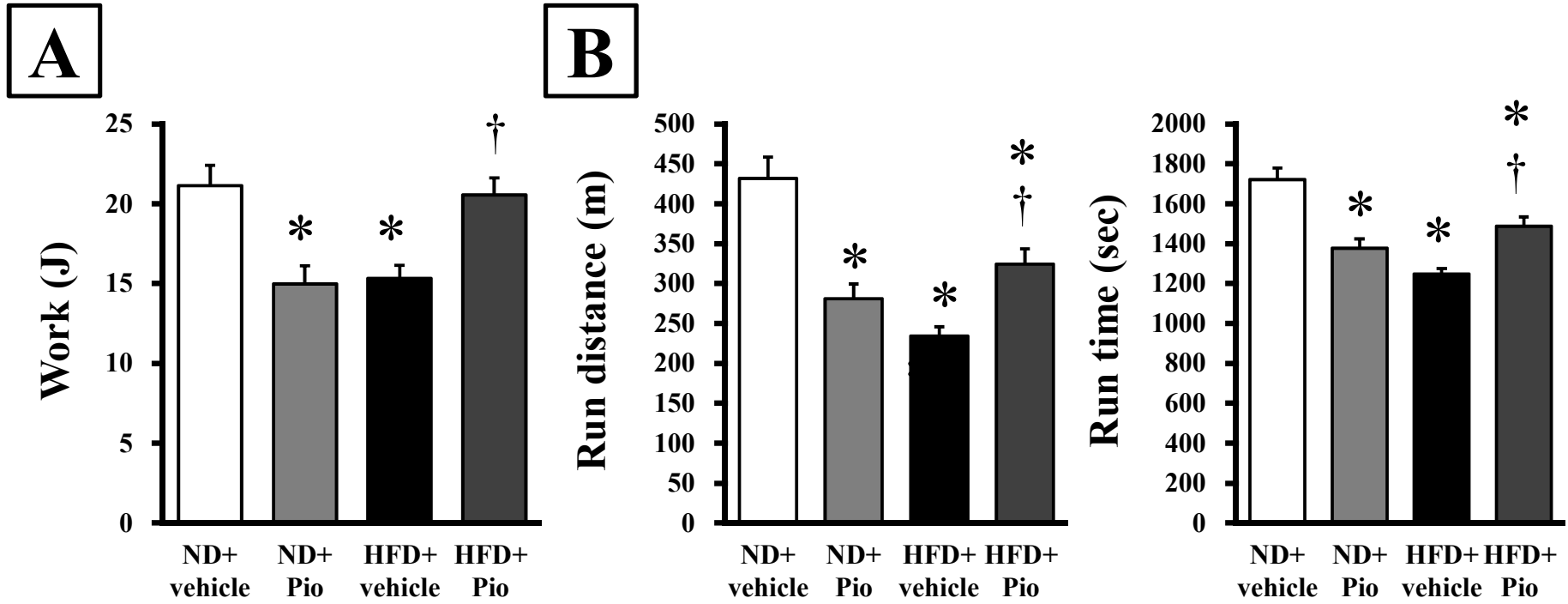
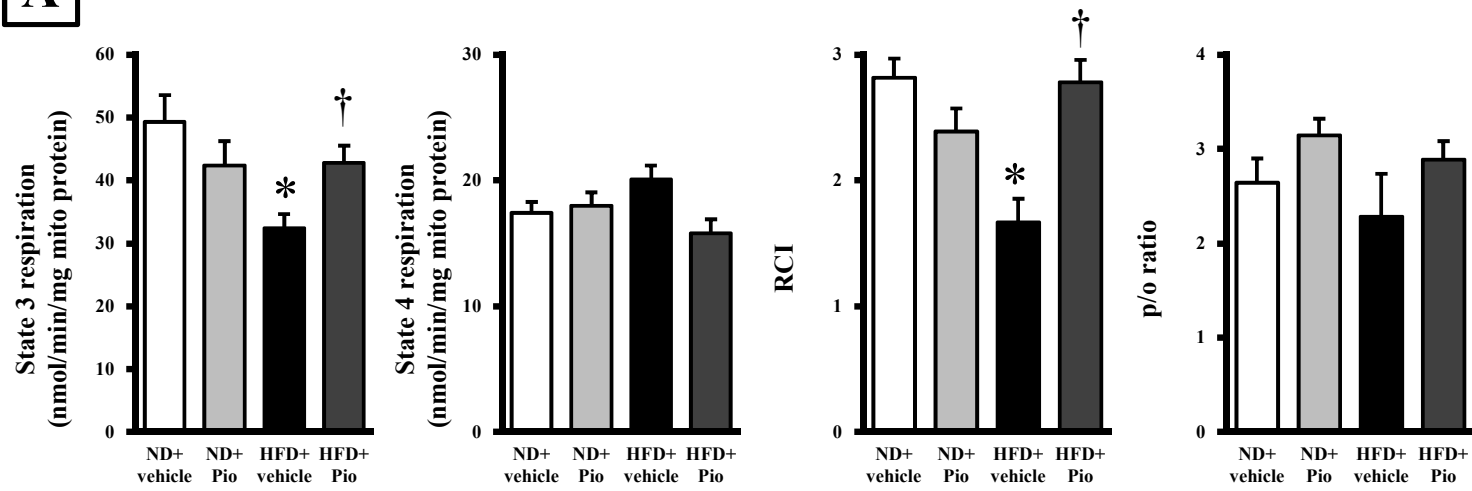
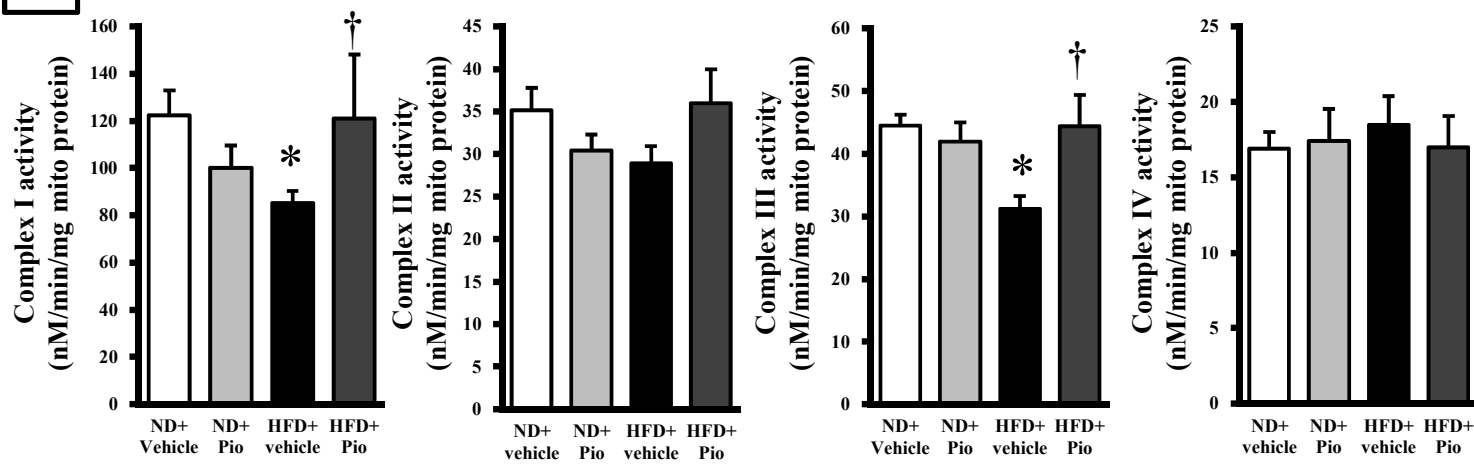
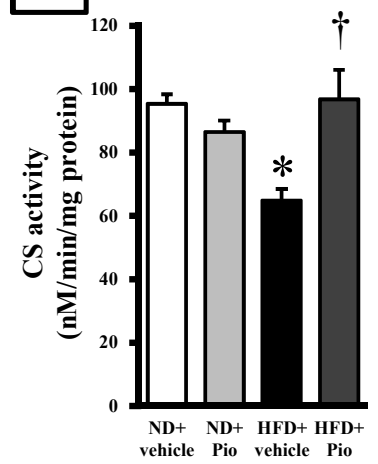


Figure 2

A**B****C****Figure 3**

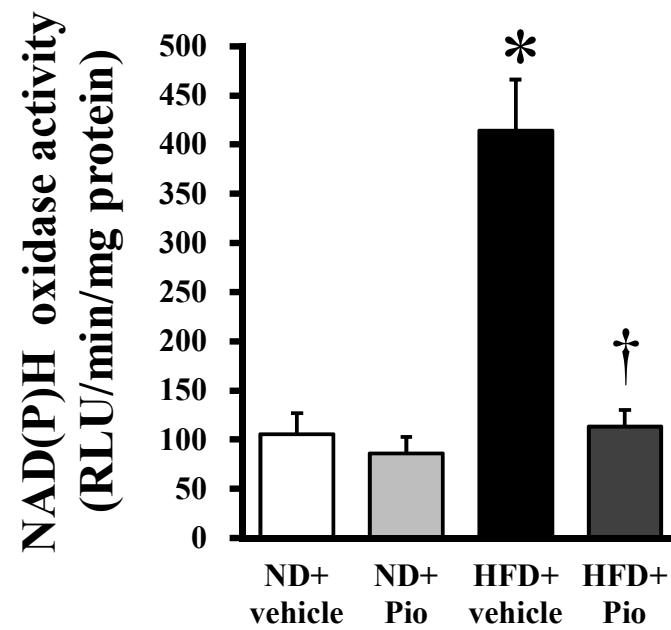


Figure 4

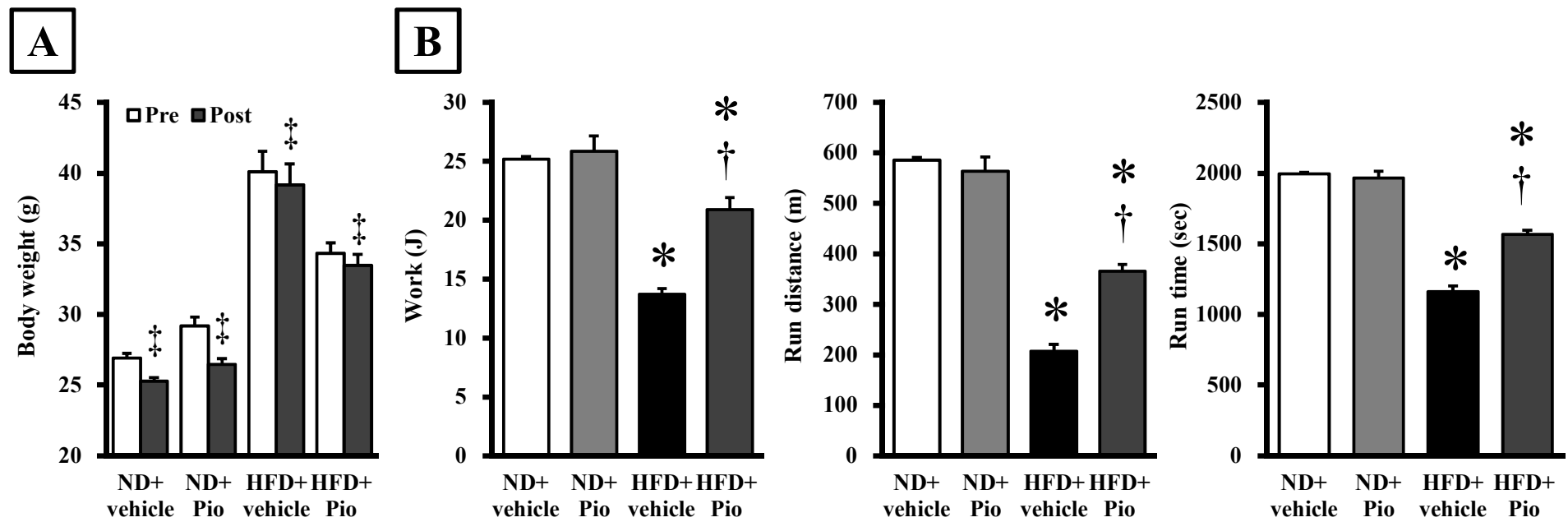


Figure 5