# 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).

77 Furthermore, the activation of NAD(P)H oxidase was due to activation of the
78 renin-angiotensin system (RAS) in the skeletal muscle, and angiotensin II type 1
79 receptor blocker (ARB) partially improved the limited exercise capacity (Takada et al.,
80 2013). The NAD(P)H oxidase-induced enhancement of oxidative stress was also
81 demonstrated in skeletal muscle from patients with type 2 diabetes (Roberts et al., 2006).
82 Therefore, RAS-dependent activation of NAD(P)H oxidase plays an important role in
83 the limited exercise capacity in HFD-induced diabetic mice.

84 NAD(P)H oxidase activity can be increased by high fatty acid levels and activation
85 of RAS, as well as by high glucose, insulin and insulin resistance (Yang and Kahn,
86 2006). NAD(P)H oxidase activity and the expression levels of the NAD(P)H subunit
87 have been shown to be activated in the skeletal muscle of insulin resistance-induced
88 diabetes mice (Bonnard et al., 2008; Takada et al., 2013; Yokota et al., 2009). Therefore,
89 insulin resistance may also play an important role in NAD(P)H oxidase and impaired
90 mitochondrial function, leading to the lowered exercise capacity in HFD-induced type 2
91 diabetic mice. We thus hypothesized that the insulin-sensitizing drug, pioglitazone (Pio),
92 could ameliorate the activated NAD(P)H oxidase and lowered exercise capacity in these
93 mice. The purpose of the present study was to determine whether the administration of
94 Pio to HFD-induced diabetic mice can ameliorate the impaired mitochondrial function
95 and the lowered exercise capacity.
96

## 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-amylene 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 $O_2$ 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 $O_2$ 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).

169

170 *2.7. NAD(P)H oxidase activity in skeletal muscle*

171 NAD(P)H oxidase activity was measured in the homogenates isolated from
172 hindlimb skeletal muscle by the lucigenin assay after the addition of NAD(P)H (300
173 μmol/L) as previously described (Suga et al., 2014; Takada et al., 2013; Yokota et al.,
174 2009).

175

176 *2.8. Administration of amiloride*

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

184

185 *2.9. Statistical analysis*

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

192

193 **3. Results**

194 *3.1. Animal characteristics*

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

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

215

216 *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 $O_2$ consumption in the skeletal muscle*

Exercise capacity is largely dependent on mitochondrial $O_2$ consumption, which is energy production, in the skeletal muscle. Therefore, mitochondrial $O_2$ 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 $O_2$ 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**).

241 CS activity was also significantly decreased in the HFD+vehicle compared with
242 ND+vehicle group, and this decrease was also inhibited by Pio (**Fig. 3C**).
243
244 *3.5. NAD(P)H oxidase activity in the skeletal muscle*
245 NAD(P)H oxidase activity was significantly increased in the skeletal muscle from
246 the HFD+vehicle compared with the ND+vehicle mice, and this change was completely
247 inhibited by Pio (**Fig. 4**).
248
249 *3.6. Effects of Pio-associated fluid retention on exercise capacity*
250 Pio significantly increased body weight, and decreased exercise capacity in ND
251 mice (**Table 1** and **Fig. 2**), which may have been due to Pio-associated fluid retention.
252 Body weight was significantly lower after amiloride treatment in all groups (**Fig. 5A**).
253 Similarly to the results in the group without amiloride treatment (**Fig. 2**), exercise
254 capacity was decreased in the HFD+vehicle compared with ND+vehicle mice, and the
255 decrease was ameliorated in HFD+Pio mice (**Fig. 5B**). Importantly, Pio did not affect
256 exercise capacity in ND mice under treatment with amiloride (**Fig. 5B**).
257
258

## 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

283 animals (de Las Heras et al., 2013; Escande et al., 2010; Lee et al., 2012; Safwat et al.,
284 2013; Zhang et al., 2010). Adiponetin (Iwabu et al., 2010; Lee et al., 2012; Lin et al.,
285 2013; Safwat et al., 2013) and sirtuin-1 (sirt-1) (de Las Heras et al., 2013; Escande et al.,
286 2010; Price et al., 2012) in particular have been widely investigated. Iwabu et al.
287 reported that adiponectin regulated exercise capacity by the increases in mitochondria
288 content and function in the skeletal muscle via the activation of AMP
289 kinase/sirt-1/peroxisome proliferator-activated receptor gamma (PPARγ) coactivator
290 1-alpha (Iwabu et al., 2010). It is well known that Pio binds to and activates the ligands
291 of PPARγ, which regulates adiponectin or sirt-1. (Kumagai et al., 2013; Lin et al., 2013).
292 Kumagai et al. reported that administration of Pio increased blood adiponectin levels in
293 KKAy diabetic mice (Kumagai et al., 2013). Furthermore, Dutchak et al. reported that
294 Rosiglitazone increased adiponectin via an increase of FGF-21 in adipocytes from
295 stromal vascular cells (Lin et al., 2013). Therefore, Pio might improve the lowered
296 exercise capacity and impaired mitochondrial function through an increase in
297 adiponectin. Sirt-1 is a key factor regulating mitochondrial function, but our previous
298 study showed that there was no difference between ND mice and HFD mice in the gene
299 expression of sirt-1 in skeletal muscle (Takada et al., 2013). Therefore, sirt-1 might not
300 have been associated with mitochondrial dysfunction in the HFD mice used in the
301 present study.

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

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

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

325 Second, the association between fatty acid metabolism and mitochondrial function in
326 HFD-induced diabetes is controversial. Our and other studies reported that HFD-feeding
327 induced insulin resistance and intramuscular lipid accumulation through the increase in
328 gene expression of lipid transportation and the decreases in the expression of
329 mitochondrial biogenesis related genes and CS activity (Chen et al., 2011; Suga et al.,
330 2014; Yokota et al., 2009). Therefore, mitochondria in HFD-fed mice would prefer the

331 utilization of free fatty acid to other substrates, which could decrease CS activity
332 through the enhanced oxidative stress (**Fig. 3**). In contrast, Turner et al. reported that
333 HFD-feeding mice had higher mitochondrial oxidative enzyme capacities including CS
334 activity than ND-feeding mice accompanied with an increase in blood level of free fatty
335 acid (Turner et al., 2007). Furthermore, the increase in free fatty acid induced the
336 increases in mitochondrial biogenesis and enzyme activities (Garcia-Roves et al., 2007;
337 Hancock et al., 2008). It has been also reported that HFD increases angiogenesis in the
338 skeletal muscle through the increase in fatty acid oxidation (Silvennoinen et al., 2013).
339 The discrepancy between our and these data would be due to the difference in blood
340 level of free fatty acid, which might be caused by the different HFD components (22.3%
341 saturated, 66.5% monounsaturated, and 10.4% polyunsaturated fatty acid profile vs.
342 31.4%, 35.5%, and 33.1%, respectively). Although we could not clearly explain this
343 issue, we think that the difference in the kind of feeding and the term of feeding may be
344 associated with the difference in the results.

345 The incidence of type 2 diabetes has been steadily increasing, creating both
346 medical and social challenges in industrialized countries. The lowered exercise capacity
347 in type 2 diabetes could lead to aggravation of the disease by limiting the applicability
348 of or compliance with exercise therapy. Our present data showed that pharmacological
349 treatment with Pio performed to attenuate insulin resistance improved exercise capacity.
350 Indeed, Regensteiner et al. reported that chronic administration of another
351 thiazolidinedione, rosiglitazone, in patients with type 2 diabetes improved their exercise
352 capacity (Regensteiner et al., 2005). In another experiment, the administration of Pio for
353 an additional 4 weeks was performed in mice that had been fed an HFD for 8 weeks, in
354 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 (Schernthaner 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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517 **Figure legends**

518

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

530

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

535

536 **Fig. 3.** (**A**) The summarized data of ADP-stimulated (state 3) respiration,
537 non-ADP-stimulated (state 4) respiration, respiratory control index (RCI) and the ratio
538 of ATP amount to consumed $O_2$ during state 3 (P/O) ratio in the isolated mitochondria in
539 glutamate and malate (n=6-7 for each group), (**B**) mitochondrial electron transport chain
540 (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 ± 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 ± 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 ± S. E. M. **P*<0.05 vs. ND; †*P*< 0.05 vs. HFD.

**Table 1. Animal Characteristics**

| | ND+ vehicle | ND+ Pio | HFD+ vehicle | HFD+ Pio |
|---|---|---|---|---|
| *Body weight and organ weight* | | | | |
| 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 |
| *Biochemical measurements* | | | | |
| 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.

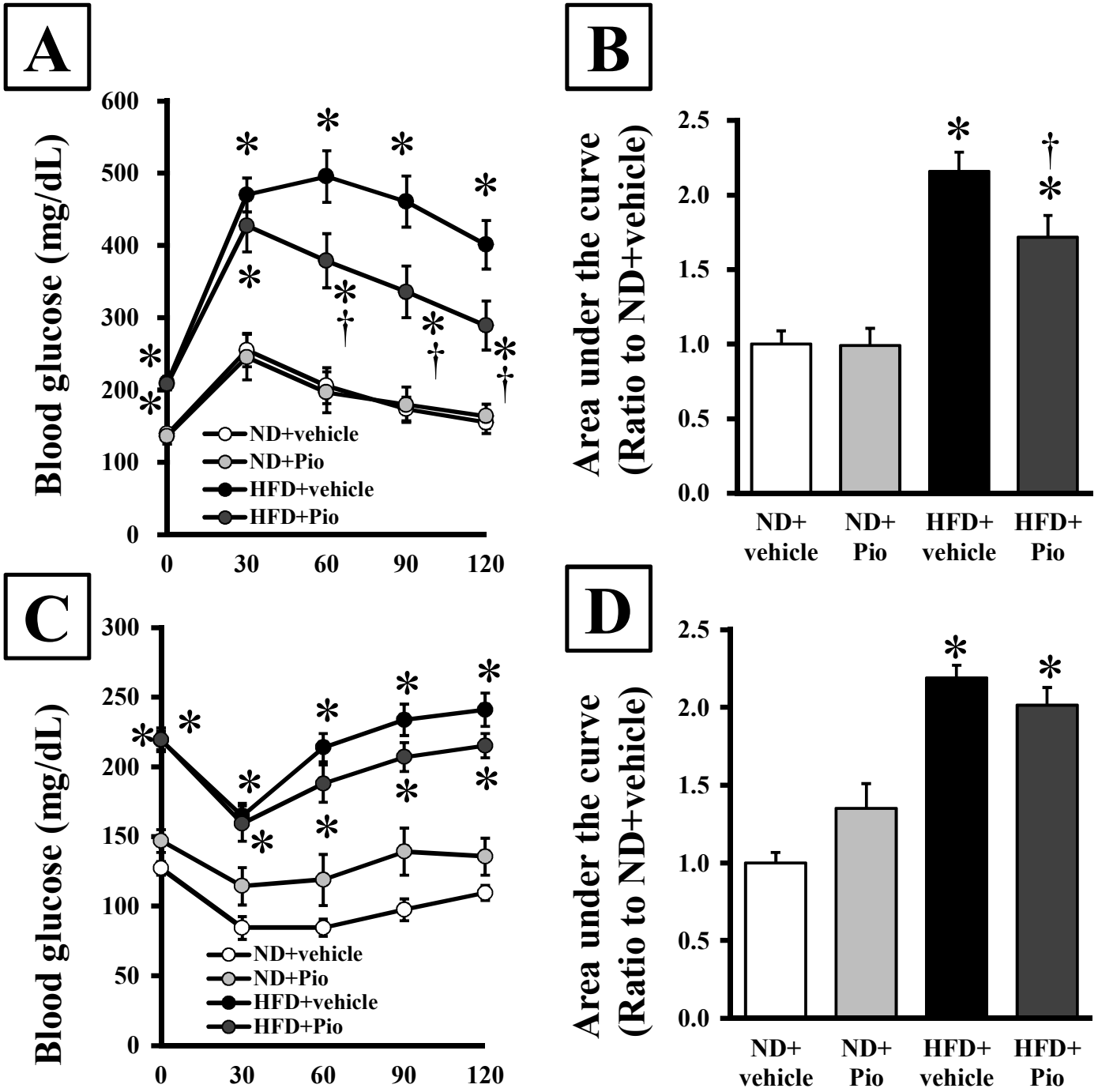
A
Blood glucose (mg/dL)
600
500
400
300
200
100
0
0
30
60
90
120
ND+vehicle
ND+Pio
HFD+vehicle
HFD+Pio
B
Area under the curve
(Ratio to ND+vehicle)
2.5
2.0
1.5
1.0
0.5
0.0
ND+ vehicle
ND+ Pio
HFD+ vehicle
HFD+ Pio
C
Blood glucose (mg/dL)
300
250
200
150
100
50
0
0
30
60
90
120
ND+vehicle
ND+Pio
HFD+vehicle
HFD+Pio
D
Area under the curve
(Ratio to ND+vehicle)
2.5
2.0
1.5
1.0
0.5
0.0
ND+ vehicle
ND+ Pio
HFD+ vehicle
HFD+ Pio

**Figure 1**

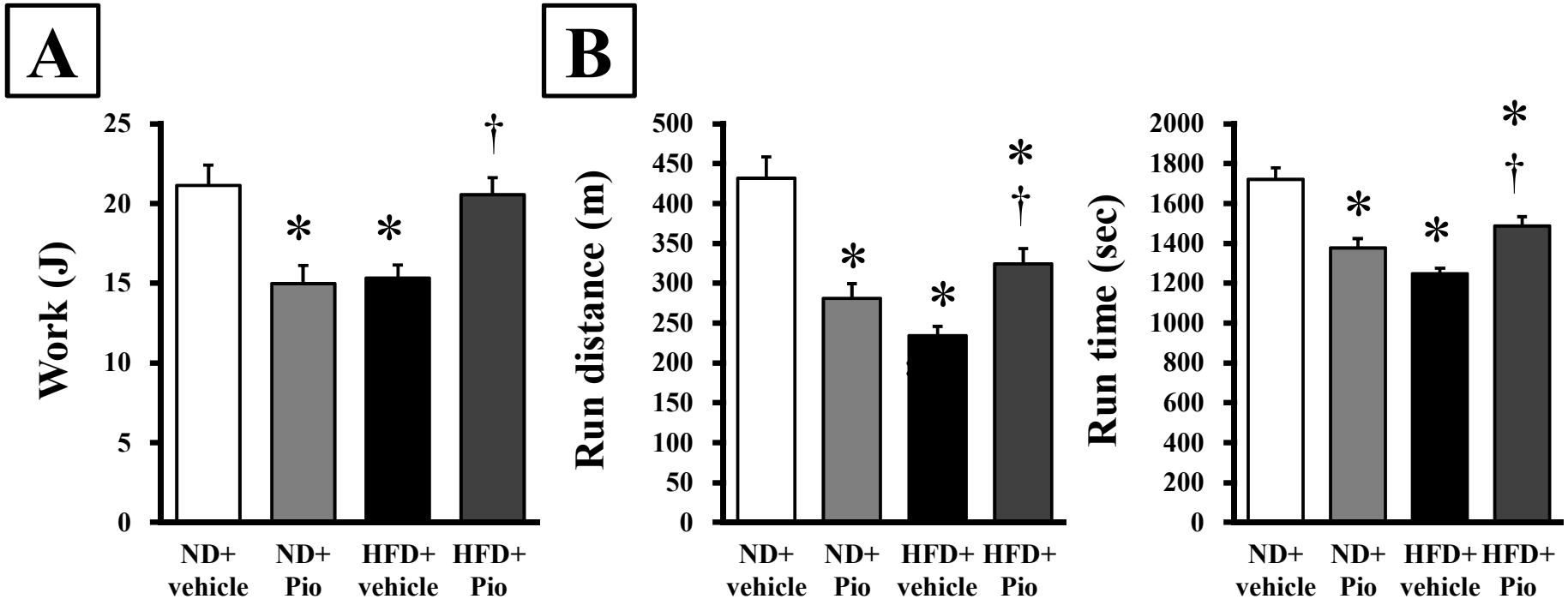
A
Work (J)
25
20
15
10
5
0
*
*
†
ND+ vehicle
ND+ Pio
HFD+ vehicle
HFD+ Pio
B
Run distance (m)
500
450
400
350
300
250
200
150
100
50
0
*
*
*
†
Run time (sec)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
*
†

**Figure 2**

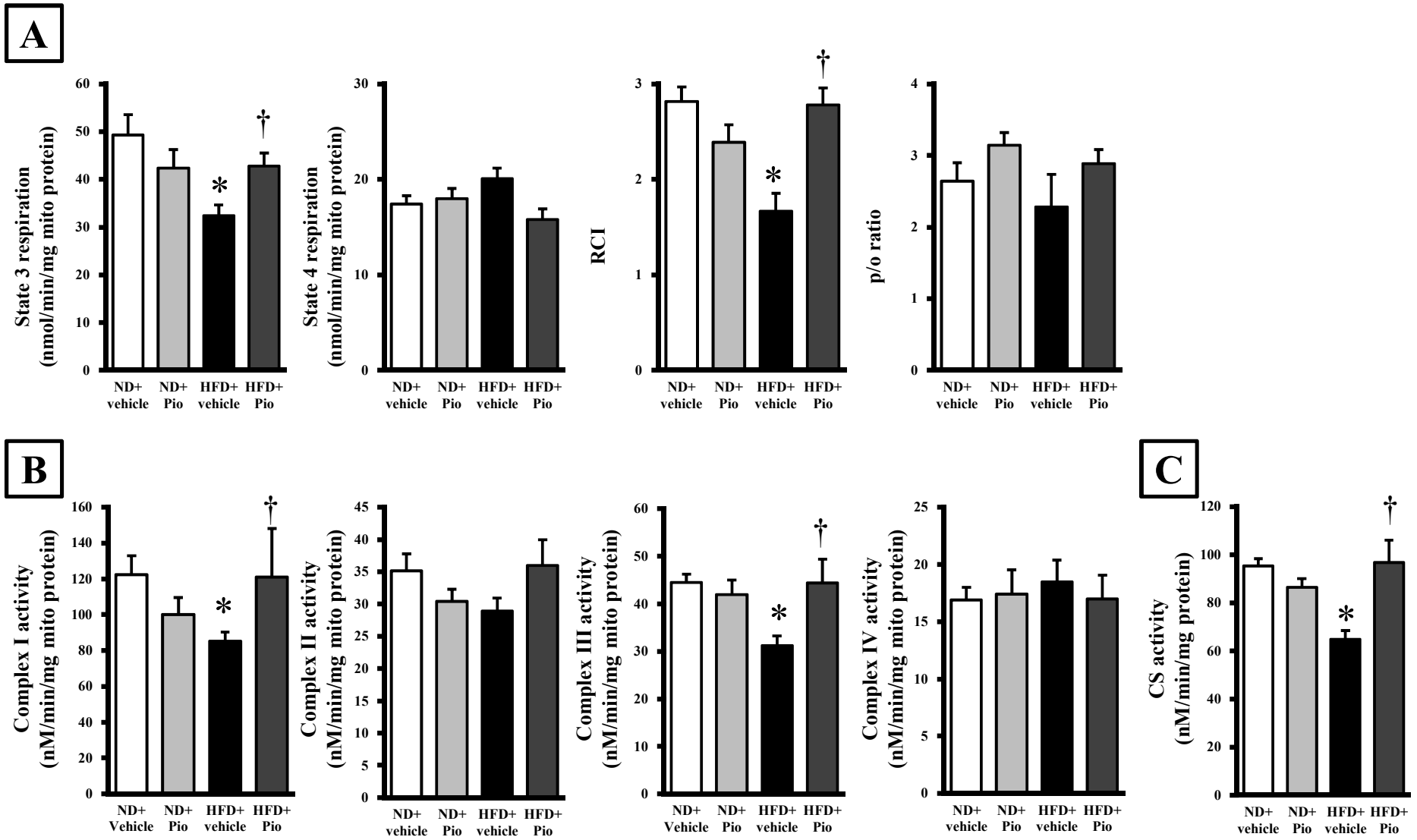
A
State 3 respiration (nmol/min/mg mito protein)
State 4 respiration (nmol/min/mg mito protein)
RCI
p/o ratio
ND+ vehicle
ND+ Pio
HFD+ vehicle
HFD+ Pio
B
Complex I activity (nM/min/mg mito protein)
Complex II activity (nM/min/mg mito protein)
Complex III activity (nM/min/mg mito protein)
Complex IV activity (nM/min/mg mito protein)
C
CS activity (nM/min/mg protein)
*
†

**Figure 3**

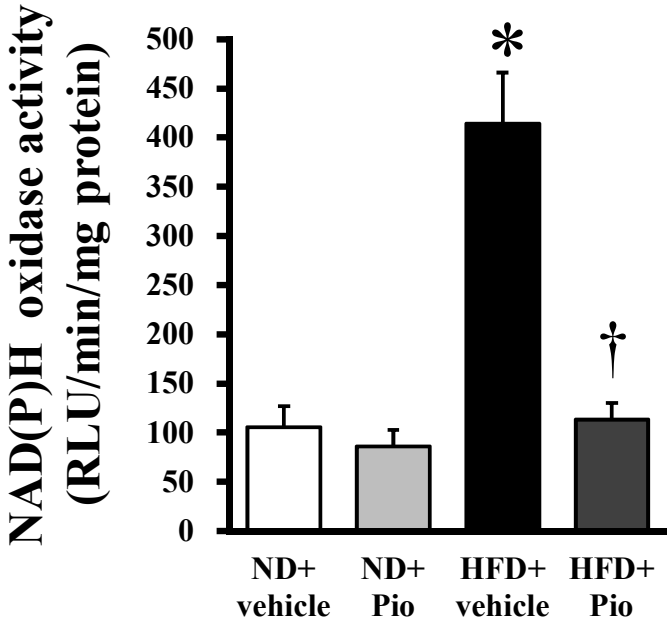
NAD(P)H oxidase activity
(RLU/min/mg protein)
500
450
400
350
300
250
200
150
100
50
0
*
†
ND+ vehicle
ND+ Pio
HFD+ vehicle
HFD+ Pio

**Figure 4**

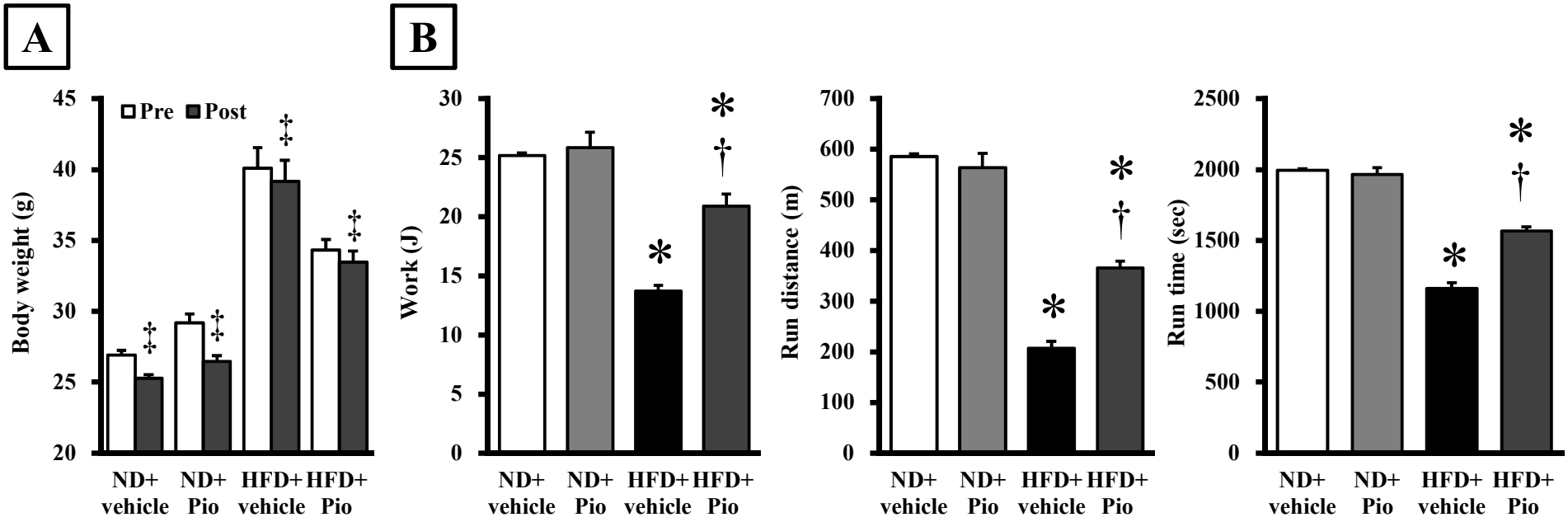
A
B
Pre
Post
Body weight (g)
45
40
35
30
25
20
‡
Work (J)
30
25
20
15
10
5
0
*
†
Run distance (m)
700
600
500
400
300
200
100
0
Run time (sec)
2500
2000
1500
1000
500
0
ND+ vehicle
ND+ Pio
HFD+ vehicle
HFD+ Pio

**Figure 5**