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Oxidative stress in skeletal muscle impairs mitochondrial respiration and limits exercise capacity in type 2 diabetic mice

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Yokota T, Kinugawa S, Hirabayashi K, Matsushima S, Inoue N, Ohta Y, Hamaguchi S, Sobirin MA, Ono T, Suga T, Kuroda S, Tanaka S, Terasaki F, Okita K, Tsutsui H. Oxidative stress in skeletal muscle impairs mitochondrial respiration and limits exercise capacity in type 2 diabetic mice. *Am J Physiol Heart Circ Physiol* 297: H1069–H1077, 2009. First published July 17, 2009; doi:10.1152/ajpheart.00267.2009.—Insulin resistance or diabetes is associated with limited exercise capacity, which can be caused by the abnormal energy metabolism in skeletal muscle. Oxidative stress is involved in mitochondrial dysfunction in diabetes. We hypothesized that increased oxidative stress could cause mitochondrial dysfunction in skeletal muscle and make contribution to exercise intolerance in diabetes. C57/BL6J mice were fed on normal diet or high fat diet (HFD) for 8 wk to induce obesity with insulin resistance and diabetes. Treadmill tests with expired gas analysis were performed to determine the exercise capacity and whole body oxygen uptake ($\dot{V}O_2$). The work (vertical distance × body weight) to exhaustion was reduced in the HFD mice by 36%, accompanied by a 16% decrease of peak $\dot{V}O_2$. Mitochondrial ADP-stimulated respiration, electron transport chain complex I and III activities, and mitochondrial content in skeletal muscle were decreased in the HFD mice. Furthermore, superoxide production and NAD(P)H oxidase activity in skeletal muscle were significantly increased in the HFD mice. Intriguingly, the treatment of HFD-fed mice with apocynin [10 mmol/l; an inhibitor of NAD(P)H oxidase activation] improved exercise intolerance and mitochondrial dysfunction in skeletal muscle without affecting glucose metabolism itself. The exercise capacity and mitochondrial function in skeletal muscle were impaired in type 2 diabetes, which might be due to enhanced oxidative stress. Therapies designed to regulate oxidative stress and maintain mitochondrial function could be beneficial to improve the exercise capacity in type 2 diabetes.

exercise intolerance; insulin resistance; mitochondrial function; oxygen uptake; superoxide

CLINICAL AND EXPERIMENTAL evidence has demonstrated that obesity leads to insulin resistance, which contributes to the enhanced risk of developing type 2 diabetes as well as atherosclerotic cardiovascular diseases (9). It has been shown that, in patients with type 2 diabetes, the exercise capacity is limited (29) and the lower exercise capacity is an independent and strong predictor for mortality (32). Exercise intolerance is generally believed to be due to the abnormalities in the energy metabolism in skeletal muscle (26). Moreover, mitochondrial fatty acid β-oxidation and mitochondrial respiration were impaired in skeletal muscle from patients with type 2 diabetes (23). These results raise the possibility that diabetes may adversely affect mitochondrial function in skeletal muscle and lead to exercise intolerance. However, it has been difficult to evaluate the impact of diabetes, per se, on the exercise capacity in patients because of concomitant diseases such as hypertension and atherosclerotic vascular diseases. To overcome these limitations in clinical studies, we employed the animal model of diabetes fed on high fat diet (HFD) in the present study (33).

The pathogenesis of skeletal muscle dysfunction in type 2 diabetes remains undefined. Systemic oxidative stress has been reported to be increased in type 2 diabetes (28). NAD(P)H oxidase, an important source of superoxide ($O_2^{\bullet -}$) generation, is also present in skeletal muscle (12) and can be activated by glucose, insulin, and fatty acids (35). Recently, $O_2^{\bullet -}$ generated via NAD(P)H oxidase has been demonstrated to cause mitochondrial dysfunction in vascular smooth muscle cells (6). Moreover, it has been reported that the mitochondrial function and structure were impaired in skeletal muscle from HFD-fed rodents with the altered oxidative stress markers (3, 11, 20). Therefore, increased oxidative stress in skeletal muscle may impair mitochondrial function and limit the exercise capacity in type 2 diabetes.

The purpose of this study is to determine *1*) whether the exercise capacity is limited in diabetic mice induced by HFD, *2*) whether mitochondrial respiration is impaired and oxidative stress is enhanced in skeletal muscle, and *3*) whether the inhibition of oxidative stress can ameliorate HFD-induced mitochondrial dysfunction in skeletal muscle and the limited exercise capacity.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice (8 to 12 wk of age) were housed in an animal room under controlled condition on a 12-h:12-h light/dark cycle. Mice were fed on either normal diet (ND; CE-2; CLEA Japan, Tokyo, Japan) containing 4.2% fat and 54.6% carbohydrate or HFD (HFD32; CLEA Japan) containing 32.0% fat and 29.4% carbohydrate for 8 wk. HFD-fed mice were further divided into two groups with or without 10 mmol/l acetovanillone (apocynin; Sigma-Aldrich, St. Louis, MO), an inhibitor of NAD(P)H oxidase activation, in drinking water. The concentration of apocynin in the present study was chosen on the basis of previous study (13). Thus the present study was performed in the following three groups of mice: *1*) ND (n = 10), *2*) HFD (n = 14), and *3*) HFD + apocynin (n = 14). 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

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care guideline for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine.

Eight weeks after treatment, exercise tests were performed. After that, blood samples were collected, all mice were euthanized, and organ weight was measured. Echocardiographic studies and the measurement of hemodynamics were performed in a subgroup of mice ($n = 4$ each). Intraperitoneal glucose tolerance tests were performed in another subgroup of mice ($n = 8$ each) to exclude the effects of repeated fasting on the exercise capacity and metabolic parameters. Because the amount of skeletal muscle samples was limited, they were divided into the experiments for mitochondrial oxygen consumption ($n = 9$ or 10), complex activity ($n = 4$ or 5), electron microscopy ($n = 3$), the histological analysis ($n = 4$), and those for the biochemical assay including O_2^- production ($n = 5$) and NAD(P)H oxidase activity ($n = 4$).

Biochemical measurements and organ weight. After animals fasted for 6 h, blood samples were collected from inferior vena cava before euthanization under anesthesia with tribromoethanol-amylene hydrate (Avertin; 2.5% wt/vol, 10 μl/g ip). Plasma insulin levels were measured by ELISA kit (Morinaga Institute of Biochemical Science, Yokohama, Japan). Total cholesterol, triglyceride, and nonesterified fatty acid (NEFA) were measured by using enzymatic assays using Cholesterol E-test, Triglyceride E-test, and NEFA C-test, respectively (Wako Pure Chemical Industries, Osaka, Japan). Epididymal fat, liver, and unilateral hindlimb skeletal muscle were then excised and weighed.

Intraperitoneal glucose tolerance test. For glucose tolerance test, mice were fasted for 6 h and were given an intraperitoneal injection of glucose (1 mg/g) in purified water. Blood samples were drawn from the tail vein before and 15, 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).

Echocardiographic and hemodynamic measurements. Echocardiographic and hemodynamic measurements were performed under light anesthesia with avertin intraperitoneally and spontaneous respiration as previously described (21).

Treadmill testing and whole body exercise capacity. Mice were treadmill tested to measure indexes defining whole body exercise capacity as previously described with minor modifications (17). At the time of treadmill testing, each mouse was placed on a treadmill enclosed by a metabolic chamber through which air flow of constant speed (1 l/min) is passing (Oxymax 2; Columbus Instruments, Columbus, OH). Oxygen (O_2) and carbon dioxide (CO_2) gas fractions were monitored at both the inlet and output ports of the metabolic chamber. Basal measurements were obtained over a period of 10 min. Mice were then provided with a 10-min warm-up period at 6 m/min at zero degree. After animals warmed up, the angle was fixed at 10 degrees and the speed was incrementally increased by 2 m/min every 2 min until the mouse reached exhaustion. Exhaustion was defined as spending time (10 s) on the shocker plate without attempting to reengage the treadmill. Whole body oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were automatically calculated every 10 s by taking the difference between the inlet and output gas flow. Respiratory exchange ratio (RER) was calculated as $\dot{V}CO_2$ to $\dot{V}O_2$. The work was defined as the product of the vertical running distance to exhaustion and body weight.

Mitochondrial O_2 consumption in skeletal muscle. Hindlimb skeletal muscle tissues were quickly harvested, and mitochondria were isolated as previously described with minor modifications (19). Before the measurement of O_2 consumption, the isolated mitochondrial protein concentration was measured by the BCA Protein Assay (Pierce, Rockford, IL). O_2 consumption by the isolated mitochondria was measured polarographically using an oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) in a closed and magnetically stirred glass chamber at 28°C, as previously described (19).

After a 1-min equilibration period, mitochondrial respiration was initiated by the addition of 2.5 mmol/l glutamate and malate as substrates. ADP-stimulated (state 3) respiration was determined after adding ADP (40 μmol/l). Non-ADP-stimulated (state 4) respiration was measured in the absence of ADP phosphorylation and validated by oligomycin (2 mg/l), an ATPase inhibitor. 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 ATP amount to consumed O_2 during state 3. Therefore, RCI indicates overall mitochondrial respiratory activity and P/O ratio indicates efficiency of ATP synthesis.

Mitochondrial complex activity in skeletal muscle. The specific enzymatic activity 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 (31).

Histology and transmission electron microscopy. Skeletal muscle was excised and fixed in 4% paraformaldehyde and embedded in paraffin for histological analysis. Skeletal muscle samples were cut in 5-μm thick sections and stained using Masson's trichrome. Morphological analysis of muscle fiber cross-sectional area was performed at least in 100 cells from each mouse.

Hindlimb skeletal muscle (tibialis anterior muscle) was fixed in 3% glutaraldehyde with 0.1 mmol/l phosphate buffer (pH 7.2) for 3 h at 4°C and postfixed in 1% osmium tetroxide with 0.1 mmol/l phosphate buffer (pH 7.2) for 90 min at 4°C and then serially dehydrated in ethanol and embedded in epoxy resin. Sections were cut on an LKB ultramicrotome and consecutive ultrathin sections were mounted on copper grids. Ultrathin sections were stained with 3% uranyl acetate and 0.2% lead citrate. Examinations were conducted with an electron microscope (H-7100; Hitachi, Tokyo, Japan). Quantification of areas of subsarcolemmal and intermyofibrillar mitochondria was performed as previously described (3).

$O_2^{\bullet-}$ production and NAD(P)H oxidase activity in skeletal muscle. The chemiluminescence elicited by $O_2^{\bullet-}$ in the presence of lucigenin (5 μmol/l) was measured in hindlimb skeletal muscle using a luminometer (AccuFLEX Lumi 400; Aloka, Tokyo, Japan) as previously described with some modifications (2). To validate that the chemiluminescence signals were derived from $O_2^{\bullet-}$, the measurements were also performed in the presence of tiron (20 mmol/l), a cell-permeant, nonenzymatic scavenger of $O_2^{\bullet-}$.

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 μmol/l) as previously described with some modifications (1).

Statistical analysis. Data are expressed as means ± SE. Between-group comparison of means was performed by one-way ANOVA followed by *t*-tests. The Bonferroni correction was applied for multiple comparisons of means. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Animal characteristics. Table 1 shows animal characteristics in each group. Body weight was significantly increased in the HFD mice compared with the ND mice, which was accompanied by the significant increase in the epididymal fat, liver, and skeletal muscle weight. Fasting blood glucose and plasma insulin levels were significantly increased in the HFD mice. Triglyceride was also significantly increased in the HFD mice, but total cholesterol and NEFA were comparable between the ND and the HFD mice. Blood glucose levels during an intraperitoneal glucose tolerance test were significantly increased in the HFD mice compared with the ND mice (Fig. 1). These results showed that HFD feeding for 8 wk induced type 2 diabetes with characteristics of obesity and insulin resistance.

Table 1. *Animal characteristics*

	ND	HFD	HFD + apocynin
n	10	14	14
Body weight, g	31±1	39±1*	39±1*
Organ weight			
Epididymal fat, mg	399±48	2,035±54*	1,916±82*
Liver, mg	1,193±88	1,497±90*	1,415±65*
Skeletal muscle, mg	909±29	1,082±31*	1,057±39*
Biochemical measurements			
Blood glucose, mg/dl	108±7	207±16*	180±16*
Insulin, ng/ml	0.28±0.05	1.11±0.33*	1.22±0.37*
Total cholesterol, mg/dl	55±3	74±12	78±11
Triglyceride, mg/dl	69±5	125±16*	119±18*
NEFA, mEq/l	0.37±0.05	0.47±0.05	0.50±0.08

Values are means ± SE. ND, normal diet; HFD, high fat diet; NEFA, nonesterified fatty acid. *$P < 0.05$ vs. ND.

Table 2. *Echocardiographic and hemodynamic data*

	ND	HFD	HFD + apocynin
n	4	4	4
Echocardiographic data			
Heart rate, beats/min	481±12	468±24	494±15
LV end-diastolic diameter, mm	3.5±0.1	3.3±0.1	3.5±0.1
Fractional shortening, %	33.5±1.1	37.5±3.0	33.5±1.8
IVS thickness, mm	0.70±0.03	0.75±0.06	0.77±0.04
LV posterial wall thickness, mm	0.72±0.00	0.81±0.03	0.80±0.05
Hemodynamic data			
Heart rate, beats/min	473±15	462±18	462±18
Systolic aortic pressure, mmHg	95±7	99±4	99±2
Diastolic aortic pressure, mmHg	67±4	67±3	66±2
LVEDP, mmHg	1.4±0.8	2.2±0.6	1.8±0.5
LV dP/dt_{max}, mmHg/s	10,144±824	9,164±1,470	8,690±805
LV dP/dt_{min}, mmHg/s	6,634±285	6,728±626	7,427±596
τ, ms	8.0±0.9	7.5±0.6	8.4±0.6

Values are means ± SE. LV, left ventricular; IVS, interventricular septum; LVEDP, LV end-diastolic pressure; dP/dt, change in pressure over time; τ, time constant needed for relaxation of 50% maximal LV pressure to baseline value.

Importantly, the HFD + apocynin mice showed no significant differences from the HFD mice in these parameters (Table 1 and Fig. 1), indicating that apocynin did not affect diabetic status.

Cardiac function. The echocardiographic and hemodynamic data are shown in Table 2. There were no significant differences in all parameters among the three groups.

Whole body exercise capacity. Figure 2*A* shows a representative plot of $\dot{V}O_2$ corresponding to each workload during exercise in an individual ND, HFD, and HFD + apocynin mouse. The work to exhaustion was decreased in the HFD compared with the ND mouse, which was accompanied by a decrease of peak $\dot{V}O_2$. These changes were ameliorated in the HFD + apocynin mouse. The running distance (Fig. 2*B*) and work (Fig. 2*C*) to exhaustion were significantly decreased in the HFD compared with the ND and were significantly improved in the HFD + apocynin mice. Coincident with the limited exercise capacity, peak $\dot{V}O_2$ and $\dot{V}CO_2$ corrected by body weight were significantly decreased in the HFD compared with the ND, and this decrease was ameliorated in the HFD + apocynin mice (Table 3). Peak RER values were greater than 1 and did not differ among the three groups (Table 3), indicating that treadmill tests were performed until exhaustion beyond anaerobic threshold in all groups of mice. Even after peak $\dot{V}O_2$ was corrected by skeletal muscle weight to exclude the influence of the differences in body composition, it

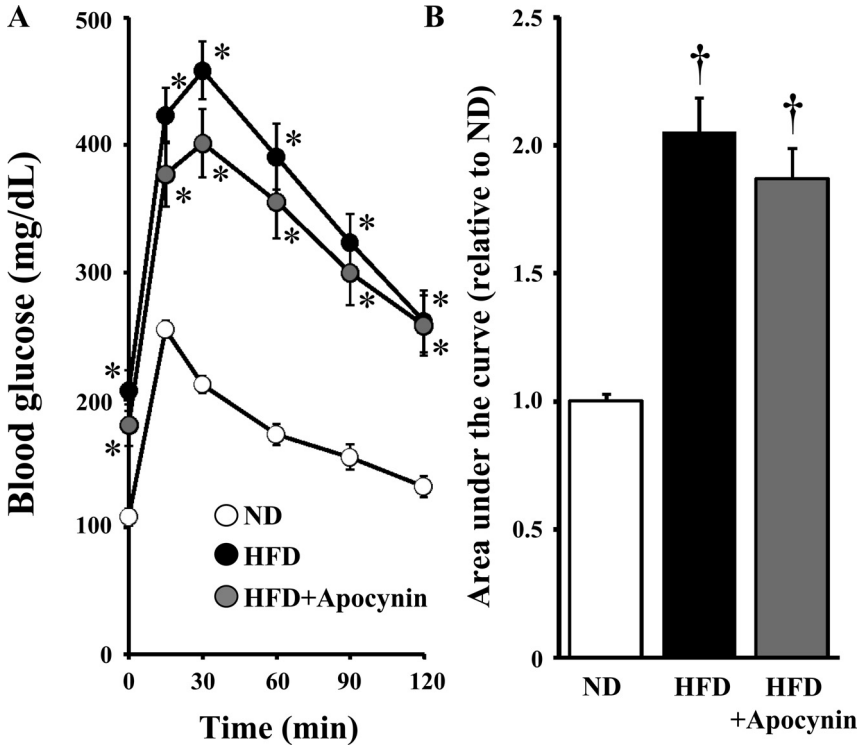

Fig. 1. *A*: blood glucose levels during intraperitoneal glucose tolerance test in the normal diet (ND; ○; $n = 8$), high fat diet (HFD; ●; $n = 8$), and HFD + apocynin (gray circles; $n = 8$) mice. *B*: area under the curve of blood glucose levels during intraperitoneal glucose tolerance test in the ND (white column), HFD (black column), and HFD + apocynin (gray column) mice. Data are expressed as means ± SE. Experiments were performed after 8 wk of feeding in all groups. *$P < 0.05$ vs. ND at each time point; †$P < 0.05$ vs. ND.

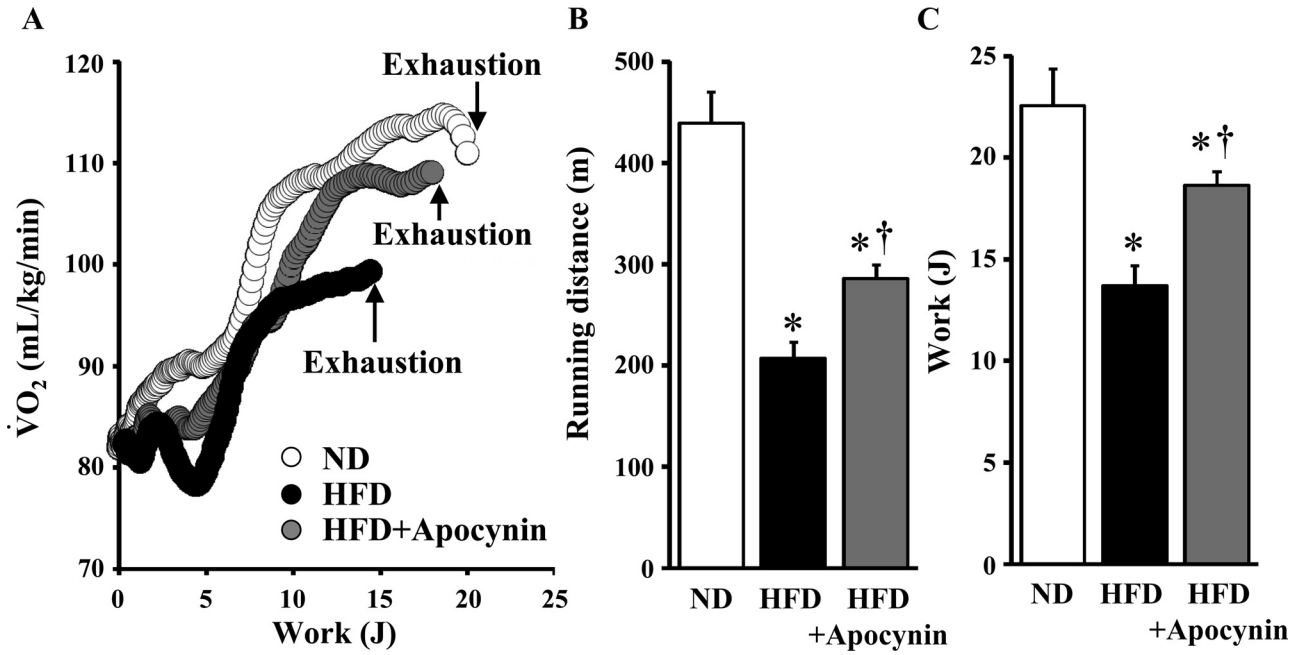

Fig. 2. *A*: representative graphs plotted for oxygen uptake ($\dot{V}O_2$) at each workload during exercise in the ND (○), HFD (●), and HFD + apocynin (gray circles) mouse. The summarized data of the running distance (*B*) and work (*C*) to exhaustion in the ND ($n = 10$), HFD ($n = 14$), and HFD + apocynin ($n = 14$) mice are shown. Data are expressed as means ± SE. Experiments were performed after 8 wk of feeding in all groups. *$P < 0.05$ vs. ND; †$P < 0.05$ vs. HFD.

was significantly decreased in the HFD compared with the ND and was significantly improved in the HFD + apocynin mice (Table 3).

Mitochondrial O_2 consumption in skeletal muscle. Figure 3*A* shows representative recordings of O_2 consumption in the mitochondria isolated from skeletal muscle measured by the oxygen electrode. State 3 respiration was decreased in mitochondria from the HFD mouse compared with the ND mouse and improved in mitochondria from the HFD + apocynin mouse. There was no difference in state 4. Summarized data demonstrated that state 3 respiration was significantly decreased in the HFD compared with the ND mice (Fig. 3*B*) without any changes in state 4 respiration (Fig. 3*C*). RCI was significantly decreased in the HFD compared with the ND mice (Fig. 3*D*). P/O ratio did not differ between groups (Fig. 3*E*). The HFD + apocynin had significantly improved state 3 respiration and RCI compared with the HFD mice.

Mitochondrial complex activity in skeletal muscle. Coincident with impaired mitochondrial respiratory activity in the HFD mice, mitochondrial ETC complex I and III activities were significantly decreased in the HFD mice compared with the ND mice, which was ameliorated by apocynin (Fig. 4, *A* and *C*). There were no significant differences in complex II and IV activities among the three groups (Fig. 4, *B* and *D*).

Fiber cross-sectional area and mitochondrial content in skeletal muscle. Histological analysis revealed that there was no difference in muscle fiber cross-sectional area among the ND, HFD, and HFD + apocynin mice (2,807 ± 281 vs. 2,689 ± 208 vs. 2,913 ± 167 μm^2; P = not significant). Areas of subsarcolemmal and intermyofibrillar mitochondria in skeletal muscle were significantly decreased in the HFD compared with the ND mice (Fig. 5). Chronic administration of apocynin significantly ameliorated the decrease in the areas of mitochondria in the HFD mice. The ultrastructure of mitochondrial morphology, such as cristae and matrix, was not damaged in the HFD mice.

$O_2^{\bullet -}$ production and NAD(P)H oxidase activity in skeletal muscle. $O_2^{\bullet -}$ production and NAD(P)H oxidase activity measured by lucigenin chemiluminescence were significantly increased in skeletal muscle from the HFD compared with the ND mice, and their changes were inhibited by apocynin (Fig. 6).

Table 3. *Expired gas analysis*

	ND	HFD	HFD + apocynin
n	10	14	14
Peak $\dot{V}O_2$/body weight, ml·kg^{-1}·min^{-1}	114±4	96±2*	106±3*†
Peak $\dot{V}O_2$/skeletal muscle weight, ml·kg^{-1}·min^{-1}	3,851±123	3,451±10*	3,880±104†
Peak $\dot{V}CO2$/body weight, ml·kg^{-1}·min^{-1}	120±4	99±2*	106±3†
Peak RER	1.07±0.02	1.05±0.02	1.02±0.03

Values are means ± SE. RER, respiratory exchange ratio. *$P < 0.05$ vs. ND; †$P < 0.05$ vs. HFD.

DISCUSSION

The major findings of the present study were that the exercise capacity was limited in the diabetic mice induced by HFD feeding for 8 wk. This limitation of maximal exercise capacity was accompanied by a decrease of peak $\dot{V}O_2$. Coincident with these alterations, mitochondrial respiratory activity, ETC complex I and III activities were decreased in skeletal muscle from the HFD mice. Moreover, skeletal muscle mitochondrial content was decreased in the HFD mice. Importantly, chronic administration of apocynin into the HFD mice significantly ameliorated the limited exercise capacity as well as

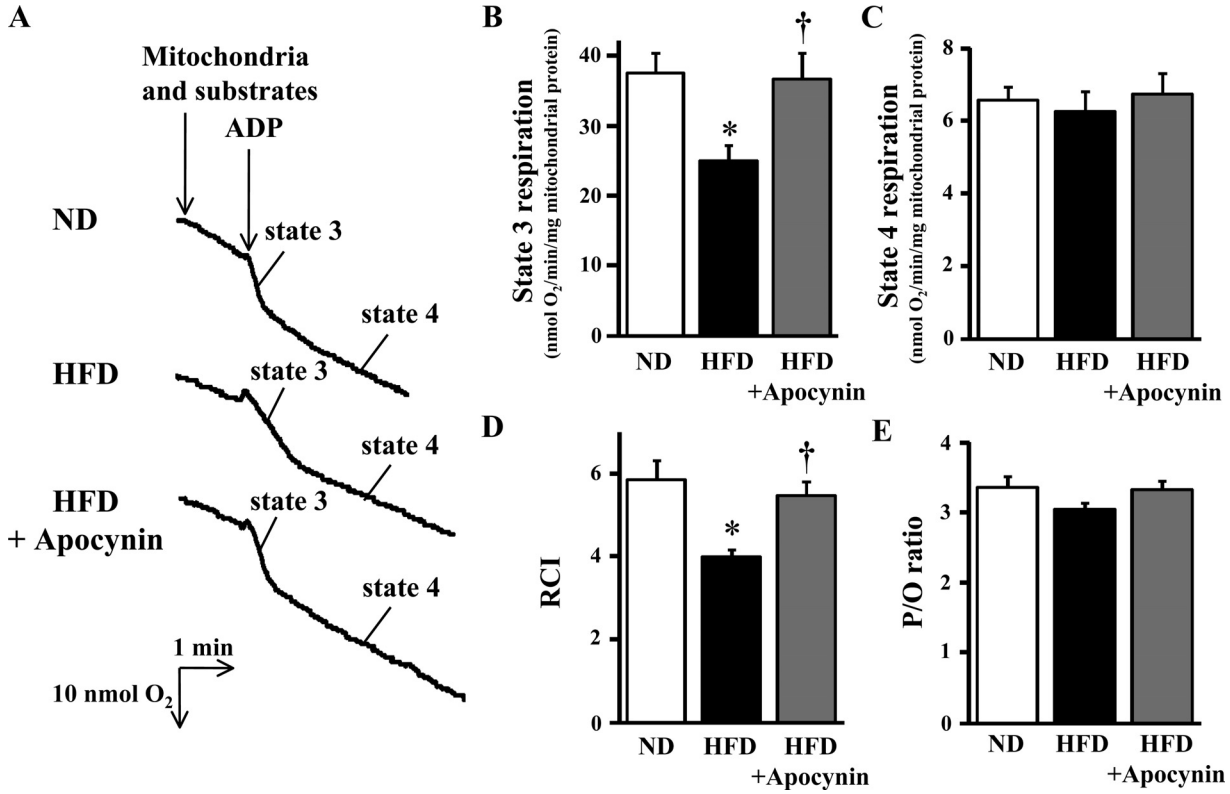

Fig. 3. *A*: representative recordings of ADP-stimulated (state 3) and non-ADP-stimulated (state 4) O_2 consumption in the mitochondria isolated from skeletal muscle in the presence of substrates. The summarized data of state 3 respiration (*B*), state 4 respiration (*C*), respiratory control index (RCI; *D*), and the ratio of ATP amount to consumed O_2 during state 3 (P/O) ratio (*E*) in 3 groups of mice ($n = 9$ to 10 for each group) are shown. Data are expressed as means ± SE. Experiments were performed after 8 wk of feeding in all groups. *$P < 0.05$ vs. ND; †$P < 0.05$ vs. HFD.

mitochondrial dysfunction in skeletal muscle without affecting glucose metabolism or body fat. Therefore, increased oxidative stress may cause mitochondrial dysfunction in skeletal muscle and contribute to exercise intolerance in diabetes.

The exercise capacity was reduced in the mice fed on HFD for 8 wk. This finding was supported by the decreased running distance and work to exhaustion (Fig. 2), which was associated with lowered peak $\dot{V}O_2$ (Table 3). Peak $\dot{V}O_2$ was significantly lowered in the HFD mice even after the correction of its value by skeletal muscle weight to exclude the influence of the increased body weight in these mice (Table 3). The methods to evaluate the exercise capacity of rodents using treadmill tests and expired gas analysis in mixing chamber were well validated by the previous study (17). The treadmill exercise protocol used in the present study was designed so that the mice could quickly attain a plateau, reaching their maximal $\dot{V}O_2$ before exhaustion, which has been confirmed by a linear increase of $\dot{V}O_2$ corresponding to each workload during exercise in all three groups of mice (Fig. 2*A*). Importantly, the peak RER at the endpoint of exercise judged by exhaustion were above 1 in all groups and did not differ among groups (Table 3), indicating that the measurement of the exercise capacity and peak $\dot{V}O_2$ was valid in our studied mice despite the differences in body weight.

HFD induced obesity, associated with insulin resistance and type 2 diabetes in the present study (Table 1 and Fig. 1). Our data of the reduced exercise capacity and depressed peak $\dot{V}O_2$ in the HFD-fed mice are consistent with previous studies in patients with diabetes (29). However, clinical studies in patients with diabetes could not completely exclude the influence of concomitant hypertension or atherosclerotic cardiovascular diseases. The present study has overcome these limitations in clinical studies by using the animal model of diabetes fed by HFD and clearly demonstrated that diabetes, per se, could limit the exercise capacity.

Based on the fact that $\dot{V}O_2$ during exercise reflects O_2 consumption in skeletal muscle (18), alterations in skeletal muscle are to a large extent responsible for the limited exercise capacity in the HFD mice. The abnormal energy metabolism in skeletal muscle is well expected to limit the exercise capacity. The present study demonstrated mitochondrial state 3 respiration and ETC complex activities were reduced (Figs. 3 and 4). These findings are supported by the previous studies in patients with insulin resistance or type 2 diabetes, in which mitochondrial ATP production and state 3 respiration were decreased in skeletal muscle (23, 27). Moreover, mitochondrial oxidative phosphorylation genes including ubiquinol cytochrome *c* reductase binding protein, a subunit of complex III, were downregulated in skeletal muscle from patients with diabetes, and their expression levels were positively correlated with peak $\dot{V}O_2$ (24). Accordingly, the metabolic abnormalities in the mitochondria from skeletal muscle can well explain a decrease in the exercise capacity and peak $\dot{V}O_2$ in the diabetic mice (Fig. 2 and Table 3).

The present study demonstrated that oxidative stress was enhanced in skeletal muscle from the mice model of type 2 diabetes (Fig. 6), which has been confirmed also in patients with type 2 diabetes (28) and also in an animal model (34).

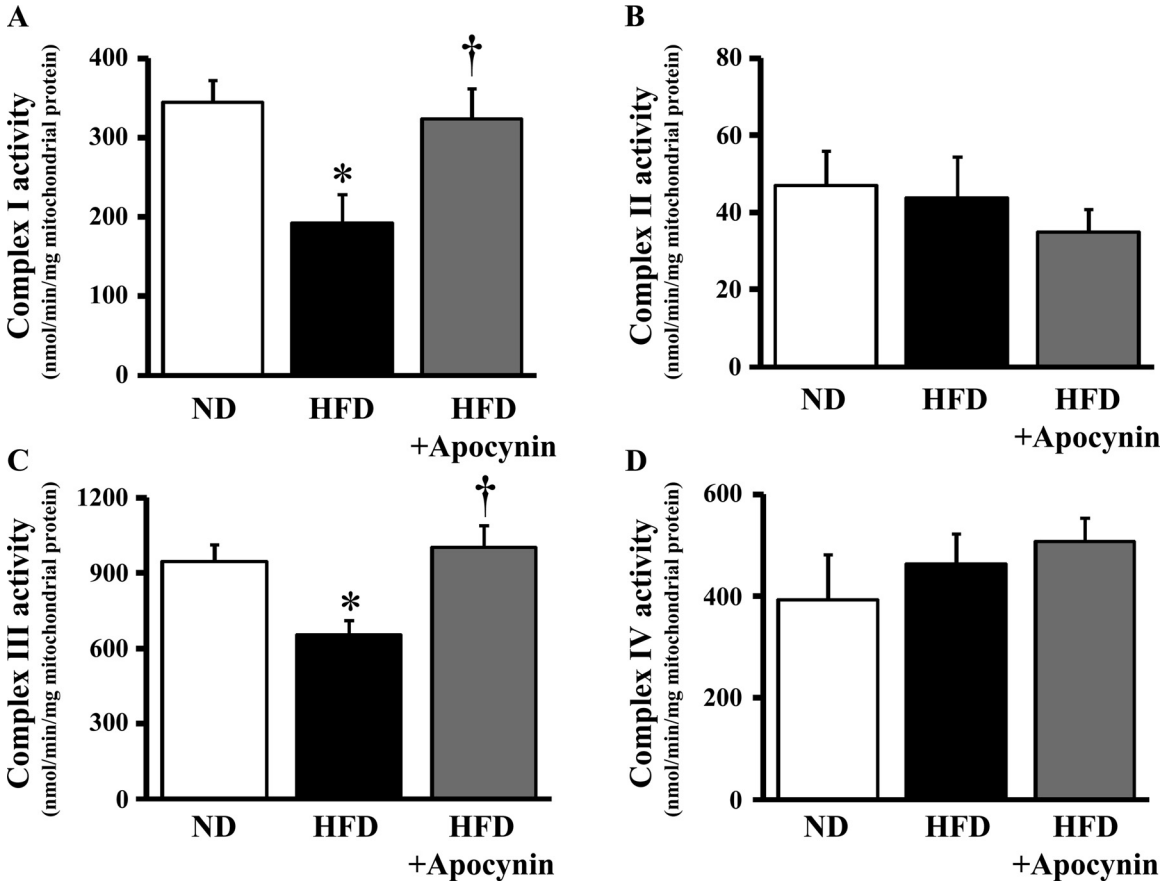

Fig. 4. Mitochondrial electron transport chain complex I (*A*), II (*B*), III (*C*), and IV (*D*) emzymatic activities in skeletal muscle from 3 groups of mice ($n = 4$ to 5 for each). Data are expressed as means ± SE. Experiments were performed after 8 wk of feeding in all groups. *$P < 0.05$ vs. ND; †$P < 0.05$ vs. HFD.

Moreover, oxidative stress in type 2 diabetes has been shown to be attributable to $O_2^{\bullet -}$ derived from NAD(P)H oxidase (30). The various stimulations, such as high glucose, insulin, and free fatty acids, have been demonstrated to activate NAD(P)H oxidase in vitro (35). Therefore, the increased blood glucose and plasma insulin may activate NAD(P)H oxidase in skeletal muscle from the HFD mice.

The most significant finding of the present study is that chronic administration of apocynin into HFD-fed mice improved exercise intolerance (Fig. 2 and Table 3) and ameliorated mitochondrial dysfunction as well as reduced mitochondrial content in skeletal muscle (Figs. 3–5). In contrast, apocynin did not affect the exercise capacity in the ND-fed mice (data not shown). These results suggested that enhanced oxidative stress via NAD(P)H oxidase activation in the skeletal muscle might be involved in exercise intolerance in diabetes. They are in agreement with the previous study from our laboratory that the exercise capacity was reduced in conditions in which $O_2^{\bullet -}$ was increased in heterozygous manganese superoxide dismutase gene knockout mice (17). The exercise capacity has been reported to be decreased also in rats fed vitamin E-deficient diets (7). On the other hand, apocynin did not affect fasting blood glucose, insulin, NEFA levels, and blood glucose levels during an intraperitoneal glucose tolerance test in the HFD-fed mice despite normalization of mitochondrial function in skeletal muscle (Fig. 1 and Table 1). These results are in agreement with the previous study by Bonnard et al. (3) that mitochondrial dysfunction did not precede insulin resistance in diet-induced diabetic mice. However, they are not consistent with previous human and animal studies that mitochondrial dysfunction in skeletal muscle is involved in the pathogenesis of insulin resistance and type 2 diabetes (14, 20, 27). Therefore, it has not been established that mitochondrial dysfunction is a major cause of insulin resistance. Therefore, the present study has extended these previous studies showing that oxidative stress and mitochondrial dysfunction in skeletal muscle play a crucial role not only in the development of insulin resistance but also exercise intolerance in type 2 diabetes.

It has been reported that the mitochondrial function and structure were impaired in skeletal muscle from HFD feeding rodents with the altered oxidative stress markers (3, 11, 20). However, the mechanisms for mitochondrial dysfunction by NAD(P)H oxidase-dependent $O_2^{\bullet -}$ in skeletal muscle are not fully understood. The decrease of mitochondrial ETC complex I and III activities can potentially be explained by direct oxidative damage to mitochondrial complexes (6). Mitochondria can be the primary target for oxidative damage when reactive oxygen species (ROS) production exceeds the capacity of the endogenous ROS scavenging system. $O_2^{\bullet -}$ easily can impair these ETC complexes because they include iron-sulfur center. In addition, oxidative damage to mitochondrial DNA can also result in the decrease in ETC complex activities. Furthermore, the impaired mitochondrial DNA may adversely

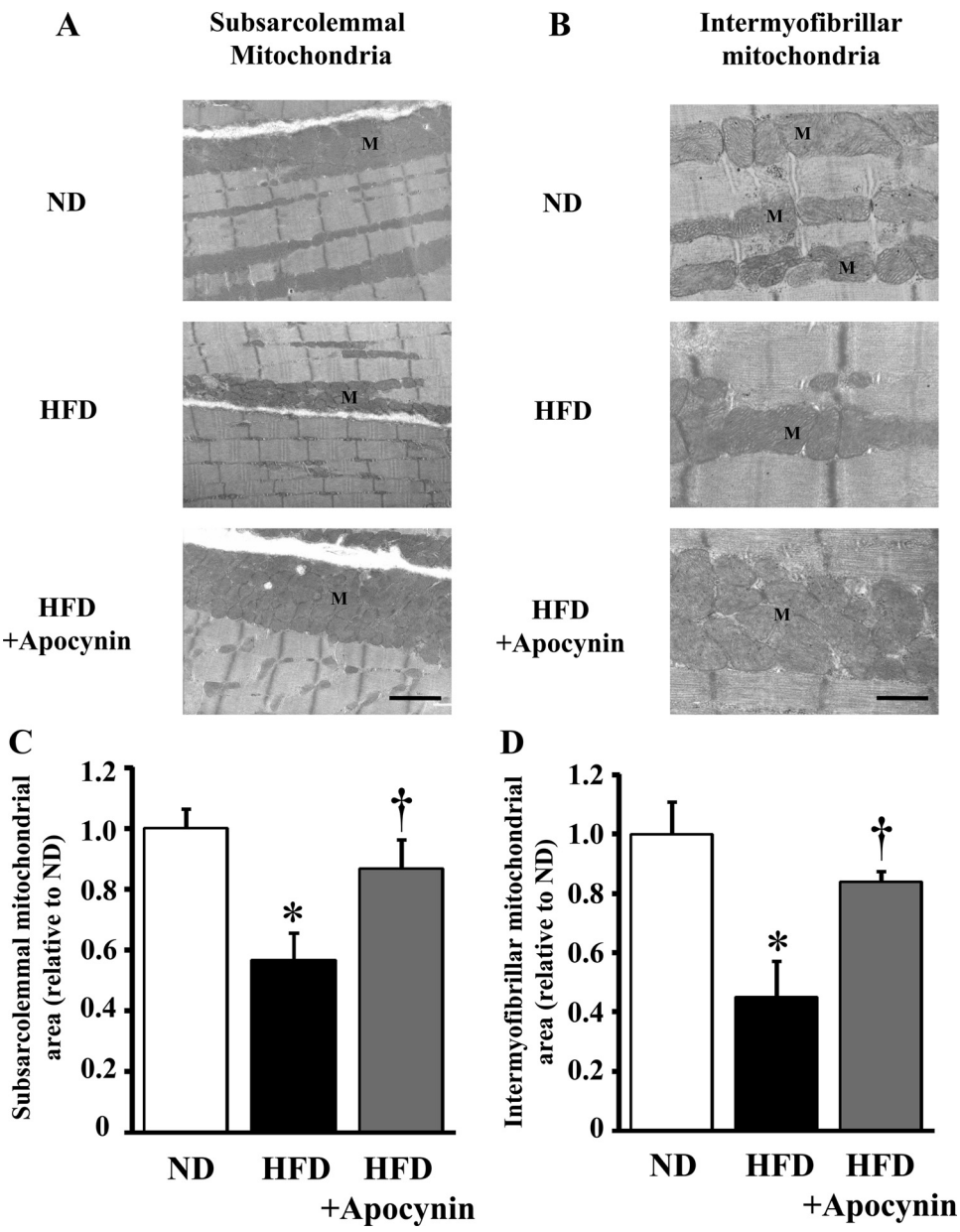

Fig. 5. Representative transmission electron microscopic images of subsarcolemmal (*A*; magnification, ×10,000; scale bar = 3 μm) and intermyofibrillar (*B*; magnification, ×30,000; scale bar = 1 μm) mitochondria in tibialis anterior muscle. Quantification of subsarcolemmal (*C*) and intermyofibrillar (*D*) mitochondrial area (analysis of 5 images in 3 mice for each group) is shown. M, mitochondria. Data are expressed as means ± SE. Experiments were performed after 8 wk of feeding in all groups. *$P < 0.05$ vs. ND; †$P < 0.05$ vs. HFD.

affect mitochondrial biogenesis. Indeed, coincident with mitochondrial dysfunction, subsarcolemmal and intermyofibrillar mitochondrial content in skeletal muscle was also reduced in the HFD mice (Fig. 5). These results were consistent with the previous data of the diet-induced insulin resistant mice (3).

Apocynin completely improved mitochondrial respiration (Fig. 3) and complex activities (Fig. 4) and inhibited $O_2^{\bullet-}$ production and NAD(P)H oxidase activity in skeletal muscle (Fig. 6), whereas it partially increased exercise capacity in HFD mice (Fig. 2). This implies that other mechanisms can be involved in the decreased exercise capacity in HFD mice. First, cardiac reserve adaptation to exercise can influence the exercise capacity and peak $\dot{V}O_2$. However, our echocardiographic and hemodynamic studies demonstrated that blood pressure and left ventricular systolic and diastolic function were preserved in the HFD mice (Table 2). Moreover, histopathological changes of myocardium such as myocyte hypertrophy, interstitial fibrosis, and atherosclerotic vascular changes were not observed in the HFD mice (data not shown). Accordingly, cardiac structure and function at rest can contribute very little to exercise intolerance in the HFD mice. Second, $O_2^{\bullet-}$ could impair nitric oxide-dependent vascular relaxation (22), which may decrease skeletal muscle blood flow especially during exercise and limit the exercise capacity. However, Ojaimi et al. (25) demonstrated that peak $\dot{V}O_2$ was not decreased in endothelial nitric oxide synthase (eNOS) knockout mice during maximal exercise, indicating that oxygen delivery to limb skeletal muscle was preserved even in the eNOS knockout mice. Therefore, skeletal muscle blood flow could have little influence on the exercise capacity in mice. Finally, skeletal muscle atrophy can cause a limitation of exercise capacity possibly through the induction of apoptosis. However, the contribution of muscle atrophy seems to be minor because skeletal muscle weight was increased in the HFD-induced

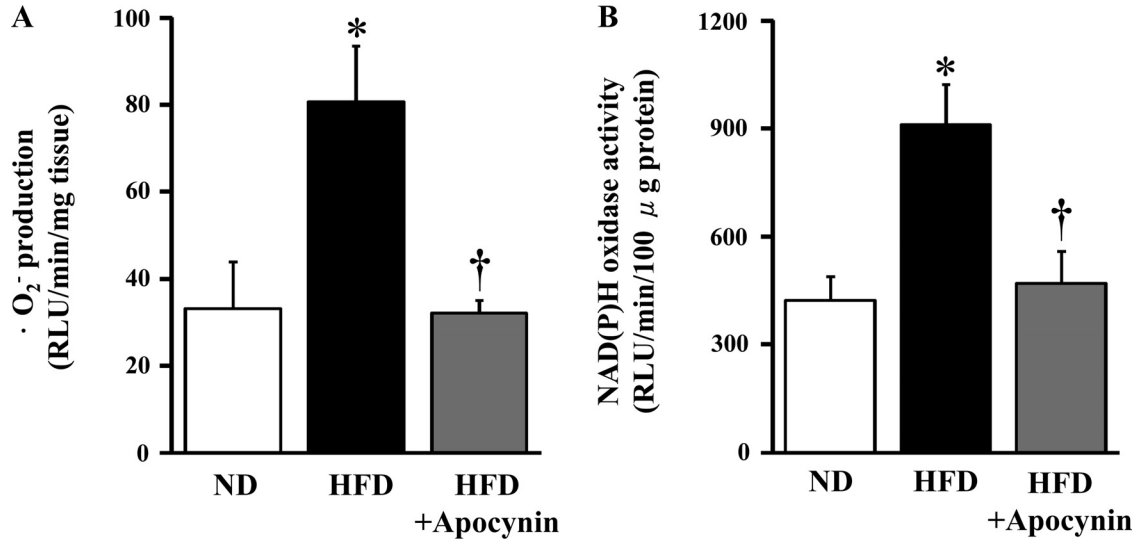

Fig. 6. Superoxide ($O_2^{\bullet -}$) production (*A*; $n = 5$ for each group) and NAD(P)H oxidase activity (*B*; $n = 4$ for each group) in skeletal muscle from 3 groups of mice. Data are expressed as means ± SE. Experiments were performed after 8 wk of feeding in all groups. RLU, relative light unit. *$P < 0.05$ vs. ND; †$P < 0.05$ vs. HFD.

diabetic mice (Table 1). Histological analysis revealed that there was no difference in muscle fiber cross-sectional area among the ND mice, the HFD mice, and the HFD + apocynin mice. A previous report has shown that the lipid accumulation was increased in skeletal muscle from HFD mice (15). Therefore, the increase in skeletal muscle weight would be due to the increased fat content rather than the increased skeletal muscle mass. Importantly, the effects on the exercise capacity were ameliorated by apocynin, indicating that structural changes of skeletal muscle did not play a major role. Finally, we could not completely exclude the effect of the increase in body weight on the exercise capacity in the HFD mice despite calculating the work to minimize the difference in body weight. HFD-induced obesity might partly be involved in the decreased exercise capacity.

An explosive increase of patients with type 2 diabetes is a growing medical as well as public health problem in industrialized countries. Therefore, the development of novel preventive and therapeutic approaches for diabetes is an important goal for further investigation. The first line in the prevention and treatment of diabetes is the lifestyle intervention including physical exercise and diet. However, the present study demonstrated that mitochondrial function was impaired in the HFD-fed mice, which might well explain the limited exercise capacity in patients with type 2 diabetes (29). Exercise training can increase mitochondrial oxidative capacity in skeletal muscle and delay or prevent diabetes by increasing insulin sensitivity. However, the limited exercise capacity might prevent the completion of proper and endurance exercise in patients with type 2 diabetes. Moreover, it has been known that lower exercise capacity and physical inactivity are independent predictors for cardiovascular morbidity and mortality in patients with type 2 diabetes (32). Therefore, therapies designed to regulate oxidative stress and maintain mitochondrial function in skeletal muscle are expected to increase the exercise capacity, which might be beneficial in the treatment of diabetes and the prevention of cardiovascular disease and death.

There are several limitations that should be acknowledged in the present study. First, even though there were no significant differences in cardiac function at rest among groups, we could not completely exclude the contribution of abnormal function during exercise. Nevertheless, we were unable to assess these changes in mice during exercise due to technical difficulty. Second, we could not completely exclude the effect of other sources of $O_2^{\bullet -}$ on the exercise capacity. Apocynin has been widely used as an inhibitor of NAD(P)H oxidase activation both in tissue preparations and in in vivo (8, 13, 16). However, a recent report using the cultured cells indicated that apocynin might predominantly act as an antioxidant (10). In this in vitro study, the inhibitory action of apocynin for NAD(P)H oxidase activation was restricted to myeloperoxidase (MPO)-expressing leukocytes and not demonstrated in MPO-free vascular cells. However, apocynin could inhibit the activation of NAD(P)H oxidase even in MPO-free skeletal muscle in in vivo situations. With the assumption that apocynin might act as an antioxidant, our results would support the concept that the enhanced oxidative stress in skeletal muscle contributed to the limited exercise capacity in type 2 diabetic mice. Finally, the induction of diabetes largely depends on the treatment duration and fat percentage in the diet and the species examined. In fact, Chalkley et al. (5) reported that long-term (10 mo) polyunsaturated fat feeding leads to insulin resistance but not diabetes in Wistar rats. Iossa et al. (11) reported that HFD feeding for 2 wk did not induce insulin resistance and diabetes. Our HFD mice had the elevated blood glucose levels during an intraperitoneal glucose tolerance test (Fig. 1). Therefore, we consider that our HFD model had obesity and also diabetes. However, the limited exercise capacity and mitochondrial dysfunction seen in our HFD mice were associated with the diabetic status and not with the increase in body weight per se in this model. Finally, the limited exercise capacity in the HFD-fed mice might be caused by the reduced physical activity. However, no significant difference has been reported in the spontaneous locomotor movement between control and HFD-induced obese mice (4).

In conclusion, the present study demonstrated that the HFD-fed obese mice with diabetes had the limited exercise capacity with the impaired mitochondrial function in skeletal muscle,

which were associated with an increase of $O_2^{\bullet -}$ in skeletal muscle. In light of the protective effects of apocynin on the abnormalities in the exercise capacity and mitochondrial function, the present study supports the notion that HFD induces insulin resistance as well as oxidative stress in skeletal muscle, which may cause mitochondrial dysfunction and ultimately lead to exercise intolerance in diabetes.

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