Combination of Exercise Training and Diet Restriction Normalizes Limited Exercise Capacity and Impaired Skeletal Muscle Function in Diet-Induced Diabetic Mice

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Exercise training (EX) and diet restriction (DR) are essential for effective management of obesity and insulin resistance in diabetes mellitus. However, whether these interventions ameliorate the limited exercise capacity and impaired skeletal muscle function in diabetes patients remains unexplored. Therefore, we investigated the effects of EX and/or DR on exercise capacity and skeletal muscle function in diet-induced diabetic mice. Male C57BL/6J mice that were fed a high-fat diet (HFD) for 8 weeks were randomly assigned for an additional 4 weeks to 4 groups: control, EX, DR, and EX+DR. A lean group fed with a normal diet was also studied. Obesity and insulin resistance induced by a HFD were significantly but partially improved by EX or DR and completely reversed by EX+DR. Although exercise capacity decreased significantly with HFD compared with normal diet, it partially improved with EX and DR and completely reversed with EX+DR. In parallel, the impaired mitochondrial function and enhanced oxidative stress in the skeletal muscle caused by the HFD were normalized only by EX+DR. Although obesity and insulin resistance were completely reversed by DR with an insulin-sensitizing drug or a long-term intervention, the exercise capacity and skeletal muscle function could not be normalized. Therefore, improvement in impaired skeletal muscle function, rather than obesity and insulin resistance, may be an important therapeutic target for normalization of the limited exercise capacity in diabetes. In conclusion, a comprehensive lifestyle therapy of exercise and diet normalizes the limited exercise capacity and impaired muscle function in diabetes mellitus. **(*Endocrinology* 155: 68–80, 2014)**

Physical inactivity and excessive calorie intake are well-established risk factors for type 2 diabetes (1). Therefore, lifestyle interventions, including exercise training (EX) and diet restriction (DR), which can reduce body weight and improve insulin resistance in diabetic patients (2–7), are the first-line treatment in the prevention and treatment of type 2 diabetes.

Diabetes is often associated with a limited exercise capacity (8, 9), which is an independent predictor of cardiovascular morbidity and mortality in patients with type 2

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Abbreviations: AUC, area under the curve; CS, citrate synthase; CSA, cross-sectional area; DR, diet restriction; EM, electron microscopy; ETC, electron transport chain; EX, exercise training; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFD, high-fat diet; H_2O_2, hydrogen peroxide; IMF, intermyofibrillar; IPGTT, ip glucose tolerance test; IPITT, ip insulin tolerance test; L-DR, long-term DR; L-ND, long-term ND; MET, metformin; mtDNA, mitochondrial DNA; NADH, reduced nicotinamide adenine dinucleotide hydrate; ND, normal diet; NEFA, nonesterified fatty acid; O_2^-, superoxide; PIO, pioglitazone; ROS, reactive oxygen species; SS, subsarcolemmal; TBARS, thiobarbituric acid reactive substances; TCA, tricarboxylic acid; UCP, uncoupling protein; VO_2, oxygen uptake.

diabetes (10, 11). The determinants of exercise capacity are multifactorial. However, it is generally believed to be impaired in the presence of abnormalities in the skeletal muscle energy metabolism, which largely depends on mitochondrial function (12, 13). EX improves the limited exercise capacity and impaired muscle mitochondrial function in type 2 diabetic patients (14, 15). In contrast, the effects of DR on those impairments remain unclear.

The pathogenesis of impaired mitochondrial function in the skeletal muscle of type 2 diabetic patients has not been fully determined. Recent studies have shown that the production of reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), increases in the skeletal muscle of type 2 diabetic animal models (16–19). Furthermore, we have clearly shown that the pharmacological inhibition of ROS can attenuate the limited exercise capacity and impaired muscle mitochondrial function (18, 19). Therefore, therapies designed to increase mitochondrial function and decrease oxidative stress in the skeletal muscle could be beneficial for improving the exercise capacity in type 2 diabetic patients.

In the present study, we investigated whether EX and/or DR could improve the limited exercise capacity and impaired skeletal muscle function in diet-induced type 2 diabetic mice.

Materials and Methods

Animals

Male C57BL/6J mice were purchased from Hokudo. The mice were housed in an animal room under controlled condition on a 12-hour light, 12-hour dark cycle. All mice were handled daily for at least 1 week before beginning the experiment. 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 in Hokkaido University Graduate School of Medicine.

Study design

Study 1: Effects of EX and/or DR in diet-induced diabetic mice

The mice were fed either a normal diet (ND) (CE-2; CLEA Japan) or a high-fat diet (HFD) (HFD32; CLEA Japan) for 8 weeks. The macronutrient compositions of the ND and HFD are listed in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. After 8 weeks after feeding, the HFD-fed mice were randomly assigned to the following 4 groups: control, EX, DR, and their combination (EX+DR). These mice were treated for an additional 4 weeks. EX consisted of swimming for 90 minutes, 5 days per week, as described previously (20, 21). The water temperature during swimming was maintained at 34°C–35°C with a water heater system, and the mice were carefully towel dried immediately after each exercise session and placed in a warm environment to avoid physiological responses and health problems related to cold stress. DR involved replacing HFD with ND (22, 23). The daily energy intake was restricted by approximately 40%–50% in the DR (6.5 ± 0.1 kcal/d per mouse) and DR+EX (6.8 ± 0.1 kcal/d per mouse) groups compared with that during the HFD feeding (12.3 ± 0.2 kcal/d per mouse). The ND group continued with the ND for the entire 4-week duration.

Study 2: Effects of DR combined with an insulin-sensitizing drug in diet-induced diabetic mice

Mice fed with a HFD for 8 weeks were randomly assigned to the following 3 groups: DR alone, DR with metformin (MET) (300 mg/kg·d), or DR with pioglitazone (PIO) (20 mg/kg·d). MET was administrated in drinking water and PIO was incorporated into the diet. These mice were treated for an additional 4 weeks. The ND group was also studied for 12 weeks.

Study 3: Effects of long-term DR (L-DR) in diet-induced diabetic mice

Mice fed with a HFD for 8 weeks were randomly assigned to either long-term control or L-DR for an additional 8 weeks. The long-term ND (L-ND) group was also studied for 16 weeks.

Organ weight and histology

Epididymal and mesenteric fats and skeletal muscle were excised and weighed. Epididymal fat was fixed in 4% paraformaldehyde, embedded in paraffin for histological analysis, and stained with hematoxylin eosin. Quantification of cross-sectional area (CSA) in adipocytes was performed at least 100 cells from each mouse using NIH ImageJ software. Gastrocnemius muscle was freeze on dry ice and stained with reduced nicotinamide adenine dinucleotide hydrate (NADH), as previously described (18). Immunohistochemistry in gastrocnemius muscle was performed by using a rabbit polyclonal antinitrotyrosine antibody (Millipore).

Blood biochemistry

Plasma insulin was measured by ELISA kit (Morinaga Institute of Biochemical Science). Total cholesterol, triglyceride, and nonesterified fatty acid (NEFA) were determined by using enzymatic assays using Cholesterol E test, Triglyceride E test, and NEFA C test, respectively (Wako Pure Chemical Industries).

Intraperitoneal glucose tolerance test (IPGTT) and ip insulin tolerance test (IPITT)

IPGTT and IPITT were performed on 6-hour-fasted mice. Mice were given an ip injection of glucose (1 mg/g) or insulin (0.5 mU/g). Blood samples were drawn from the tail vein before and indicated time point after injection. Blood glucose levels were determined using a glucometer (Glutest Ace R; Sanwa Kagaku Kenkyusho).

Exercise capacity

The mice were tested on a treadmill to measure indices defining exercise capacity, as previously described (18, 19, 24). Briefly, each mouse was placed on a treadmill enclosed by a metabolic chamber (Oxymax 2; Columbus Instruments). O_2 and 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 minutes. The mice were then provided a 10-minute warm-up period at 6 m/min at 0° inclination. After the warmed-up, the inclination angle was fixed at 10°, and the speed was incrementally increased by 2 m/min every 2 minutes, until the mouse reached exhaustion. Oxygen uptake (VO_2) was calculated automatically every 10 seconds. Exhaustion was defined as the inability of the mouse to return to running within 10 seconds after direct contact with the shocker plate. Exercise capacity was assessed by measuring work (the product of the vertical running distance and body weight) and peak VO_2.

Mitochondrial function

The ATP levels in the skeletal muscle were measured using a luciferase/luciferin assay kit (Toyo Ink) according to the manufacturer's protocol. The luciferase/luciferin luminescent was recorded with a luminometer (AccuFLEX Lumi 400; Aloka). Muscle ATP levels were normalized to the level of ND.

To assess mitochondrial function in the skeletal muscle, mitochondria were isolated as descried previously (19, 24). All procedures for mitochondrial isolation were performed at 4°C. Briefly, muscle sample were minced and homogenized in isolation buffer (220mM sucrose, 1mM EDTA, and 10mM HEPES-KOH; pH 7.4). The homogenate were centrifuged at 800*g* for 10 minutes. The pellet was discarded, and the supernatant was centrifuged at 12 000*g* for 10 minutes to obtain the mitochondria pellet. The mitochondrial pellet was washed twice and centrifuged at 12 000*g*, and the supernatant was discarded. The final mitochondrial pellet was resuspended in a minimal isolation buffer and stored in aliquots at −80°C.

The enzyme activities of citrate synthase (CS), isocitrate dehydrogenase, and malate dehydrogenase in tricarboxylic acid (TCA) cycle were spectrophotometrically measured as described previously (25).

The enzyme 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), complex IV (cytochrome *c* oxidase), and ATP synthase were spectrophotometrically measured in the isolated mitochondria as described previously (19, 24).

Transmission electron microscopy (EM)

The skeletal muscle sample was fixed in 3% glutaraldehyde with 0.1mM phosphate buffer (pH 7.2) for 3 hours at 4°C and postfixed in 1% osmium tetroxide with 0.1mM phosphate buffer (pH 7.2) for 90 minutes at 4°C. The sample were then serially dehydrated in ethanol and embedded in epoxy resin. Sections were cut on an LKB ultramicrotome (LKB), and consecutive ultrathin sections were mounted on copper grids. The ultrathin sections were stained with 3% uranyl acetate and 0.2% lead citrate. Examinations were conducted with an EM (H-7100; Hitachi). Quantifications of CSA and density of the subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria subpopulation in the skeletal muscle were performed using ImageJ software. The CSA of mitochondria in each population were calculated as the average of the area with at least 50 mitochondria on 5 images. The density of SS mitochondria was evaluated at the maximal width in the part with the accumulated part of mitochondria. The density of IMF mitochondria was assessed as the percent area of mitochondria located per 100 μm^2.

Real-time PCR and mitochondrial DNA (mtDNA) analysis

Total RNA was extracted from frozen muscle samples with QuickGene-810 (FujiFilm) according to the manufacture's instructions. The extracted RNA was quantified spectrophotometrically at 260 nm. cDNA was synthesized with the high-capacity cDNA reverse transcription kit (Applied Biosystems). Reverse transcription was performed at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds, and then cooled at 4°C. cDNA samples were stored at −20°C until subsequent amplification.

TaqMan quantitative real-time PCR was performed with the 7300 real-time PCR system (Applied Biosystems) with TaqMan Gene Expression Master Mix (Applied Biosystems). TaqMan primers and proves used in the present study are listed in Supplemental Table 2. After 2 minutes at 50°C and 10 minutes at 95°C, the PCR amplification was performed for 40 cycles, with each cycle at 95°C for 15 seconds denaturing step and 60°C for 1 minute annealing/extending step. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. mRNA expression was normalized to GAPDH expression levels in each sample.

To determine mtDNA content, DNA was isolated from frozen muscle samples using the DNAeasy tissue kit (QIAGEN). DNA was quantified spectrophotometrically at 260-nm wavelength. The DNA was used to amplify cytochrome *b* and GAPDH using quantitative real-time PCR. The cytochrome *b* primer probe was generated by using the following sequence: forward primer TATTCCTTCATGTCGGACGA, reverse primer AAATGCTGTGGCTATGACTG, and probe ACCTGAAACATTGGAGTACTTCTACTG.

Oxidative stress

Plasma H_2O_2 levels were measured using the Amplex Red (Molecular Probes), according to the manufactures' instructions.

O_2^- production in the skeletal muscle was measured using lucigenin chemiluminescence as described previously (18, 19, 24). Briefly, a freshly muscle sample was placed in a modified Krebs/HEPES buffer and preincubated to equilibrate for 10 minutes at 37°C. Muscle sample was then transferred in 5μM lucigenin solution and further incubated for 10 minutes. After the incubation, O_2^- production was measured using a luminometer and observed total relative light unit for 5 minutes. To validate that the chemiluminescence signals were derived from O_2^-, the measurements were also performed in the presence of tiron (10mM), a cell-permeant O_2^- scavenger. The O_2^- production was calculated from the difference between conditions with and without tiron.

The degree of lipid peroxidation in the skeletal muscle was determined through biochemical assay of thiobarbituric acid reactive substances (TBARS) by thiobarbituric acid test, as described previously (18, 26, 27). 1,1,3,3,-Tetraehoxypropane was used as standard in this assay.

Reduced nicotinamide adenine dinucleotide phosphate hydrate [NAD(P)H] oxidase activity in the skeletal muscle was measured by the lucigenin-enhanced chemiluminescence method (18, 19, 24, 26, 27). The muscle homogenate (100 μg) was added

in 5μM lucigenin solution. The chemiluminescence signals was immediately recorded after the addition of 100μM NADPH for 3 minutes. NAD(P)H oxidase activity was calibrated by subtracting a background signal.

Statistical analysis

Statistical analysis for comparisons among groups was performed using one-way ANOVA. Post hoc comparisons were made by Tukey's test. The level of significance was set at $P < .05$. All statistical tests were performed using SPSS 13.0 for Windows software.

Results

Combination of EX and DR normalizes obesity and insulin resistance in diet-induced diabetic mice

The mice were fed an ND or an HFD for 8 weeks, after which the HFD-fed mice showed a significantly greater increase in body weight than the ND-fed mice (Supplemental Figure 1A). The HFD also caused a significantly greater increase in the fasting blood glucose level and responses of blood glucose during IPGTT than the ND (Supplemental Figure 1B). After 4 weeks of lifestyle interventions, the HFD further increased body weight significantly as compared with that after 8 weeks of feeding, whereas it was significantly decreased by EX, DR, and EX+DR (Figure 1A). The decrease in body weight was greater in the DR group than in the EX group. However, neither DR nor EX reversed body weight to the level seen in the ND group. In contrast, body weight was completely normalized in the EX+DR group. The fat weight (percent of body weight, sum of epididymal fat and mesenteric fat) and adipocyte CSA were consistent with body weight (Figure 1, B and C, and Supplemental Table 3). No significant difference was observed in the skeletal muscle weight (rectus femoris and gastrocnemius) among all 5 groups (Supplemental Table 3). The HFD caused a significantly greater increase in heart weight and left ventricular diastolic dimension than the ND (Supplemental Tables 3 and 4). However, there was no difference in cardiac function among all 5 groups, as determined by the ejection fraction (Supplemental Table 4).

The HFD caused a significantly greater increase in fasting blood glucose, plasma insulin, triglyceride, total cholesterol, and NEFA levels than the ND (Figure 1, D–F). The EX significantly decreased blood glucose but did not affect other. The DR significantly decreased blood glucose, insulin, and total cholesterol levels and showed a tendency to decrease triglyceride and NEFA. Again, these blood parameters significantly decreased in the EX+DR groups in the same levels to that seen with ND. Responses of blood glucose during IPGTT and IPITT were evaluated by area under the curve (AUC) analysis (Figure 1, G and H). The AUCs of IPGTT and IPITT in the HFD were significantly higher than those in ND and significantly lowered by EX, DR, and EX+DR. The AUCs of IPGTT and IPITT in the DR group were significantly lower than those in the EX group. However, they were significantly higher than those in the ND group. The AUCs of IPGTT and IPITT were similar in the ND and EX+DR groups.

Combination of EX and DR normalizes the limited exercise capacity and impaired muscle mitochondrial function in diet-induced diabetic mice

At 8 weeks after feeding, exercise capacity showed a significantly greater reduction in the HFD-fed mice than the ND-fed mice (Supplemental Figure 2, A and B). After 4 weeks of lifestyle interventions, the HFD further decreased exercise capacity significantly as compared with that after 8 weeks of feeding ($P < .001$), whereas it was significantly increased by EX, DR, and EX+DR (Figure 2, A and B). There was no difference in the exercise capacities of the EX and DR groups, both of which did not improve the exercise capacity to the level seen in the ND group. In contrast, the exercise capacity was completely normalized in the EX+DR group.

Mitochondrial function in the skeletal muscle is closely related to exercise capacity (11, 12). The ATP level in the skeletal muscle was significantly lower in the HFD group than in the ND group. The EX+DR caused a significant increase in the muscle ATP level (Figure 3A). The enzyme activities of CS and malate dehydrogenase, key enzymes of TCA cycle, showed a significantly greater decrease in the HFD group than in the ND group (Figure 3B). These enzyme activities were partially increased by EX and completely normalized by EX+DR. The enzyme activities of complex I and III in the mitochondrial ETC showed a significantly greater decrease in the HFD group than in the ND group. These enzyme activities were completely normalized by EX+DR (Figure 3C). The enzyme activity of ATP synthase, namely a complex V, showed a significantly greater decrease in the HFD group than in the ND group. This enzyme activity tended to increase with EX+DR. No significant difference was observed in the complex II and IV activities among all 5 groups. NADH histochemical reaction in the skeletal muscle was decreased in the HFD group compared with the ND group, and it was completely reversed by EX+DR (Figure 3D). Gene expressions related to mitochondrial function, including TCA cycle (*Cs* and *Idh2*) and ETC (*Cox4i1* and *Atp5b*), showed a significantly greater decrease in the HFD group than in the ND group (Figure 3E). However, those gene downregulations were significantly reversed by EX+DR.

In the previous studies, diet-induced diabetic mice not only showed mitochondrial dysfunction but also mito-

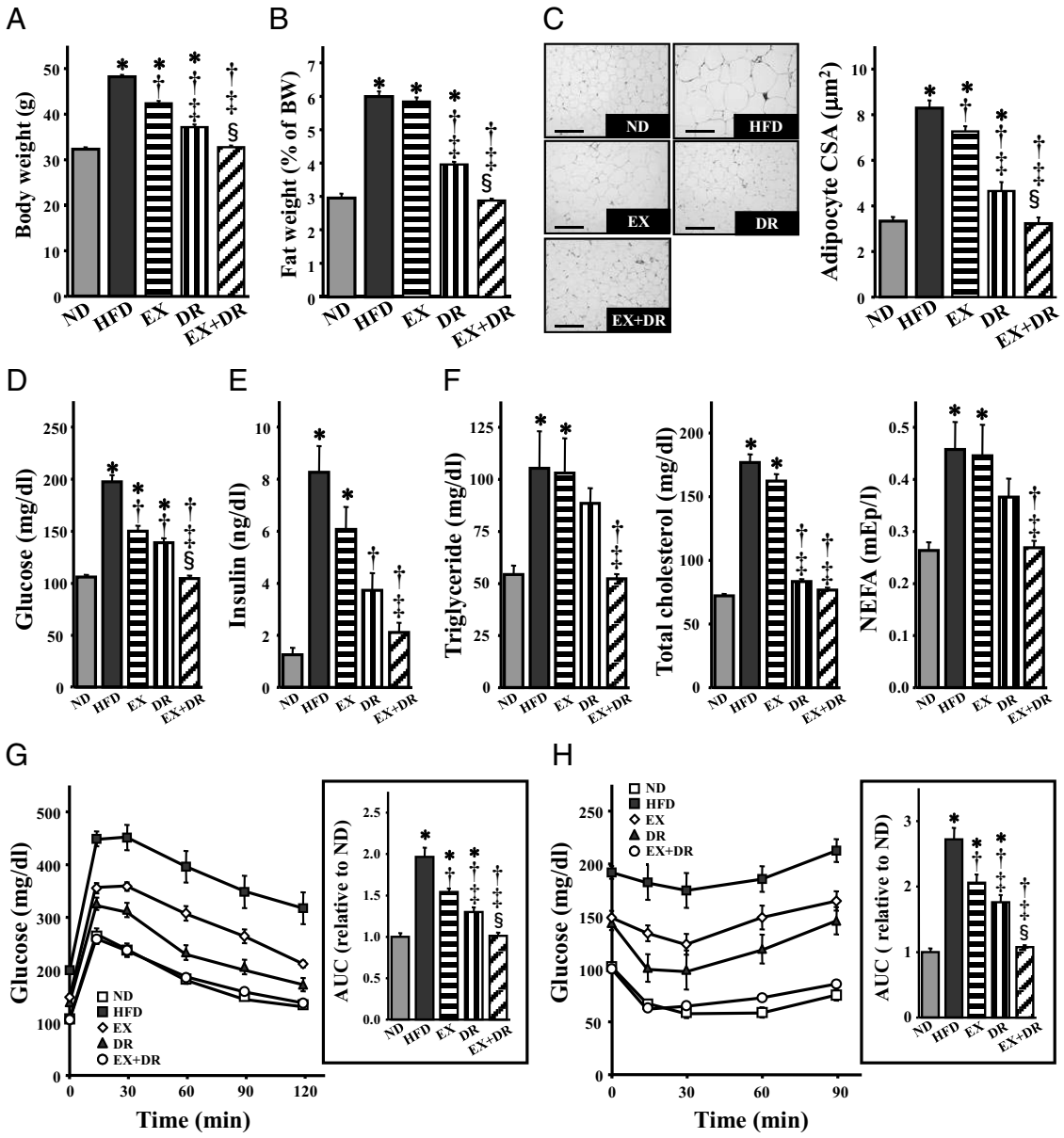

Figure 1. Combination of EX and DR normalizes obesity and insulin resistance in diet-induced diabetic mice. Body weight (A, n = 19), visceral fat weight (B, n = 19), representative photomicrograph of adipocytes in epididymal fatpad (C, left; scale bar = 300 μm), summary data of CSA of adipocytes (C, right; n = 9), fasting blood glucose (D, n = 19), plasma insulin (E, n = 11), and lipid profiles (F, n = 11), glucose levels during IPGTT and IPITT (F and G, left, n = 10) and their AUC (F and G, right) after a 4-week intervention of EX and/or DR in diet-induced diabetic mice. Results are presented as means ± SE. BW, body weight. *, $P < .05$ vs ND; †, $P < .05$ vs HFD; ‡, $P < .05$ vs EX; §, $P < .05$ vs DR.

chondrial morphological abnormality, such as reductions of mitochondrial size and content (16, 19). Representative transmission EM images of SS and IMF mitochondria in the skeletal muscle are shown in Figure 4A. SS and IMF mitochondrial sizes showed a significantly greater reduction in the HFD group than in the ND group and were significantly increased in the EX+DR groups (Figure 4B). The SS mitochondrial density was significantly decreased in the HFD and DR groups as compared with the ND group (Figure 4C). There was no significant difference in the IMF mitochondrial density among all 5 groups. The SS mitochondrial protein showed a significantly greater decrease in the HFD and DR groups as compared with the ND group and was significantly increased in the EX+DR group (Supplemental Figure 3). The IMF mitochondrial protein also showed a significantly greater decrease in the HFD group than in the ND group. The relative amount of mtDNA, which represents mitochondrial content, showed a significantly greater reduction in the HFD group than in the ND group and was significantly increased in the EX+DR group (Figure 4D). Gene expressions related to mitochondrial biogenesis (*Pgc-1α*, *Ppar-α*, and *Mfn1*) showed a significantly greater decrease in the HFD group than in the ND group, and those

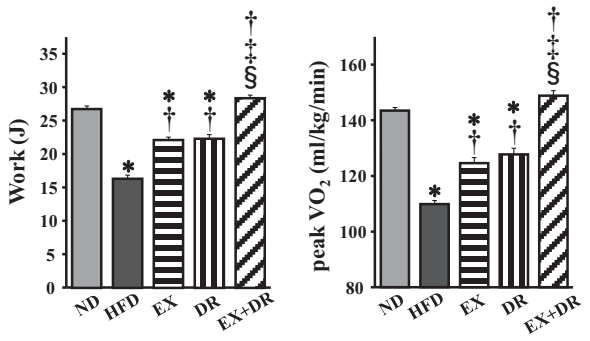

Figure 2. Combination of EX and DR normalizes the limited exercise capacity in diet-induced diabetic mice. Work (left) and peak VO_2 (right) measured by treadmill test after a 4-week intervention of EX and/or DR in diet-induced diabetic mice (n = 19). Results are presented as means ± SE. *, $P < .05$ vs ND; †, $P < .05$ vs HFD; ‡, $P < .05$ vs EX; §, $P < .05$ vs DR.

gene down-regulations were significantly reversed in the EX+DR group (Figure 4E).

It has been previously reported that the energy substrate metabolism in the skeletal muscle is impaired in diabetes patients (28), and it may be closely related to the limited exercise capacity and impaired muscle mitochondrial function (29). Glycogen content in the skeletal muscle was not affected by the HFD (Supplemental Figure 4A). In contrast, triglyceride content in the skeletal muscle showed a significantly greater increase in the HFD group than in the ND group, and it was significantly reduced by DR and EX+DR but not by EX alone (Supplemental Figure 4B). The number of intramyocellular lipid droplets in the skeletal muscle was consistent with the triglyceride content (Supplemental Figure 5). The enzyme activity of hexokinase, a key enzyme of glucose uptake, showed a significantly greater decrease in the HFD group than in the ND group, and it was completely normalized in the EX+DR group (Supplemental Figure 6A). Moreover, the enzyme activity of β-hydroxyacyl coenzyme A dehydrogenase, a key enzyme of fatty acid β-oxidation, showed a significantly greater decrease in the HFD group than in the ND group and was completely normalized by EX and EX+DR but not by DR alone (Supplemental Figure 6C). No significant differences in the gene expressions were noted in term of energy substrate metabolisms among all 5 groups (Supplemental Figure 6, B and D).

Combination of EX and DR normalizes the enhanced oxidative stress in the skeletal muscle in diet-induced diabetic mice

Previous studies have shown that oxidative stress is increased in the skeletal muscle of type 2 diabetic mice with HFD feeding (16–19). Furthermore, we have previously shown that the enhanced muscle oxidative stress is closely related to limited exercise capacity and impaired mitochondrial function (18, 19, 24, 30). Plasma H_2O_2 levels showed a significantly greater increase in the HFD group than in the ND group. These levels were not affected in the EX group, partially reduced in the DR group, and completely reduced in the EX+DR group (Figure 5A). The levels of plasma TBARS showed the same results (Supplemental Figure 7). O_2^- production, TBARS, and NAD(P)H oxidase activity in the skeletal muscle showed a significantly greater increase in the HFD group than in the ND group. These levels were partially reduced with EX and DR and were completely reduced with EX+DR (Figure 4, B–D). Nitrotyrosine immunohistochemical reaction in the skeletal muscle was increased in the HFD group compared with the ND group, and it was completely reduced by EX+DR (Figure 4E). Gene expressions of NAD(P)H oxidase subunits (*gp91phox, p22phox, p40 phox, p47phox,* and *p67phox*) showed a significantly greater increase in the HFD group than in the ND group, and those gene up-regulations were significantly decreased in the EX, DR, and EX+DR groups (Figure 5F). There were no significant differences in the gene levels of *Nox 1* and *4* among all five groups.

There were no significant differences in the enzyme activities of antioxidant, superoxide disumtase, catalase, and glutathione peroxidase in the skeletal muscle among all 5 groups (Supplemental Figure 8, A–C). Moreover, gene expressions related to antioxidant enzyme did not differ among all 5 groups (Supplemental Figure 8D).

Normalization of obesity and insulin resistance do not require improving exercise capacity and skeletal muscle function in diet-induced diabetic mice

The limited exercise capacity and impaired mitochondrial function in type 2 diabetes is often involved in obesity and insulin resistance (8, 28, 29). We investigated whether the normalization of obesity and insulin resistance could completely reverse the limited exercise capacity and impaired skeletal muscle function in diet-induced diabetic mice. First, the mice treated with DR were given an insulin-sensitizing drug, with either PIO or MET. The body weight in the DR+MET group, but did not in the DR+PIO group, showed a significantly greater decrease than that in the DR group (Figure 6A). However, it did not decrease body weight to the level seen in the ND group. In contrast, AUCs of IPGTT and IPITT were completely reversed in both the DR+MET and DR+PIO groups (Figure 6, B and C). However, both drugs did not affect the exercise capacity (Figure 6D). Moreover, mitochondrial function and oxidative stress assessed by CS activity and NAD(P)H oxidase activity in the skeletal muscle were not ameliorated by the insulin sensitivity drugs (Figure 6, E and F).

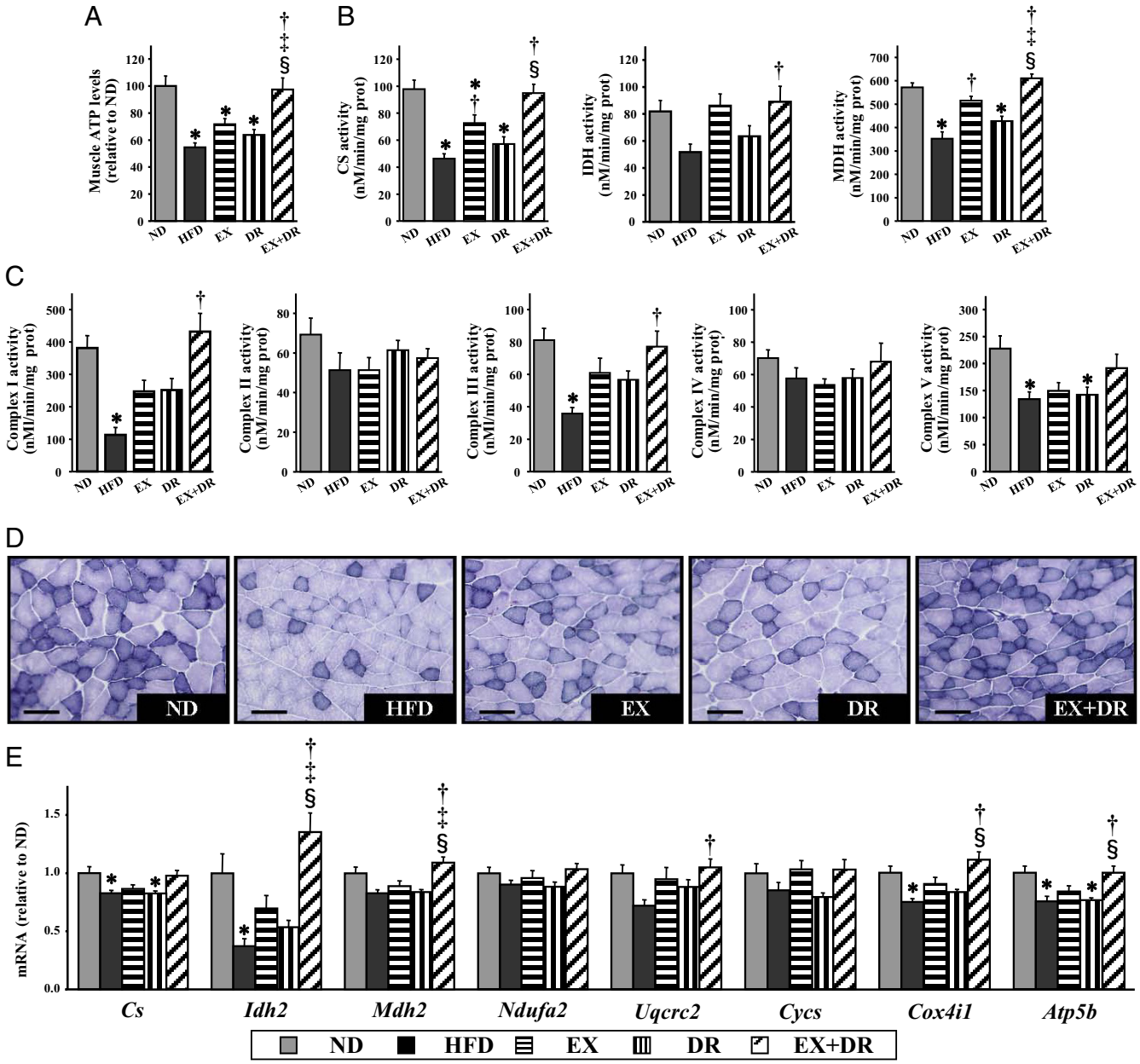

Figure 3. Combination of EX and DR normalizes the impaired mitochondrial function in the skeletal muscle in diet-induced diabetic mice. The ATP level (A, n = 12), enzymatic activities of TCA cycle (B, n = 12) and ETC (C, n = 11), NADH activity staining (D), and mRNA levels of gene associated with TCA cycle and ETC (E, n = 11) after a 4-week intervention of EX and/or DR in diet-induced diabetic mice. Results are presented as means ± SE. IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase. *, $P < .05$ vs ND; †, $P < .05$ vs HFD; ‡, $P < .05$ vs EX; §, $P < .05$ vs DR.

Next, we examined the effect of a long-term intervention of DR. The body and fat weights in the L-DR group reached levels equal to those in the L-ND group (Figure 7, A and B). Furthermore, AUC of IPGTT was completely reversed in the L-DR group to the normal level of the L-ND group (Figure 7C). However, L-DR did not normalize the exercise capacity and skeletal muscle function (Figure 7, D–F).

Discussion

In the present study, we showed that limited exercise capacity in HFD-induced diabetic mice can be partially improved by either EX or DR, and the combination of both can completely normalized it. The improvement was associated with the normalization of impaired mitochondrial

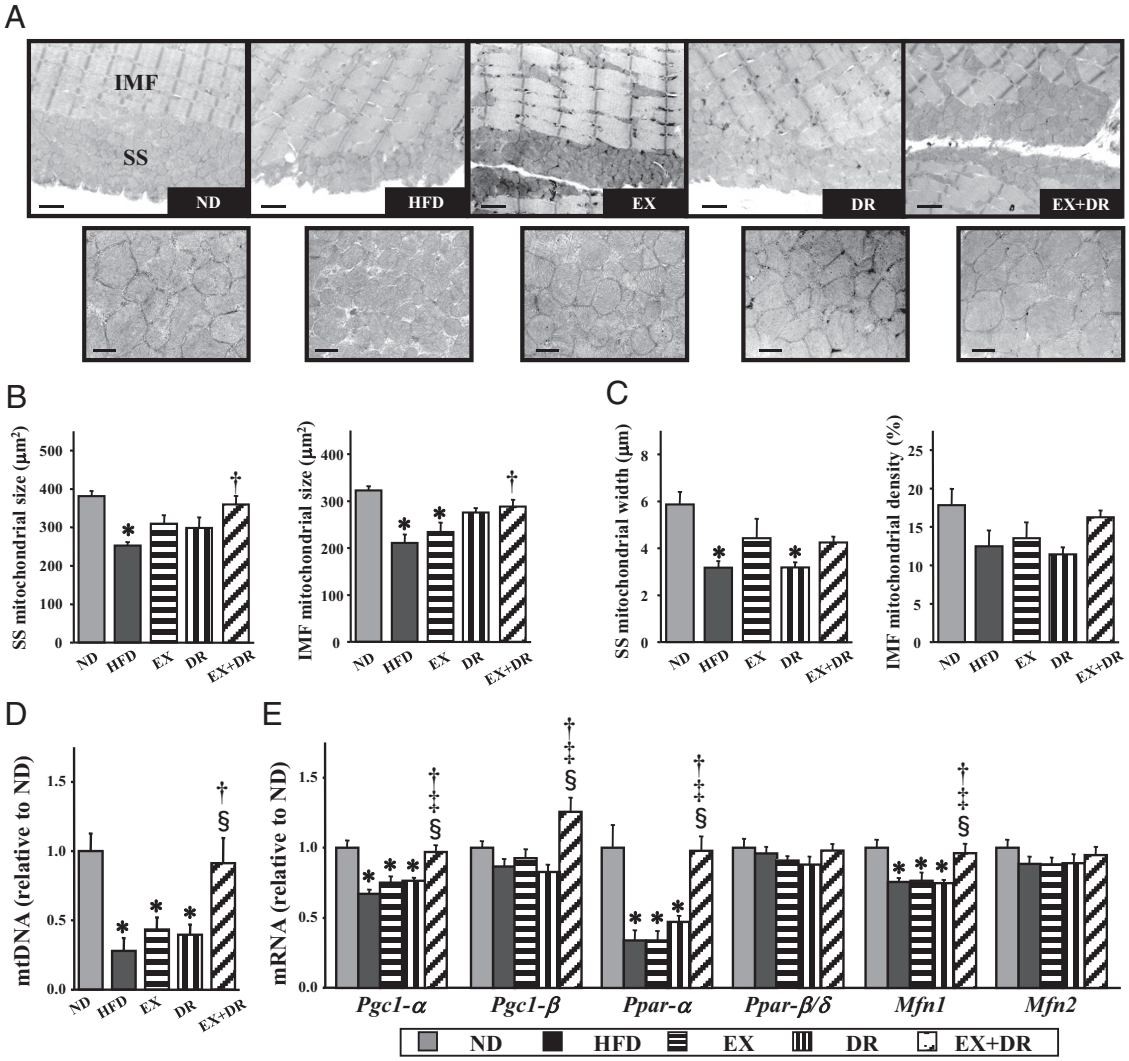

Figure 4. Combination of EX and DR normalizes mitochondrial morphological abnormality in the skeletal muscle in diet-induced diabetic mice. Representative transmission EM images (A, upper; scale bar = 2 μm; lower; scale bar = 500 μm), summary data of size (B, n = 5) and density (C; n = 5) in SS and IMF mitochondria, mtDNA (D; n = 8), and mRNA levels of gene associated with mitochondrial biogenesis (E, n = 11) after a 4-week intervention of EX and/or DR in diet-induced diabetic mice. Results are presented as means ± SE. *, $P < .05$ vs ND; †, $P < .05$ vs HFD; ‡, $P < .05$ vs EX; §, $P < .05$ vs DR.

function and enhanced oxidative stress in the skeletal muscle of diabetic mice. Furthermore, we found that normalization of body weight and insulin sensitivity was not required to improve the exercise capacity in diabetic mice. These findings suggest that skeletal muscle function, rather than obesity and insulin resistance, plays an important role in improving limited exercise capacity in diabetes.

Combined intervention of exercise and diet is beneficial for treating obesity and insulin resistance (5–7). Furthermore, clinical studies have reported that the combined intervention also improved the exercise capacity for obese or diabetic patients (6, 7, 31). However, there is little information about the effects of EX and/or DR on skeletal muscle function, such as mitochondrial function and oxidative stress. Toledo et al (31) reported that the combined intervention of exercise and diet improved exercise capacity and skeletal muscle mitochondrial function in obese patients significantly more than the single intervention of diet alone. However, it remains unclear whether EX and/or DR could reduce the enhanced oxidative stress in the skeletal muscle of diabetes. Oxidative stress is a strong factor causing mitochondrial dysfunction (32, 33). In fact, oxidative stress increases in the skeletal muscle of type 2 diabetic mice and is associated with mitochondrial dysfunction (16–19). We found that NAD(P)H oxidase-derived O_2^- production increased in the skeletal muscle from HFD-induced diabetic mice, which was closely associated with the limited exercise capacity as well as impaired mitochondrial function (18, 19). Furthermore, treatment with an inhibitor of NAD(P)H oxidase, apocynin, significantly improved the limited exercise capacity and impaired skeletal muscle in diabetic mice (19). The

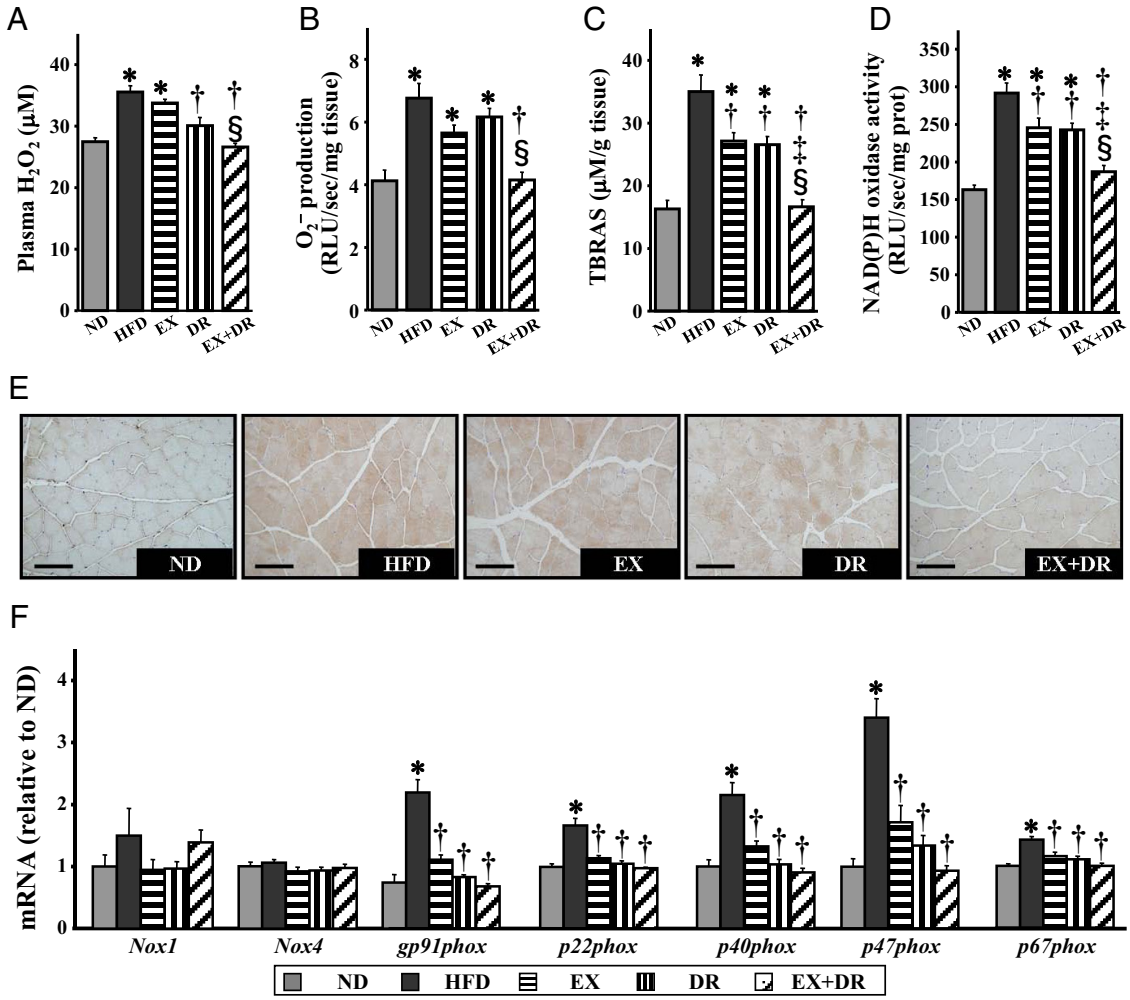

Figure 5. Combination of EX and DR normalizes the increased oxidative stress in diet-induced diabetic mice. Plasma H_2O_2 (A, n = 12), O_2^- production (B, n = 6), TBARS (C, n = 13), NAD(P)H oxidase activity (D, n = 12), protein nitrotyrosin staining (E), and mRNA levels of gene associated with NAD(P)H oxidase subunits (F, n = 11) in the skeletal muscle after a 4-week intervention of EX and/or DR in diet-induced diabetic mice. Results are presented as means ± SE. *, $P < .05$ vs ND; †, $P < .05$ vs HFD; ‡, $P < .05$ vs EX; §, $P < .05$ vs DR.

present study showed that the enhanced oxidative stress in the skeletal muscle of diabetic mice was reduced to normal by the combined intervention, and it was parallel to the improvements in exercise capacity and muscle mitochondrial function. The activity of mitochondrial ETC complex I and III, which are the main source for ROS production (32, 33), was decreased in the skeletal muscle of diabetic mice, and it was resumed to normal by the combined intervention. Therefore, these results suggest that the inhibition of oxidative stress by the combined intervention is closely related to the improvement in exercise capacity and muscle mitochondrial function. We, as well as other researchers, have previously shown that the oxidative stress in the skeletal muscle reduced the exercise capacity in gene-manipulated mice (30, 34). We reported that the exercise capacity was reduced in heterozygous manganese superoxide disumtase gene knockout mice, in which O_2^- production in the skeletal muscle was increased (30). Thus, oxidative stress in the skeletal muscle may be a strong factor for regulation of exercise capacity and mitochondrial function in diabetes.

In the present study, single intervention with EX or DR partially reversed obesity and insulin resistance in diabetic mice. These improvements were greater with DR than with EX. However, they were not normalized. This raised a possibility that the improvement in the exercise capacity and skeletal muscle function in type 2 diabetic mice may be associated with the improvement in obesity and insulin resistance. Therefore, we examined whether the normalization of obesity and insulin resistance could completely reverse the limited exercise capacity and impaired skeletal muscle function in diabetic mice. However, the exercise capacity and mitochondrial function did not show complete recovery even when insulin resistance was normalized by DR combined with an insulin-sensitizing drug. Moreover, normalization of body weight and insulin re-

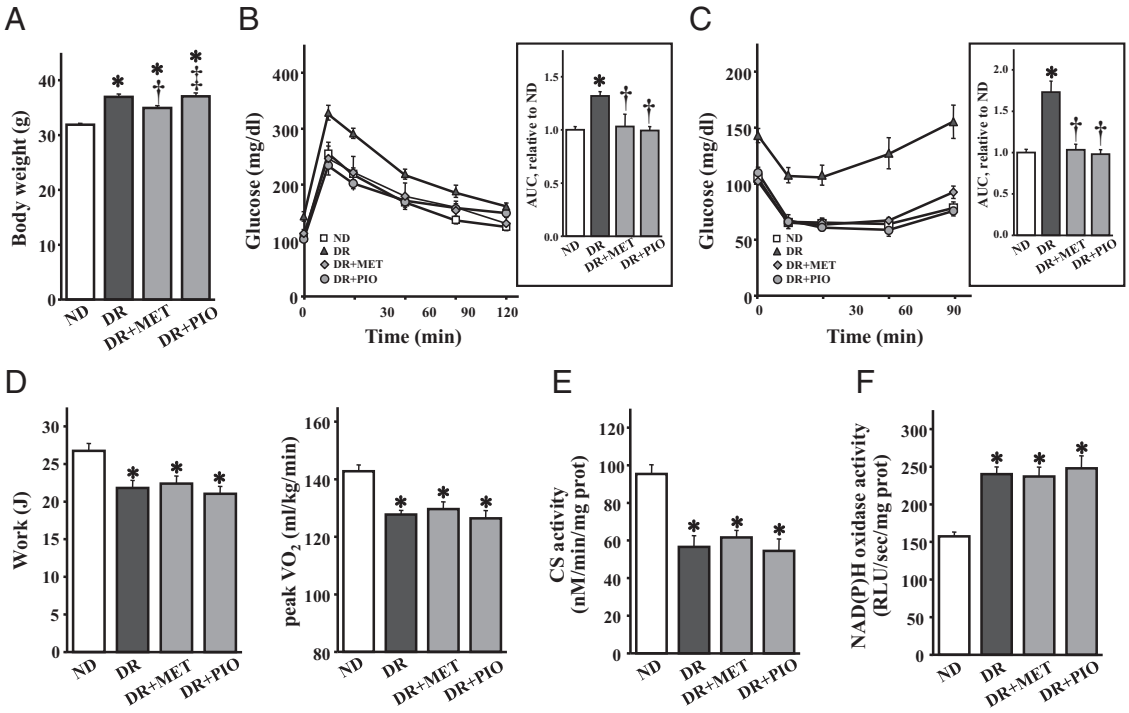

Figure 6. Normalization of insulin resistance does not require improving exercise capacity and skeletal muscle function in diet-induced diabetic mice. Body weight (A, n = 10), glucose levels during IPGTT and IPITT (B and C, left, n = 5) and their AUC (B and C, right), exercise capacity (D, n = 8), CS activity (E, n = 8), and NAD(P)H oxidase activity (F, n = 7) in the skeletal muscle after a 4-week intervention of DR with an insulin sensitizing drug in diet-induced diabetic mice. Results are presented as means ± SE. *, $P < .05$ vs ND; †, $P < .05$ vs DR; ‡, $P < .05$ vs DR+MET.

sistance by long-term DR did not completely reverse the exercise capacity and mitochondrial function. However, we could not exclude the effects of an even longer period of DR. Therefore, obesity and insulin resistance may be partially involved in the limited exercise capacity and impaired mitochondrial function in diabetic mice. Importantly, consistent with exercise capacity and mitochondrial function, DR with an insulin-sensitizing drug or long-term intervention did not affect the enhanced oxidative stress in the skeletal muscle of diabetic mice. In our previous study, treatment with antioxidants significantly improved the exercise capacity and mitochondrial function without affecting obesity and insulin resistance (18, 19). Thus, oxidative stress may be an important target for improving exercise capacity and mitochondrial function in type 2 diabetes.

In the present study, therapy with exercise or diet alone was effective in the improving exercise capacity, and the effects of these interventions were similar. Single therapy with EX had a greater effect on the mitochondrial function in the skeletal muscle, whereas single therapy with DR had a greater effect on obesity, insulin resistance, and hyperlipidemia. EX is known to improve energy metabolism in the skeletal muscle of diabetic subjects as well as the normal population (15), and therefore, it may improve exercise capacity via a favorable effect on the skeletal muscle mitochondrial function, which consequently leads to the partial improvement of obesity and insulin resistance. In contrast, the effects of DR on exercise capacity and skeletal muscle function in diabetes have been poorly understood. We have previously reported that abnormal lipid accumulation in the skeletal muscle is closely related to the limited exercise capacity and impaired muscle energy metabolism in patients with metabolic syndrome (13). Moreover, it has been previously determined that diet intervention in diabetic patients could reduce lipid accumulation in the skeletal muscle, which occurs independently of an improvement in mitochondrial function (31, 35). In the present study, we found that DR ameliorated excessive lipid accumulation in the skeletal muscle of diabetic mice. Therefore, DR may improve exercise capacity via a favorable alternation in substrate metabolism, especially lipid metabolism, as a consequence of the improvement in obesity and insulin resistance. Thus, EX and DR may improve exercise capacity in diabetic mice via partially different mechanisms, and a combined intervention may have an additive effect.

Several limitations of the present study need to be acknowledged. First, we showed that the effects of EX and/or DR on the exercise capacity and mitochondrial function were associated with decrease in oxidative stress. However, we were unable to elucidate the cause and effect relationship. Second, we showed that single therapy with exercise or diet could partially improve exercise capacity;

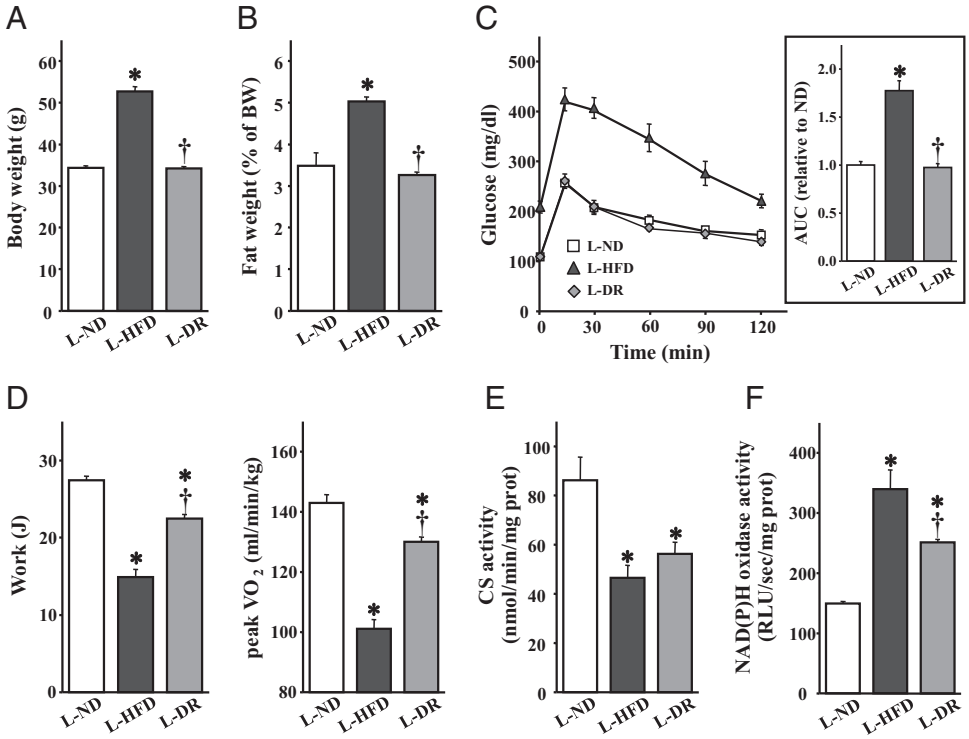

Figure 7. Normalization of obesity and insulin resistance do not require improving exercise capacity and skeletal muscle function in diet-induced diabetic mice. Body weight (A, n = 8), fat weight (B, n = 8), glucose levels during IPGTT and its AUC (C, n = 8), exercise capacity (D, n = 8), CS activity (E, n = 6), and NAD(P)H oxidase activity (F, n = 6) in the skeletal muscle after an 8-week intervention of DR in diet-induced diabetic mice. Results are presented as means ± SE. *, $P < .05$ vs L-ND; †, $P < .05$ vs long-term control (L-HFD).

however, the difference in the biological mechanisms between exercise and diet on exercise capacity and skeletal muscle function could not be determined in detail. Third, the limited exercise capacity in diabetes is often associated with cardiac dysfunction (36). Paulino et al (37) showed that the improvement in exercise capacity by EX and/or DR in diet-induced diabetic rats is associated with increased cardiac function. In the present study, however, cardiac function in diet-induced diabetic mice did not decrease, as shown by echocardiography. Thus, the cardiac function of the mice had an insignificant effect on the results of present study. Finally, swimming training is often considered an activity with the beneficial effects of cold exposure. Cold exposure increases the expression of cold stress-inducible factors, such as uncoupling protein (UCP) in the adipose tissue (38), and an increase in these factors is important in the regulation of obesity and insulin resistance via an increase in the energy expenditure in humans and rodents (39, 40). Thus, we measured the gene expression of the UCP family in white and brown adipose tissues after a single swimming session. No significant difference in the UCP family gene expressions were noted in both adipose tissues between the mice who had participated in the swimming exercise and those who did not (Supplemental Figure 9). This finding suggests that acute exposure to water does not increase cold stress-inducible factors. However, we did not measure the gene expression of the UCP family in the adipose tissues after prolonged swimming training, because chronic EX also increases gene expression of UCP family in both adipose tissues (41). Thus, we could not completely exclude the effect of cold exposure on some of the beneficial effects of swimming training.

Lifestyle intervention is the recommended first-line treatment in patients with type 2 diabetes. Clinically, the improvement in glucose tolerance in many diabetic patients could be achieved by using a variety of drug therapies. In the present study, we showed that insulin-sensitizing drugs could not effectively improve the limited exercise capacity in diabetes despite normalization of insulin resistance. Moreover, we determined that intervention with either exercise or diet could not completely improve the limited exercise capacity, which is independent of obesity and insulin resistance. In contrast, we clearly provided the significance of a combination of EX and DR in improving exercise capacity. Given the close association between exercise capacity and prognosis, the combination of EX and DR would be necessary for the treatment of diabetes.

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