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PPARδ Is Required for Exercise to Attenuate Endoplasmic Reticulum Stress and Endothelial Dysfunction in Diabetic Mice

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Physical activity has profound benefits on health, especially on cardiometabolic wellness. Experiments in rodents with trained exercise have shown that exercise improves vascular function and reduces vascular inflammation by modulating the balance between nitric oxide (NO) and oxidative stress. However, the upstream regulator of exercise-induced vascular benefits is unclear. We aimed to investigate the involvement of peroxisome proliferator–activated receptor δ (PPARδ) in exercise-induced vascular functional improvement. We show that PPARδ is a crucial mediator for exercise to exert a beneficial effect on the vascular endothelium in diabetic mice. In *db/db* mice and high-fat diet–induced obese mice, 4 weeks of treadmill exercise restored endothelium-dependent vasodilation of aortas and flow-mediated vasodilation in mesenteric resistance arteries, whereas genetic ablation of Ppard abolished such improvements. Exercise induces AMPK activation and subsequent PPARδ activation, which help to reduce endoplasmic reticulum (ER) and oxidative stress, thus increasing NO bioavailability in endothelial cells and vascular tissues. Chemical chaperones 4-phenylbutyric acid and tauroursodeoxycholic acid decrease ER stress and protect against endothelial dysfunction in diabetic mice. The results demonstrate that PPARδ-mediated inhibition of ER stress contributes to the vascular benefits of exercise and provides potentially effective targets for treating diabetic vasculopathy.

Cardiovascular disease (CVD), which is among the top causes of mortality and disability worldwide, is attributed primarily to the high prevalence of obesity and diabetes (1). Existing epidemiological studies have long highlighted the protective benefits of exercise against CVD in healthy individuals and in patients with hypertension, type 2 diabetes, or chronic heart disease (2,3). Physical exercise, with its vasoprotective outcomes, has been suggested to rival or even outperform CVD medication (4,5).

The endoplasmic reticulum (ER) is a crucial organelle responsible for protein folding, maturation, quality control, and trafficking (6). Only properly folded proteins can be destined to cellular organelles or cell surface; nevertheless, misfolded or unfolded proteins are retained in the ER to eventually be degraded (7). Disruption of the aforementioned processes causes retention of newly synthesized unfolded proteins in the ER, and this condition is defined as ER stress. In response to ER stress, the three branches of the unfolded protein response are activated through the initiation of three ER membrane–associated proteins—PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6)—and engaged in complex downstream signaling pathways (6,8). The transcriptional factors downstream of these proteins collectively induce unfolded protein response target genes involved in protein synthesis,

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oxidative stress, inflammation, and apoptosis. ER stress markers are activated at atherosusceptible arterial sites (9) in *ob/ob* mice and in mice fed a high-fat diet (10). Of note, many risk factors that induce endothelial dysfunction and vascular inflammation, such as high glucose (11), free fatty acid (12), and oxidized and glycated LDL (13), can also induce ER stress, suggesting that ER stress may be a potential mechanism underlying diabetic vasculopathy.

Although in humans it is well known that exercise training benefits endothelial function, the underlying cellular mechanisms are not fully elucidated. In mice, the increase of nitric oxide (NO) bioavailability and reduction of reactive oxygen species (ROS) level by moderate exercise inhibit the development of atherosclerosis in mice (14). Exercise reduces oxidative stress by inducing antioxidative enzymes, including uncoupling protein 1, mitofusin-2, and Nrf2 (15,16). Of note, exercise augments shear stress in the blood vessels, which is the primary stimulant for the release of NO in endothelial cells (17). The vascular benefits of exercise are mainly focused on shear stress–induced vasodilatation, which is largely attributed to the increment of NO production and release as well as diminishment of ROS formation, but detailed signaling pathways remain to be further explored.

Studies on the effect of exercise on metabolic reprogramming in skeletal muscle have revealed several transcription factors and serine-threonine kinases (18,19), including AMPK, which is the major enzyme responsible for glucose homeostasis and energy balance and important for endothelial cell function (20,21). AMPK has been reported to interact with peroxisome proliferator–activated receptors (PPARs) to improve lipid metabolism during exercise (22). AMPK activation improves vascular and metabolic functions because it is a natural suppressor of ER stress (13), and it forms a transcriptional complex with PPARδ to induce the transcription of several PPAR target genes in skeletal muscle (23). We have shown that PPARδ is an essential mediator for metformin (AMPK activator) to reduce ER stress and protect endothelial function in the aortas of obese mice (24), which led us to postulate that the vascular benefit of exercise also depends on PPARδ. The purpose of the current study was to evaluate the impact of exercise training on vascular ER stress and to investigate whether exercise training ameliorates endothelial dysfunction in diabetic mice through stimulating the AMPK-PPARδ signaling cascade with the use of *db/db* mice and *Ppard*-deficient mice.

RESEARCH DESIGN AND METHODS

Animal Experiments

The use of animals for these experiments was approved by the Animal Research Ethical Committee of the Chinese University of Hong Kong (CUHK) and was consistent with the Guide for the Care and Use of Laboratory Animals. Animals were male leptin receptor–deficient *db/db* (homozygous) and age-matched *db/m*$^+$ heterozygous mice generated from the C57BL/KsJ, male C57BL/6J mice, male *Ppard* knockout (KO) mice, and age-matched *Ppard* wild-type (WT) littermates generated from the C57BL/6N × 129/Sv background (25). All animals were supplied by the CUHK Laboratory Animal Service Center and were housed in animal holding rooms with controlled temperature (22–23°C) with an alternating 12-h light/dark cycle. Diet-induced obese (DIO) mice were generated by feeding *Ppard* KO and age-matched *Ppard* WT littermates at the age of 6 weeks a high-fat diet for 10–12 weeks (Rodent Diet with 45 kcal% fat, D12451; Research Diets, New Brunswick, NJ). Male *db/m*$^+$ and *db/db* mice at 12–14 weeks were randomly divided into several groups and administered orally with 4-phenylbutyric acid (PBA) 100 mg/kg/day (Sigma-Aldrich, St. Louis, MO), tauroursodeoxycholic acid (TUDCA) 100 mg/kg/day (Sigma-Aldrich), or vehicle for 4 weeks.

Exercise Protocol

Male *db/m*$^+$, *db/db*, *Ppard* WT, and *Ppard* KO mice aged 11–13 weeks were randomly divided into several groups and trained to run on a motorized treadmill exercise system. To allow for animal acclimatization, exercise duration and intensity were gradually increased over the first week of exercise exposure. The exercise speed was initially set at 5 m/min for 30 min and then incrementally increased to the target speed at 8 m/min for 30 min (240 m total). This exercise intensity was well below the tolerance level of the mouse (26). Mice were exercised daily, 6 days/week, for 4 weeks after 1 week of training (5 weeks total). Sedentary groups were placed on a nonmoving treadmill for the same duration as the exercise groups.

Isometric Force Measurement in Wire Myograph

After animals were sacrificed, thoracic aortas and mesentery arteries were rapidly removed and placed in oxygenated ice-cold Krebs-Henseleit solution. Segments of mouse aortas or mesenteric arteries were mounted to a Multi Wire Myograph System (Danish Myo Technology, Aarhus, Denmark), and changes in isometric tension were measured as previously described (27). Endothelium-dependent relaxation (EDR) was determined by cumulative addition of acetycholine (ACh) (3 nmol/L to 10 μmol/L) in phenylephrine (Phe) (3 μmol/L) precontracted segments. In some mesenteric rings, insulin (1 nmol/L to 1 μmol/L)-induced relaxations were also assessed. Some segments were incubated with thapsigargin (0.1 μmol/L), an ER stress inducer (Tocris Bioscience), or L-α-lysophosphatidylcholine (LPC) (30 μmol/L), a major component of oxidized LDL, for 30 min before EDR measurement. Sodium nitroprusside (SNP) (1 nmol/L to 10 μmol/L), an exogenous NO donor, was used to test endothelium-independent relaxation. All the chemicals were from Sigma-Aldrich except thapsigargin which was purchased from Tocris Bioscience.

Flow-Mediated Dilatation in Pressure Myograph

Segments of mesenteric resistance arteries (second order), dissected free of surrounding adipose tissue, were cannulated

between two glass cannulas with tip diameter <100 μm in a chamber filled with 10 mL oxygenated Krebs solution for measuring flow-mediated dilatation (FMD) (28). The cannulated artery was connected to independent Krebs solution with 1% BSA. The intraluminal pressure was monitored by the pressure myograph system (Model 110P; Danish Myo Technology), and vessel diameter were monitored by a light-inverted microscope (Axiovert 40; Carl Zeiss) with video camera and MyoVIEW software (Danish Myo Technology). Under a no-flow condition, the artery segment was subjected to a stepwise increment of 20 mmHg in intraluminal pressure from 20 to 80 mmHg at 5-min intervals at 37°C. Phe (3 μmol/L) was added to induce vasoconstriction after the vessel's diameter stabilized, and FMD was triggered by a pressure change equal to ~15 dynes/cm^2 shear stress. Passive dilatation was obtained at the end of the experiment by changing the bathing solution to Ca^{2+}-free Krebs solution with 2 mmol/L EGTA. FMD was calculated as the percentage of diameter changes: (flow-induced dilatation − Phe tone)/(passive dilatation − Phe tone).

Ex Vivo Culture of Mouse Aortas

Mouse thoracic aortic segments were dissected in sterile PBS and incubated in DMEM (Gibco, Gaithersburg, MD) supplemented with 10% FBS (Gibco) plus 100 IU/mL penicillin and 100 μg/mL streptomycin. Tunicamycin (2 μg/mL), an ER stress inducer; PBA (10 μmol/L); TUDCA (20 μmol/L); adenovirus Ad-CA-PPARδ (10^6 plaque-forming units [pfu]/aortic segment); GSK0660 (500 nmol/L); and ROS scavengers, including tempol (100 μmol/L), tiron (1 mmol/L) plus diethyldithiocarbamate (DETCA) (100 μmol/L), diphenyleneiodonium (100 nmol/L), and apocynin (100 μmol/L), were added individually into the culture medium that bathed aortic rings in an incubator at 37°C for 16 or 24 h. After the incubation period, segments were transferred to fresh Krebs solution for functional studies in a wire myograph and for Western blotting. All the chemicals in this experiment were from Sigma-Aldrich.

Primary Culture of Mouse Aortic Endothelial Cells

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Heparin (100 units/mL in PBS) was infused from the left ventricle into the circulation. Aortas were removed and dissected in sterile PBS. Incubating the aortas with collagenase type I at 37°C for 8 min detached the endothelial cells, which then were collected by centrifugation, resuspended, and cultured in endothelial cell growth medium supplemented with bovine brain extract (Lonza; Walkersville, MD) until 80–90% confluency. The identity of mouse aortic endothelial cells (MAECs) was verified by a positive staining of endothelial nitric oxide synthase (eNOS) (BD Transduction Laboratories, San Diego, CA) and a negative staining of α-smooth muscle actin (Abcam, Cambridge, U.K.).

Western Blot Analysis

Protein samples prepared from mouse aorta homogenates underwent SDS-PAGE and were transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). One percent BSA in 0.01% Tween-20 PBS was used to block nonspecific binding sites, and the membranes were probed with primary antibodies against phospho (p)-AMPKα at Thr^{172}, AMPKα (Cell Signaling Technology, Danvers, MA), ATF6 (Abcam), p-eIF2α at Ser^{52} (Invitrogen, Carlsbad, CA), eIF2α, p-JNK at Thr^{183}/Tyr^{185}, JNK (Cell Signaling), ATF3 (Santa Cruz Biotechnology, Dallas, TX), PPARδ (Cayman Chemical, Ann Arbor, MI), p-eNOS at Ser^{1177} (Abcam), p-eNOS at Ser^{633}, eNOS (BD Transduction Laboratories), caspase-3 (Abcam), and GAPDH (Ambion, Austin, TX) at 4°C overnight followed by incubation with horseradish peroxidase–conjugated secondary antibodies (DakoCytomation, Carpinteria, CA). The membranes were developed with an enhanced chemiluminescence detection system (ECL reagents; Amersham, Buckinghamshire, U.K.) and exposed on X-ray films.

Detection of ROS by Dihydroethidium Staining

Aortic segments were fresh-frozen in optimal cutting temperature compound and sectioned (10 μm) by using a Leica CM 1000 cryostat before incubation in dihydroethidium (DHE) (Ex515/Em585nm, 5 μmol/L; Invitrogen)-containing normal physiological saline solution at 37°C for 15 min. The normal physiological saline solution contained (in mmol/L) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 5 HEPES (pH 7.4). Fluorescence images were obtained with a confocal system by measuring the fluorescence intensity.

Measurement of NO Production in MAECs

Intracellular NO level was determined by using an NO-sensitive dye (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate [DAF-DA], Ex495/Em515nm; Molecular Probes) (29). The amount of NO in response to A23187 (1 μmol/L, calcium ionophore; Tocris Bioscience) was evaluated by measuring the fluorescence intensity with a confocal system. Changes in NO production were calculated as F1/F0, where F0 is the average fluorescence signals before addition of A23187, and F1 is after addition of A23187.

Statistical Analysis

All results are mean ± SEM from the various groups. Cumulative concentration-response curves were analyzed by determining the area under curve. Protein expression was quantified by densitometer (FluorChem; Alpha Innotech, San Leandro, CA) normalized to GAPDH and then compared with control. Data were analyzed using GraphPad Prism software. Comparisons among groups were made using one-way ANOVA followed by Tukey test for multiple comparison. The results were considered statistically significant between groups with $P < 0.05$.

RESULTS

Exercise Training Improves Vascular Functions of *db/db* Mice

To examine the vascular benefit of exercise, *db/db* mice and age-matched control mice were subjected to treadmill

running for 4 weeks. Regular running exercise markedly improved EDRs in *db/db* mouse aortas, whereas it had no effect on nondiabetic *db/m*$^+$ mice (Fig. 1*A* and *B*). Endothelium-independent relaxations to SNP were similar among the four groups (Fig. 1*C*). Moreover, aortas from exercised lean as well as exercised diabetic mice were protected against the effect of LPC (the major component of oxidized LDL cholesterol) (Fig. 1*D* and *E*) or thapsigargin (ER stress inducer) (Fig. 1*F* and *G*) than the sedentary control mice. We also studied the effect of exercise on small resistance arteries that are more important for vascular homeostasis than for conduit arteries. Exercise enhanced insulin-induced dilatation in main mesenteric arteries from *db/db* mice (Fig. 1*H*). The impaired FMDs in small resistance arteries from *db/db* mice were improved after exercise (Fig. 1*I* and *J*). Exercise slightly reduced the levels of total cholesterol and non-HDL cholesterol in *db/db* mice (Supplementary Table 1). Body mass, blood glucose, and plasma triglyceride concentration were unchanged.

Exercise Attenuates ER Stress and Inflammation

To investigate the mechanism of the vascular benefit of exercise, we collected aortas to determine whether NO pathway and ER stress are involved. Exercise restored the reduced phosphorylations of AMPKα at Thr^{172} and eNOS at Ser^{1177} (indicators of NO bioavailability) in aortas from *db/db* mice (Fig. 2*A*–*C*), whereas the total protein levels of AMPKα, eNOS, and p-eNOS Ser^{633} were not altered (Supplementary Fig. 1*A*–*C*). Plasma nitrite level was elevated after exercise in *db/db* mice (Supplementary Fig. 1*D*), indicating an enhanced NO bioavailability. The upregulations of ER stress markers, including phosphorylations of eIF2α at Ser^{52} and JNK at Thr^{183}/Tyr^{185}, ATF3, and cleaved ATF6, were also normalized by exercise (Fig. 2*A* and *D*). Exercise also attenuated the downregulation of

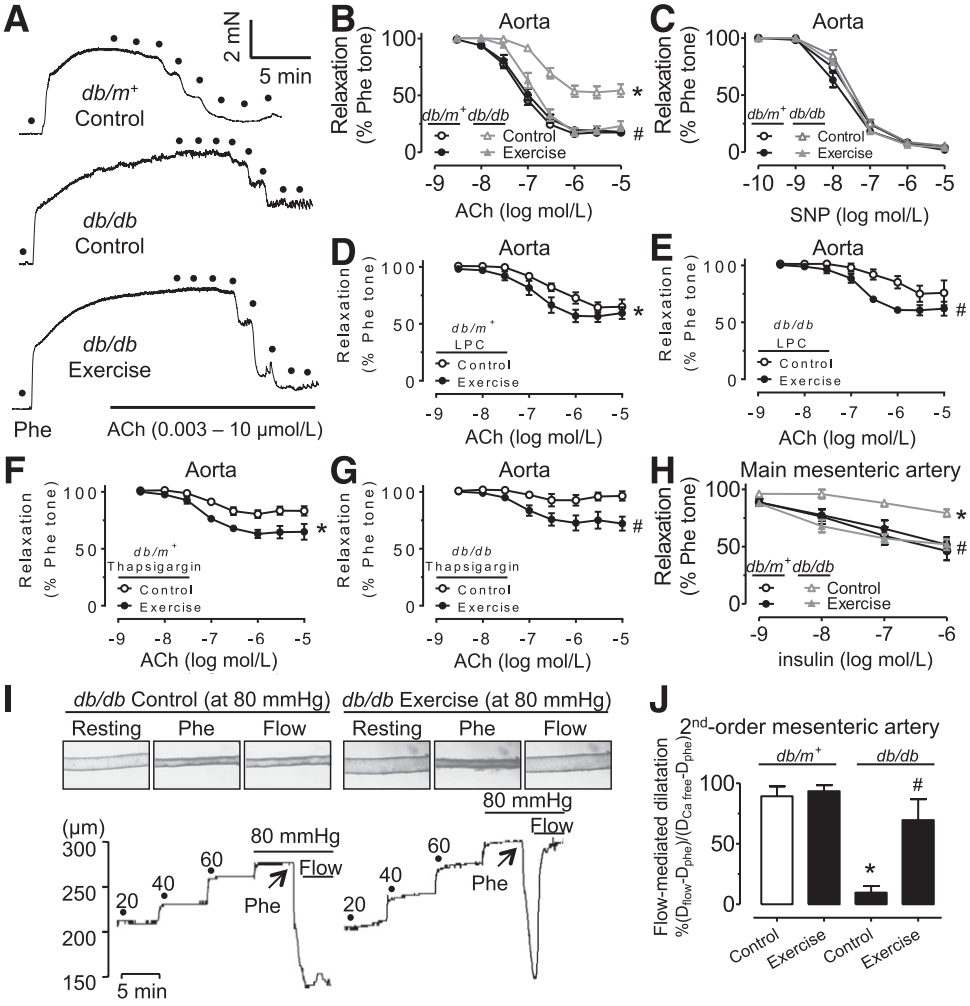

Figure 1—Vasoprotective effect of exercise in *db/db* mice. *A* and *B*: Effect of treadmill exercise on ACh-induced EDRs in mouse aortas. *C*: SNP-induced endothelium-independent relaxations in aortas. *D*–*G*: Effects of 30-min incubation with 30 μmol/L LPC and 0.1 μmol/L thapsigargin on relaxations in *db/m*$^+$ and *db/db* aortas. *H*: Insulin-induced relaxations in main mesenteric arteries from *db/db* mice. *I* and *J*: FMD in second-order mesenteric resistance arteries. Data are mean ± SEM from eight mice for each group. *$P < 0.05$ vs. *db/m*$^+$ control; #$P < 0.05$ vs. *db/db* control. See also Supplementary Fig. 1 and Supplementary Table 1.

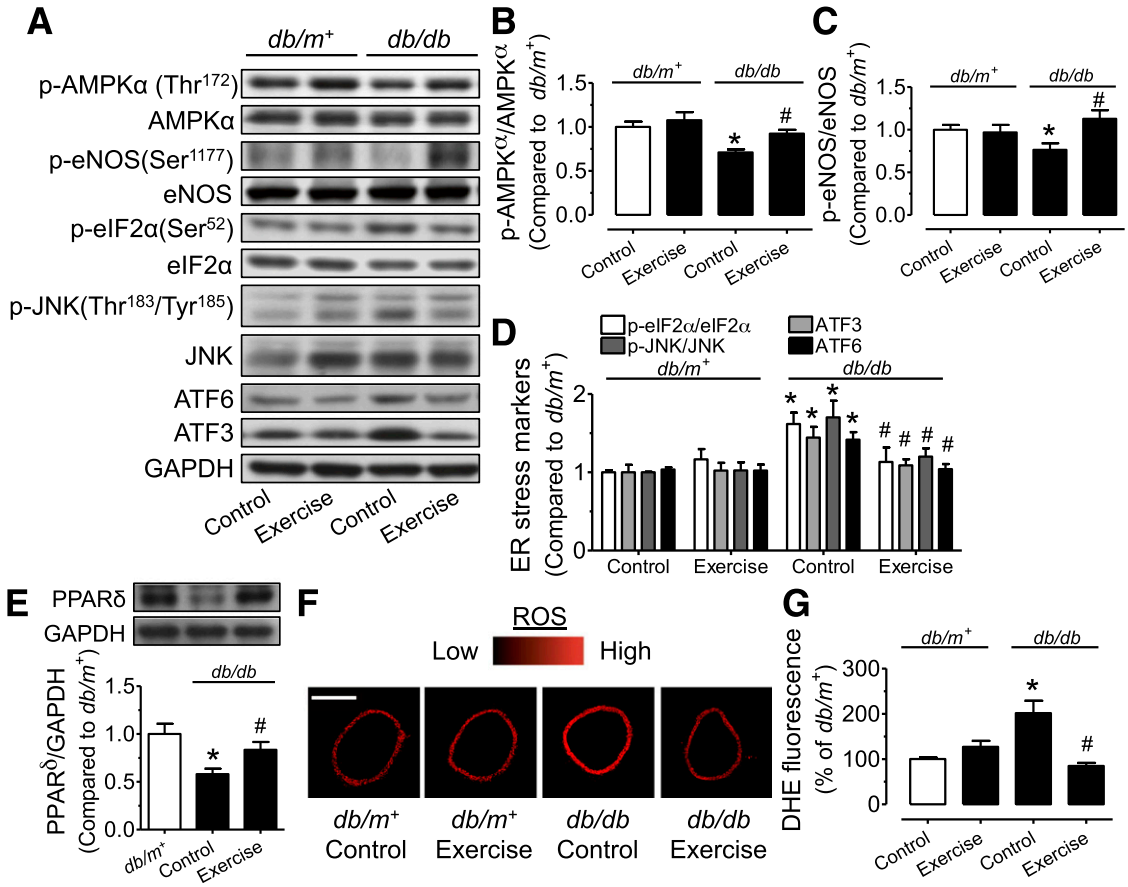

Figure 2—Exercise reduces ER stress and oxidative stress. *A–D*: Western blotting showing the phospho- and total AMPKα and eNOS, and the expressions of ER stress markers, including phospho- and total eIF2α and JNK, ATF3, and cleaved ATF6 in aortas from four groups of mice. *E*: Western blotting of PPARδ expression. *F* and *G*: Representative images and summarized graph showing DHE intensity (oxidative stress indicator) in mouse aortas. Scale bar, 0.5 mm. Data are mean ± SEM from eight mice for each group. *$P < 0.05$ vs. *db/m*$^+$ control; #$P < 0.05$ vs. *db/db* control. See also Supplementary Fig. 1.

PPARδ in aorta from *db/db* mice (Fig. 2*E*). To verify the contribution of PPARδ on vascular function, we examined EDRs by using adenovirus Ad-CA-PPARδ (30) and PPARδ antagonist GSK0660. Ad-CA-PPARδ (10^6 pfu/cell, 24 h) increased PPARδ protein expression in human umbilical vein endothelial cells (HUVECs) compared with Ad-GFP control (Supplementary Fig. 1*E*). Overexpressing PPARδ by Ad-CA-PPARδ (10^6 pfu/aortic segment, 24 h) ameliorated EDRs in aortas from *db/db* mice (Supplementary Fig. 1*F*). On the other hand, GSK0660 (500 nmol/L, 16 h) blocked the exercise-induced improvement of EDRs in *db/db* mice (Supplementary Fig. 1*G*). In addition, exercise reduced the elevated oxidative stress in *db/db* mouse aortas (Fig. 2*F* and *G*). We also looked at the effect of exercise on vascular inflammation. En face staining of the endothelial surface showed a reduction in vascular cell adhesion molecule 1 (VCAM-1), an inflammatory marker, in *db/db* mice after exercise (Supplementary Fig. 1*H* and *I*).

Induction of ER Stress Impairs Endothelial Function

Because we found that exercise reduced ER stress and improved NO bioavailability, we next examined whether the induction of ER stress directly on blood vessels is able to impair vascular function and whether inhibiting ER stress improves it in diabetic mice. We found that tunicamycin (an ER stress inducer, 16 h) impaired EDRs of C57 aortas (Supplementary Fig. 2*A*), which was reversed by cotreatment with ER stress alleviators PBA and TUDCA (Supplementary Fig. 2*B*). Induction of ER stress also generated ROS (measured by DHE fluorescence and chemiluminescence) in C57BL/6J mouse aortas, which were inhibited by PBA and TUDCA (Supplementary Fig. 2*C* and *D*). Likewise, ROS scavengers, including diphenyleneiodonium (100 nmol/L), apocynin (100 μmol/L), tempol (100 μmol/L), and tiron (1 mmol/L) plus DETCA (100 μmol/L), improved EDRs impaired by tunicamycin (Supplementary Fig. 2*E* and *F*). Of note, tiron plus DETCA and tempol did not suppress ER stress markers (Supplementary Fig. 2*G* and *H*), suggesting that ROS was induced by ER stress in our experiments. Of note, tunicamycin (2 μg/mL, 16 h) did not trigger apoptosis as measured by cleaved caspase-3 in HUVECs (Supplementary Fig. 2*I*), indicating that tunicamycin did not induce cell death in the endothelium directly. Expressions of multiple genes related to ER stress were altered

in response to tunicamycin (4 h) in HUVECs (Supplementary Fig. 3*A*). More importantly, we found that PPARδ agonist GW1516 (1 μmol/L) suppressed the expression of tunicamycin-stimulated genes, including ATF4 (downstream of the PERK/eIF2α pathway), C/EBPB (regulates genes involved in immune and inflammatory responses), and DDIT3 (promotes apoptosis), but did not affect HERPUD1 (related to protein load) or XBP1 (downstream of IRE1 pathway) in HUVECs (Supplementary Fig. 3*B*–*F*), which were involved in the ER stress pathways.

Alleviation of ER Stress Prevents Diabetes-Associated Endothelial Dysfunction

Because we found that ER stress has a direct effect on vascular function ex vivo, we wondered whether administration of an ER stress inhibitor in vivo would improve endothelial function in diabetic mice. Oral gavage of PBA and TUDCA (10 mg/kg/day) for 4 weeks enhanced EDRs in *db/db* mouse aortas (Fig. 3*A*), with no effect on endothelium-independent relaxations to SNP (Fig. 3*B*). Insulin-induced dilatations were also augmented in mesenteric arteries of *db/db* mice (Fig. 3*C*). PBA and TUDCA reduced ER stress markers (p-eIF2α, ATF3, and ATF6) in *db/db* mouse aortas (Fig. 3*D* and *E*). Metabolic parameters, including total cholesterol, triglycerides, and non-HDL cholesterol, were moderately reduced after PBA or TUDCA treatment, whereas fasting glucose, body mass, and so forth were unaltered (Supplementary Table 2). We also found that mouse aortas exposed to high glucose (30 mmol/L, 48 h) had impaired EDRs that were restored by cotreatment with PBA or TUDCA (Fig. 3*F*). In addition, both ex vivo high glucose exposure and in vivo (*db/db* mice) treatment-induced ROS production were suppressed by PBA or TUDCA in mouse aortas (Supplementary Fig. 4*A* and *B*). Similarly, NO production in response to A23187 (1 μmol/L) was blunted in primary MAECs by high glucose (Fig. 3*G* and *H*). Treatment of ER stress alleviators (10 μmol/L PBA or 20 μmol/L TUDCA) restored the decreased NO production. Likewise, tunicamycin attenuated NO production in MAECs, and this effect was inhibited by PBA or TUDCA (Supplementary Fig. 4*C* and *D*).

Exercise Ameliorates Endothelial Function and Oxidative Stress Through PPARδ-Dependent Mechanisms

Previous reports suggested that PPARδ is the major regulator of metabolic effects of exercise (23,31). However, less is known in the vasculature. We studied whether the vascular effects of exercise are blunted in *Ppard* KO mice. *Ppard* WT and KO mice were fed a high-fat diet for 12 weeks and exercised for 4 weeks. Genotyping verified the identity of *Ppard* WT and KO mice (Supplementary Fig. 5*A*). DIO *Ppard* WT and KO mice had impaired EDRs

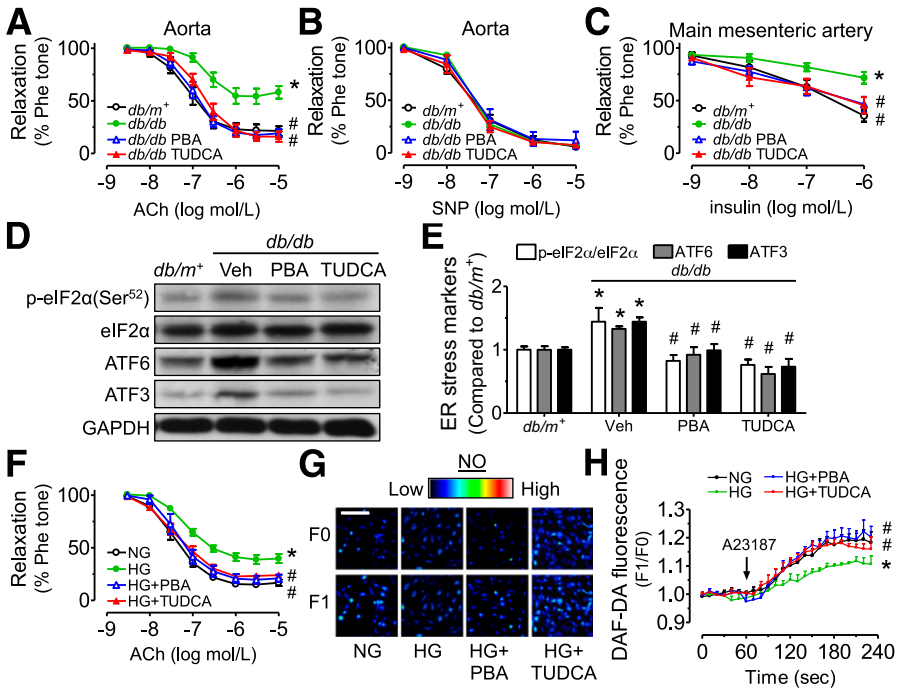

Figure 3—Alleviation of ER stress protects endothelial function in diabetic mice. *A*: Effect of oral administration of PBA or TUDCA (100 mg/kg/day, 4 weeks) on ACh-induced relaxation in *db/db* mouse aortas. *B*: SNP-induced endothelium-independent relaxations in aortas. *C*: Insulin-induced relaxations in main mesenteric arteries. *D* and *E*: The expressions of ER stress markers eIF2α, ATF3, and cleaved ATF6 in aortas from *db/db mice*. *F*: Effect of pretreatments with high glucose (HG) (30 mmol/L, 48 h) and cotreatment with PBA (10 μmol/L) or TUDCA (20 μmol/L) on ACh-induced relaxations in C57BL/6J mouse aortas. *G* and *H*: Representative images and summarized data showing that PBA (10 μmol/L) and TUDCA (20 μmol/L) rescue NO production (DAF-DA fluorescence) of primary MAECs in response to 1 μmol/L A23187 impaired by HG. Scale bar, 0.2 mm. Data are mean ± SEM from eight mice for each group (*A*–*E*) or from five experiments (*F*–*H*). **P* < 0.05 vs. *db/m*$^+$ or normal glucose (NG) (5 mmol/L); #*P* < 0.05 vs. *db/db* or HG. See also Supplementary Table 2. Veh, vehicle.

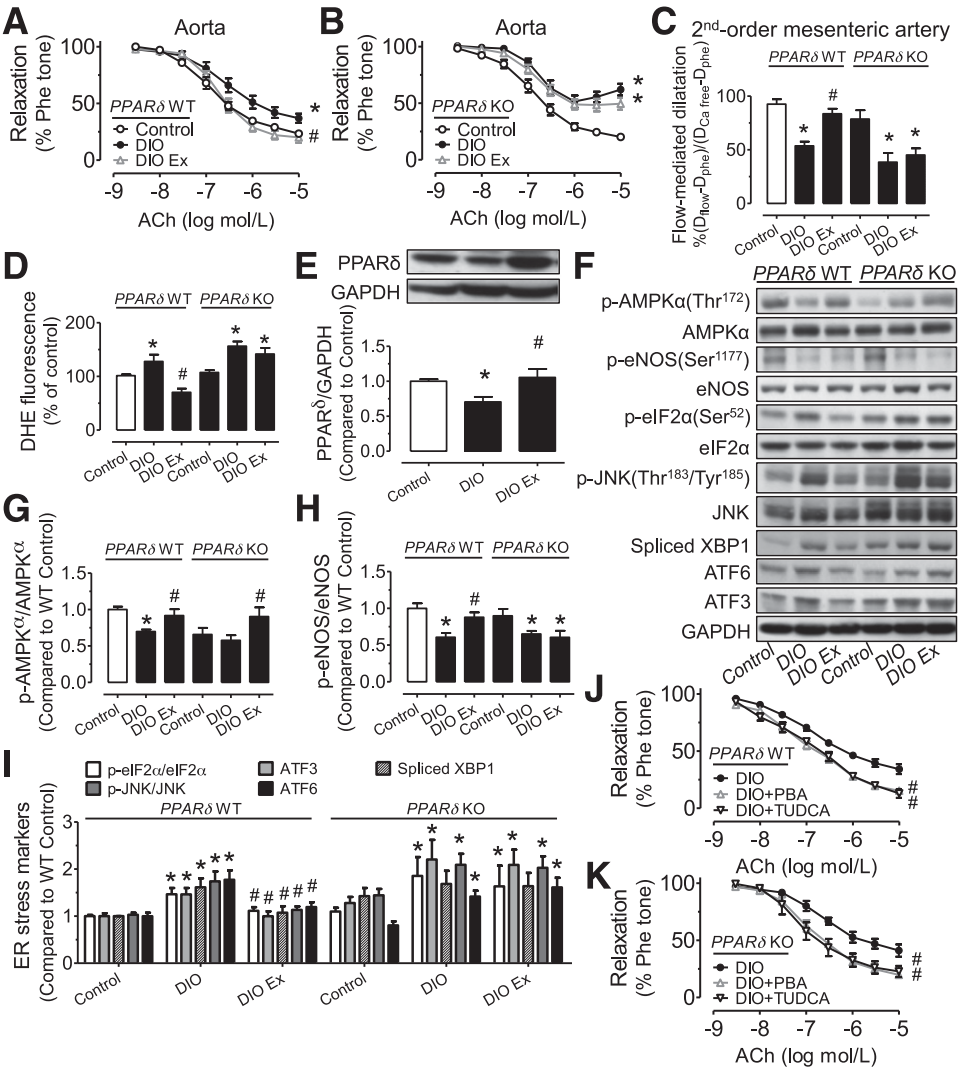

Figure 4—Role of PPARδ in exercise-induced vascular benefit in DIO mice. *A* and *B*: Effect of exercise for 4 weeks on ACh-induced relaxation of aortas of *Ppard* WT and KO mice. *C*: Effect of exercise on FMD in second-order mesenteric resistance arteries. *D*: Effect of exercise on oxidative stress in DIO mouse aortas. *E*: Western blotting of PPARδ expression in aortas from DIO mice after exercise. *F–I*: Western blotting of AMPKα, eNOS, eIF2α, spliced XBP1, JNK, ATF3, and cleaved ATF6 in mouse aortas. *J* and *K*: Effect of PBA (10 μmol/L) or TUDCA (20 μmol/L) treatment for 16 h on ACh-induced relaxations in DIO *Ppard* WT and KO mouse aortas. Data are mean ± SEM from eight mice for each group. *$P < 0.05$ vs. control; #$P < 0.05$ vs. DIO from each genotype. See also Supplementary Fig. 5 and Supplementary Table 3. Ex, exercise.

in aortas. Exercise training ameliorated endothelial dysfunction in aortas from DIO *Ppard* WT mice (Fig. 4*A*) but not from *Ppard* KO mice (Fig. 4*B*). A similar effect was observed for the FMD in mesenteric resistance arteries (Fig. 4*C*). Compared with DIO *Ppard* WT mice, the impairment of EDR was aggravated at the maximal dose of ACh (10 μmol/L) in DIO *Ppard* KO mice (Supplementary Fig. 5*B*). One possibility for the occurrence of the small contraction induced in EDR at high doses of ACh in DIO *Ppard* KO mice might be the release of endothelium-derived contracting factors under diabetic conditions (32), which remains unclear and needs future work to address this in greater detail. Exercise also reduced ROS in DIO *Ppard* WT mice but not in *Ppard* KO mice (Fig. 4*D*). Although the total cholesterol, triglycerides, and non-HDL cholesterol were lower in *Ppard* KO than in *Ppard* WT mice, no difference was found between the sedentary and exercise groups in the same genotype (Supplementary Table 3). Regular exercise for 4 weeks moderately reduced blood glucose but had no effect on either body mass or plasma lipids.

Exercise Suppresses ER Stress Through PPARδ

We wondered whether the inhibition on ER stress is also PPARδ dependent. The reduced PPARδ expression in aortas from DIO *Ppard* WT mice was enhanced with exercise (Fig. 4*E*). Exercise activated AMPK in aortas from both DIO *Ppard* WT and *Ppard* KO mice. Reduced p-eNOS and upregulations of p-eIF2α, p-JNK, spliced XBP1, ATF3, and cleaved ATF6 were all normalized in *Ppard* WT after exercise but not in *Ppard* KO mice (Fig. 4*F–I*). However, the anti-inflammatory effect of exercise was independent of

PPARδ because reduction of VCAM-1 level was observed in both DIO *Ppard* WT and *Ppard* KO mice (Supplementary Fig. 5*C*). Unlike exercise, PBA (10 μmol/L) or TUDCA (20 μmol/L) treatment for 16 h restored EDRs in aortas from both DIO *Ppard* WT and *Ppard* KO mice (Fig. 4*J* and *K*). Endothelium-independent relaxations to SNP were similar (Supplementary Fig. 5*D* and *E*). More representative blots for ER stress markers in individual animals are shown in Supplementary Figs. 6 and 7.

DISCUSSION

The current study used multiple approaches aided by the use of type 2 *db/db* mice and *Ppard* KO mice to elucidate whether PPARδ plays an essential role in exercise-induced suppression of ER stress and protection of vasodilatation. Exercise ameliorates impaired EDRs in conduit aortas and mesenteric resistance arteries in both diabetic and obese mice accompanied by increases of vascular AMPK and eNOS activities and inhibition of ER stress, vascular inflammation, and oxidative stress. Exercise in *Ppard* KO and WT mice shows that PPARδ is a significant contributor to the beneficial effects of exercise on vascular function in obese mice by attenuating ER stress and oxidative stress. Collectively, the data suggest that PPARδ acts as the major regulator in response to exercise to restore vascular homeostasis in diabetes and obesity.

Earlier work by others showed that exercise helps to prevent CVD in patients with diabetes and diabetic animals (2,3) through increasing NO bioavailability (14) and limiting oxidative stress (15,16). A later study discovered AMPK activation by exercise as a result of an increase in the cellular AMP/ATP ratio (33). Phosphorylation/activation of eNOS is known to be triggered by AMPK (34). AMPK also mitigates ROS production associated with diabetes (35,36). A genetic study in humans demonstrated that exercise induces epigenetic modifications, particularly DNA methylation changes of PRKAB1 (the gene encoding the AMPK subunit), indicating that exercise modulates AMPK in humans (37). We also show that 4-week exercise in diabetic mice enhances EDRs without changing endothelium-independent relaxation of vascular smooth muscle in both conduit and resistance arteries, and this improvement was accompanied by increased AMPK and eNOS activities and decreased ROS production in the vasculature. Chronic low-grade inflammation actively participates in the development of vascular dysfunction associated with diabetes and obesity. Atherosclerotic lesions are preferentially developed at arterial branches and curvatures where disturbed and reciprocating flow is commonly present (38,39). Disturbed flow induces expressions of proatherogenic genes in endothelial cells, mainly chemokines and adhesion molecules such as VCAM-1, intracellular adhesion molecule 1, and MCP-1 (40). Such changes are accompanied by downregulation of antioxidative enzymes and reduced production and function of vasodilators (NO and prostacyclin) (41). On the contrary, laminar flow produces an opposing effect by upregulation of antiatherogenic genes, which are beneficial for endothelial cells (42). Of note, exercise enhances laminar flow (17). Consistent with this, we show that VCAM-1 upregulation in the thoracic aortas of diabetic mice is ameliorated by exercise. However, whether PPARδ is involved in the mechanism underlying the anti-inflammatory effect of exercise in the vasculature needs further exploration.

We also looked at whether a common initiator induces vascular dysfunction by modulating NO and ROS in the vasculature and whether it is a target of physical exercise. Growing evidence suggests that ER stress is a central feature of insulin resistance in diabetes and obesity (10). Therefore, we examined all three branches of ER stress: eIF2α and ATF3 downstream of the PERK pathway, XBP1 and JNK activated by autophosphorylated IRE1, and cleaved active ATF6. Exercise is effective at suppressing all three pathways of ER stress in diabetic mice. We then show that ER stress induction directly impairs endothelial function accompanied by elevated ROS generation and reduced NO production in aortas and endothelial cells. More importantly, alleviation of ER stress in vivo and ex vivo inhibited the vascular oxidative stress and rescued endothelial function in diabetic mice. These data indicate the causative role of ER stress in vascular dysfunction of diabetic mice. Of note, ROS scavengers did not suppress ER stress markers, so ER stress in the diabetic mouse vasculature is upstream of ROS generation. The data show no obvious changes of glucose metabolism with chronic oral treatment of PBA or TUDCA, which are contradictory with a previous study that showed that PBA and TUDCA improves glucose metabolism in *ob/ob* mice (43). This may be explained by the difference in treatment duration, dosage (10 times less in the current study), and animal models used. However, PBA and TUDCA lower the plasma level of triglycerides, and both effects might partially contribute to the vasoprotective effect of ER stress inhibition. Nevertheless, the direct effect of PBA and TUDCA on isolated aortas where ambient insulin and lipid levels are constant suggests that the contribution of lipid modulation should be minimal. Exercise protects endothelial function by counteracting the heightened ER stress and oxidative stress in diabetic mice or in the presence of thapsigargin or LPC.

AMPK is a physiological suppressor of ER stress (13,44) and incorporates PPARδ to regulate gene transcription in response to exercise (23). PPARδ activation by GW1516 increases insulin sensitivity (45) and protects endothelial function in diabetes (46). Our previous study also supported that PPARδ mediates the effect of metformin (AMPK activator) to attenuate ER stress and subsequently endothelial dysfunction in aortas from obese mice (24). Indeed, we found that PPARδ was upregulated in exercised diabetic mice and that PPARδ overexpression was able to rescue ER stress–induced endothelial dysfunction. More importantly, PPARδ antagonist and PPARδ deletion abolished the effect of exercise on relaxation, ER stress, ROS, and NO bioavailability without affecting AMPK activation in arteries. This provides solid evidence

that supports PPARδ as indispensable in the cellular responses to exercise for inhibition of ER stress and oxidative stress, thus favoring NO production in endothelial cells. PPARδ agonist directly affected the suppression of the transcription of ATF4, C/EBPB, and DDIT3 but had no impact on HERPUD1 or XBP1. We cannot rule out the possibility that PPARδ may have an effect on genes related to ER stress after interacting with other activated proteins, although it does not work alone. A previous report suggested that AMPKα and PGC1α cooperatively interact with PPARδ to further induce transcriptional activity (23). ER stress alleviators restored relaxation in obese *Ppard*-deficient mice, which supports that ER stress alleviation is downstream of PPARδ activation, contributing to the vascular benefit of exercise. Excessive nutrients, such as glucose and lipids, in diabetes are common risk factors that cause vascular complications (47). Exercise reduces blood glucose and plasma cholesterol levels. Furthermore, exercise increased brown fat mass in both DIO *Ppard* WT and KO mice in the current study (data not shown), whereas browning of adipose tissue is accompanied by an increase in energy expenditure (48). The protective effect of exercise against endothelial dysfunction might be partially due to its favorable modulation of glucose and lipid metabolism. Both animal and clinical studies have reported that PPARs benefit vascular function and that PPARs can become potential useful targets for pharmaceutical intervention in the protection against vascular complications in diabetes (49–51). Given the publicized adverse effect of the PPARγ agonist rosiglitazone on cardiovascular outcomes in patients with diabetes (52,53) and the obligatory role of PPARδ on the vascular benefits of exercise, PPARδ could be a hopeful alternative target for the treatment of cardiovascular events in diabetes.

In conclusion, the current study provides new insights into the benefits of physical exercise on vascular health by reducing ER stress and oxidative stress and enhancing eNOS activity and NO production. This study also identifies PPARδ as a critical mediator for the vascular benefits of exercise to restore endothelial function in obesity and diabetes. Clinical studies have shown different outcomes of exercise and diabetic drug intervention in patients with type 2 diabetes probably due to variances of study duration, the intensification of medication overtime, and so forth (5,54). The current novel findings should help to arouse public awareness about the importance of physical activity to prevent or delay the onset of diabetes and obesity-related vasculopathy. The AMPK/PPARδ signaling cascade is a potentially effective target in response to orally active antidiabetic drugs in the treatment of vascular and metabolic diseases.

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