Saxagliptin Restores Vascular Mitochondrial Exercise Response in the Goto-Kakizaki Rat

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Abstract: Cardiovascular disease risk and all-cause mortality are largely predicted by physical fitness. Exercise stimulates vascular mitochondrial biogenesis through endothelial nitric oxide synthase (eNOS), sirtuins, and PPARγ coactivator 1α (PGC-1α), a response absent in diabetes and hypertension. We hypothesized that an agent regulating eNOS in the context of diabetes could reconstitute exercise-mediated signaling to mitochondrial biogenesis. Glucagon-like peptide 1 (GLP-1) stimulates eNOS and blood flow; we used saxagliptin, an inhibitor of GLP-1 degradation, to test whether vascular mitochondrial adaptation to exercise in diabetes could be restored. Goto-Kakizaki (GK) rats, a nonobese, type 2 diabetes model, and Wistar controls were exposed to an 8-day exercise intervention with or without saxagliptin ($10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). We evaluated the impact of exercise and saxagliptin on mitochondrial proteins and signaling pathways in aorta. Mitochondrial protein expression increased with exercise in the Wistar aorta and decreased or remained unchanged in the GK animals. GK rats treated with saxagliptin plus exercise showed increased expression of mitochondrial complexes, cytochrome c, eNOS, nNOS, PGC-1α, and UCP3 proteins. Notably, a 3-week saxagliptin plus exercise intervention significantly increased running time in the GK rats. These data suggest that saxagliptin restores vascular mitochondrial adaptation to exercise in a diabetic rodent model and may augment the impact of exercise on the vasculature.

Key Words: mitochondria, exercise, saxagliptin, type 2 diabetes, GLP-1, eNOS

(J Cardiovasc Pharmacol™ 2015;65:137–147)

INTRODUCTION

Diabetes is a leading cause of death worldwide, imparting a 3- to 5-fold excess cardiovascular disease (CVD) mortality, despite optimal control of traditional CVD risk factors.[1] Epidemiological and cohort studies demonstrate that lower CVD and all-cause mortality are predicted by increased physical fitness or increased quantity of physical activity.[2–4] We and others have demonstrated that adults and children with type 1 and type 2 diabetes (T1DM and T2DM) have impairments in functional exercise capacity, or fitness, at maximal and submaximal workloads.[5–8] Understanding the mechanism of this functional limitation has high clinical relevance.

The importance of mitochondrial function for vascular contractile function was established by Taggart and Wray and Sward et al.[9,10] More recent studies have confirmed the importance of mitochondrial function for maintenance of blood pressure in a model of sepsis-mediated hypotension[11] and a genetic model of Huntington's disease with mitochondrial dysfunction.[12] Mitochondrial calcium handling in the vascular smooth muscle cell is a postulated mechanism for the mitochondrial regulation of vascular contractile function (reviewed in Ref. 13). In diabetes, studies demonstrate changes in vascular mitochondrial structure and function including fragmentation of mitochondria in cardiac endothelial cells, increased mitochondrial reactive oxygen species production in the vasculature, and impaired contractile function.[14–16] The impact of exercise on mitochondrial regulation in the vasculature has only recently been examined in disease states.[17,18]

We and others have also shown abnormal mitochondrial responses to exercise in the vasculature of animal models of diabetes and hypertension and after manipulation of nitric oxide synthase (NOS).[19,20] Mitochondrial protein expression failed to increase with exercise in spontaneous hypertension and heart failure (SHHF) animals as compared with a significant increase in complexes I, II, and III in exercised control animals.[19] We recently reported that endothelial NOS (eNOS)/NOS enzyme activity is essential for the maintenance of basal and adaptive mitochondrial regulation in the vasculature.[21] The mechanisms contributing to impaired vascular mitochondrial exercise response in T2DM have not been fully characterized in the vasculature but may in part be explained by dysregulation of eNOS in the diabetic vasculature.[22]

NOS and nitric oxide (NO) are established regulators of mitochondrial biogenesis, and they are a critical component of the vascular response to a bout of exercise.[23–25] eNOS null mice have reductions in mitochondrial biogenesis in the

muscle and the vasculature,[21,24,26] and deletion of eNOS in brown adipocytes interferes with cyclic guanosine monophosphate induction of PGC-1α and mitochondrial biogenesis.[24] An agent capable of stimulating eNOS in the diabetic environment could be used to test the importance of eNOS for vascular mitochondrial adaptation in diabetes. Food and Drug Administration–approved drugs for the management of diabetes that could be repurposed to target eNOS include agents that regulate the gut hormone, glucagon-like peptide 1 (GLP-1). GLP-1 is an insulin secretagogue, and for the purpose of this investigation, GLP-1 also regulates mitochondrial function by stimulating cyclic adenosine monophosphate (cAMP), eNOS, and cAMP response element-binding protein and decreasing mitochondrial reactive oxygen species.[27–30] GLP-1 receptors are highly expressed in vascular tissues, and GLP-1 improves muscle blood flow through a NOS-/NO-dependent mechanism.[31] Endogenously, GLP-1 has a brief half-life and is cleaved by dipeptidyl peptidase 4 (DPP-4), an enzyme both freely circulating and located on endothelial cells. Inhibitors of DPP-4 increase circulation of endogenous GLP-1.

In this study, we hypothesized that blunted mitochondrial adaptation to exercise in the diabetic vasculature would be restored by saxagliptin, a Food and Drug Administration–approved DPP-4 inhibitor that increases circulating GLP-1, in turn activating eNOS. After initial baseline mitochondrial comparisons between a control (Wistar) and nonobese insulin-resistant [Goto-Kakizaki (GK)] rat model exposed to a bout of exercise, we administered saxagliptin in combination with an acute 8-day exercise intervention in the GK rats and measured the effects of this intervention on mitochondrial profiles. In a second 3-week exercise training intervention, we evaluated the impact of saxagliptin on running time in these animals. Our primary aim was to characterize responses to a short-term exercise exposure (low intensity, nonvoluntary treadmill exercise), concurrent with saxagliptin administration, with a focus on mitochondria adaptation in the aorta.

METHODS

Animals

Male GK (Taconic Farms, Inc) and control Wistar (Charles River Laboratories International, Inc) 18-week-old rats were used for the studies. Animals were housed with a 12:12 light cycle and provided water and food ad lib. Approval from the Institutional Animal Care and Use Committee at the Denver VA Medical Center was obtained. The GK rat is a model of nonobese T2DM, characterized by insulin resistance and mild hyperglycemia. We chose this model to fit our hypothesis and endpoints of addressing mitochondrial signaling in the absence of obesity, leptin receptor mutation, or extreme hyperglycemia.

Exercise Intervention

GK and Wistar rats were randomized into exercise and sedentary groups and subjected to a nonvoluntary exercise on a Columbus Instruments Exer 3/6 Treadmill (Columbus, OH). Acclimation took place over the course of 5 days and began by placing rats on a stationary treadmill (0% grade) for
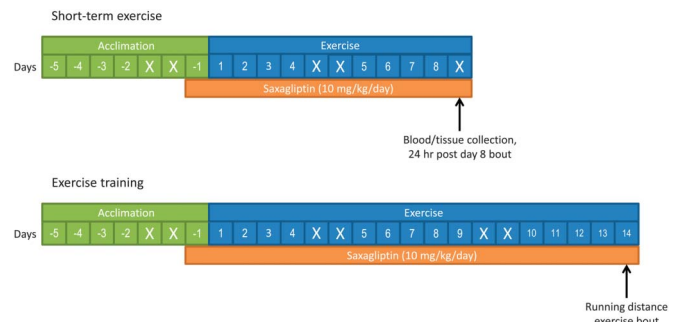
FIGURE 1. Experimental design. Rats were evaluated 8 and 13 days after exercise intervention with and without saxagliptin. In the first protocol, tissues and blood were collected 24 hours after the final exercise bout. In the second protocol, running distance was measured using an exhaustive exercise bout.

5–15 minutes on day -5 (Fig. 1) with speed set to 10 m per minute for 10 minutes on treadmill by day -1. Exercised animals were run on the treadmill (0% grade) at 15 m per minute for 30 minutes on day 1 and 2 and for 45 minutes from day 3 forward (all rats completed). The 8-day exercise paradigm was chosen based on previous short-term moderate exercise interventions in the literature examining antioxidant profiles and eNOS.[32,33]

A separate group of GK rats was used to assess the impact of saxagliptin on running distance (13 days over the course of 3 weeks, Fig. 1). On day 14, rats were initially run on the treadmill at a speed of 8 m per minute for 3 minutes, then increased to 12 m per minute for 3 minutes, and finally increased to 18 m per minute until the rat fatigued as previously defined[34] or 2 hours. Running distance was subsequently calculated.

Saxagliptin Administration

Saxagliptin (Bristol-Myers Squibb) was incorporated into Teklad Global Rodent Diet 2018 and designed with Teklad Custom Research Diets to deliver 10 mg·kg^{-1}·d^{-1} (TD.110344). This dosage was based on previous studies of saxagliptin establishing its consistent bioavailability and physical distribution in rats.[35,36] Rats were switched from Diet 2018 to the saxagliptin diet the day before the exercise intervention onset (Fig. 1). Sedentary rats in a saxagliptin treatment group received saxagliptin for the same duration as rats in the saxagliptin with exercise group. Rats not receiving saxagliptin remained on Diet 2018.

Serum/Plasma and Tissue Collection

Animals were anesthetized 24 hours after their last exercise (or sedentary, nonexercise) bout under 3.5%–4% isoflurane. Blood was taken by cardiac puncture and saved as serum or plasma at −80°C until assayed using kits for thiobarbituric acid-reactive substances (TBARS) and glucose from Cayman Chemicals, insulin and GLP-1 (7–36) from ALPCO Diagnostics, and DPP-4 activity from Enzo Life Sciences. Aortas were removed, cleaned of perivascular fat, and either used fresh for mitochondrial respiration or frozen in liquid nitrogen (<2 minutes from removal) and stored at

−80°C until further processing and analysis. For each of the analyses, the same region of the aorta was used from each animal to ensure equal pulsatile exposure.
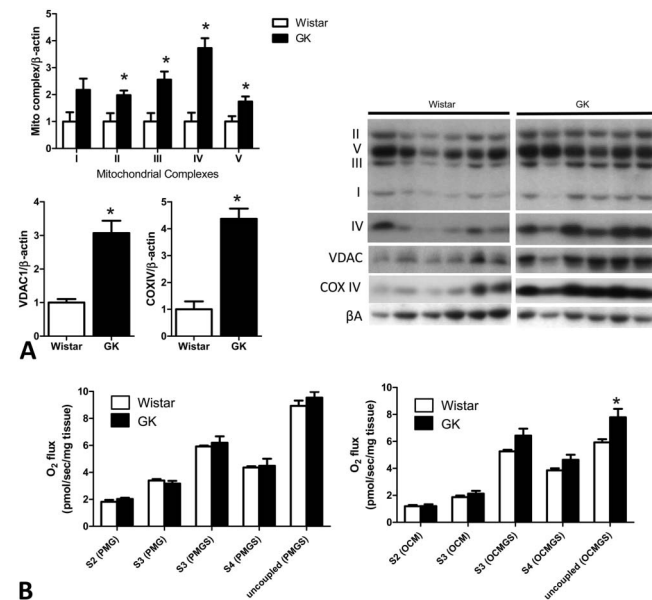
Mitochondrial Respiration

Mitochondrial respiration was measured using Oroboros Oxygraph-2k (O2k; Oroboros Instruments Corp, Innsbruck, Austria). The aortic arch was removed and placed in a mitochondrial preservation buffer [BIOPS (10 mM Ca-EGTA, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl$_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1)] and kept on ice. The aorta was cut longitudinally, and the endothelium was removed by rolling a cotton-tipped applicator. The aortic tissue was permeabilized by incubation with saponin (50 μg/mL) in BIOPS on ice on a shaker for 30 minutes. The aorta was then washed for 10 minutes on ice on a shaker in mitochondrial respiration buffer [MiR06 (0.5 mM EGTA, 3 mM magnesium chloride, 60 mM K-lactobionate, 20 mM taurine, 10 mM potassium phosphate, 20 mM HEPES, 110 mM sucrose, 1 g/L bovine serum albumin, 280 U/mL catalase, pH 7.1)]. The aorta was blotted dry for a weight (8–20 mg) and added to MiR06 that had been prewarmed to 37°C in the chamber of the O2k. Oxygen concentration in the MiR06 started at approximately 400 μM and was maintained above 250 μM. Substrates and inhibitors were added to assess respiration rates at several states. Rates were measured with the final concentrations of 5 mM pyruvate + 2 mM malate + 10 mM glutamate (PMG) + 6 mM succinate (PMGS) or 200 μM octanoylcarnitine + 1 mM malate (OCM) + 2 mM glutamate + succinate (OCMGS), 2 mM adenosine diphosphate (ADP), 10 μg/mL oligomycin, and 0.5 μM stepwise titration of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone added until maximal uncoupling (uncoupled state). Cytochrome c (10 μM) was used to determine mitochondrial membrane damage.

Western Blot Analyses

The aortic arch was pulverized in lysis buffer [M-PER (Thermo Scientific) with 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM Na$_4$P$_2$O$_7$·10H$_2$O, 1 mM Na$_3$VO$_4$, 20 mM NaF, 500 mM okadaic acid, 1% protease inhibitor cocktail (Sigma-Aldrich)] under liquid nitrogen and further homogenized using an Ultra-Turrax T8 (IKA). Protein concentration was assayed by the Bradford's method. Equal protein was resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels followed by transferring to polyvinyl difluoride (PVDF) membrane. Membranes were probed with specific antibodies from Abcam [voltage-dependent anion channel 1 (VDAC1), cytochrome c oxidase IV (COX IV), representative subunits of mitochondrial oxidative phosphorylation complexes I–V, I (subunit NDUFA9), II (subunit SDHA), III (subunit UQCRC2), IV (subunit IV), and V (subunit ATP5A), sirtuin 1 (SIRT1), and uncoupling protein 3 (UCP3)], Cell Signaling [adenosine monophosphate kinase (AMPK), SIRT3, phosphorylated AMPK, and acetylated lysine], BD Biosciences [eNOS, cytochrome c, and neuronal NOS], Santa Cruz Biotechnology [citrate synthase (CS), PPARγ coactivator 1α (PGC-1α), and phosphorylated eNOS] and Upstate/Millipore [manganese superoxide dismutase (MnSOD)]. Proteins were detected by chemiluminescence using CDP-Star Reagent (Invitrogen), and densitometric analysis performed using Quantity One (Bio-Rad). All data are normalized to β-actin (Sigma-Aldrich) protein expression.

Mitochondrial Complexes and CS Activity Assays

Lysates from pulverized descending thoracic aorta in a sucrose buffer (1:10 wt/vol; 20 mM Tris-HCl, 250 mM sucrose, 40 mM KCl, 2 mM EGTA, pH 7.4) and subjected to 3 freeze and thaw cycles were used to assay activity of respiratory chain enzyme complexes I + III, II + III, and IV and CS spectrophotometrically (as previously described with slight modifications[37]) on a BioTek Synergy H1 microplate reader. All enzyme activities were normalized to protein (assayed by Bradford's method), and complexes I + III, II + III, and IV activities were normalized to CS activity.

Mitochondrial DNA to Nuclear DNA Ratio

DNA was extracted and purified from the descending thoracic aorta using the Qiagen DNeasy Blood & Tissue Kit and following manufacturer's protocol. Mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) copy number was measured using a real-time quantitative polymerase chain reaction

FIGURE 2. Baseline mitochondrial differences in the aorta of sedentary Wistar and GK rats. A, Protein expression of representative subunits of mitochondrial complexes I–V [I (subunit NDUFA9), II (subunit SDHA), III (subunit UQCRC2), IV (subunit IV), and V (subunit ATP5A)], VDAC1, and COX IV (n = 6). Data are normalized to β-actin (βA) and expressed as mean fold change from sedentary Wistar group + SEM. B, Respiration rates of denuded and permeabilized aorta (n = 5). Data are presented as O$_2$ flux (picomole of O$_2$·s^{-1}mg^{-1} of tissue) + SEM. P, pyruvate; M, malate; G, glutamate; S, succinate; OC, octanoylcarnatine; S2, state 2 respiration; S3, state 3 respiration; S4, state 4 respiration. *P < 0.05, Student's t test.

TABLE 1. Metabolic Parameters of Wistar and GK rats, Sedentary and Exercised

	Body Weight, g	Glucose, mg/dL	Insulin, ng/mL	DPP-4 Activity, pmole/min	TBARS, μM	mtDNA/nDNA (Mt-Cyb/MyHc)
	n = 6	n = 6	n = 6	n = 3–4	n = 4–5	n = 6
Wistar						
Sedentary	525 ± 15	170 ± 16	2.64 ± 0.74	147 ± 4	11.8 ± 1.6	279.9 ± 48.9
Exercise	499 ± 15	162 ± 8	1.92 ± 0.24	124 ± 21	11.5 ± 1.6	275.7 ± 57.3
GK						
Sedentary	382 ± 13*	382 ± 69*	1.96 ± 0.39	235 ± 25*	6.7 ± 1.7*	913.5 ± 400.9*
Exercise	373 ± 5*	290 ± 30*	1.74 ± 0.20	192 ± 4*	6.0 ± 1.6*	638.9 ± 173.5*

Values are expressed as mean ± SEM.
*$P < 0.05$ animal model effect, 2-way analysis of variance.

using an Applied Biosystems 7900 Sequence Detection System. The primer and TaqMan TAMRA probe sequences for the mitochondrial Cytb gene were forward primer (ratCYTB-DNAFWD), 5′-TTCTACCATCCTCCGTGAAATCA-3′; reverse primer (ratCYTB-DNAREV), 5′-GGCCCGGAGCGAGAAG-3′, probe (ratCYTB-DNAPRB), 5′-6FAMCAACCCGCC-CACTCGTCCCCTAMRA-3′. The primer and TaqMan TAMRA probe sequences for the nuclear gene MYHC were as follows: forward primer (ratBMYHC-DNAFWD), 5′-AA-CAGGGCAGACACGGGTT-3′; reverse primer (ratBMYHC-DNAREV), 5′-TGGTCCTCCTTCACAGTCACC-3′, probe (ratBMYHC-DNAPRB), 5′-6FAMTGAGGGCCTGCAT-GGGCTGTTTACTAMRA-3′.

Power Calculation

PS: Power and Sample Size Calculation Version 3.0, 2009 (Dupont WD and Plummer WD), was used to estimate the number of animals needed to detect a 30% difference in the means of the mitochondrial complexes between intervention and control animals (SD = 0.20). We established a sample size of n = 4 in each group. To accommodate this calculation, we planned for either n = 6 or n = 12 per group or comparator to capture subtle effects and account for any unforeseen variability.

Statistics

Where appropriate, either an unpaired Student's *t* test or 2-way analysis of variance was used, and significance was set at $P < 0.05$.

RESULTS

### Baseline Mitochondrial Differences in the Aorta of GK and Wistar Rats

To examine whether baseline differences existed in the mitochondria of Wistar and GK rats' aortas, we looked at the expression of mitochondrial complexes, VDAC1 and COX IV proteins in the sedentary rats. Representative subunits of mitochondrial complexes II–V in the GK animals were all significantly higher than those in the Wistars ($P < 0.05$, Fig. 2A) while complex I only trended higher ($P < 0.1$). Expression of VDAC1 protein, a mitochondrial membrane-bound protein

used as a proxy for mitochondrial count, was significantly higher in the GK rats compared with that in the Wistar rats ($P < 0.05$, Fig. 2A). COX IV protein was more than 4-fold higher in the GK rats compared with that in the Wistar animals ($P < 0.05$, Fig. 2A).

To examine whether these differences translated to baseline functional differences, we measured the respiration of the permeabilized aortic tissue using 2 different sets of substrates. Examination of respiration in response to the carbohydrate substrates, pyruvate, malate, glutamate, and succinate (PMG and PMGS), revealed no differences in respiration states 2 (S2, ADP independent), 3 (S3, ADP dependent), and 4 (S4, ADP independent) or uncoupled respiration between Wistar and GK aorta (Fig. 2B). In contrast, examination of respiration when exposed to theoretical lipid-derived substrates, octanoylcarnitine with malate, glutamate, and succinate (OCM and OCMGS), revealed a trend for higher respiration in the GKs for OCMGS S3 and S4 ($P < 0.1$) and a significantly higher uncoupled respiration in the GKs when compared with the Wistars ($P < 0.05$, Fig. 2B). These differences in respiration between substrates for the Wistar and GK rats, along with higher mitochondrial protein (increased mitochondrial content) in the GK rats, may suggest variation in fuel utilization in the context of diabetes, favoring lipid substrate. Between an 11% and 15% increase in respiration rates in both Wistar and GK aorta was observed, indicating modest cytochrome c leakage. No differences between animal models were noted, suggesting an aortic tissue response to saponin permeabilization.

Exercise Intervention in GK and Wistar Rats

We previously reported failed mitochondrial adaptation in an obese model of T2DM.[19] To examine the contribution of diabetes separate from obesity, we used the nonobese GK rat model of insulin-resistant diabetes and the relevant Wistar control. Metabolic characteristics revealed significant model effects between GK and Wistar rats. Wistar animals had significantly higher body weights ($P < 0.05$, Table 1), and GK animals had the expected significant glucose elevations ($P < 0.05$, Table 1). No significant differences in insulin were observed between groups with or without exercise. GLP-1 concentrations were 1.4 ± 0.6 pM for Wistar sedentary animals and below the detectable limit of the assay (<0.9 pM) in the other groups
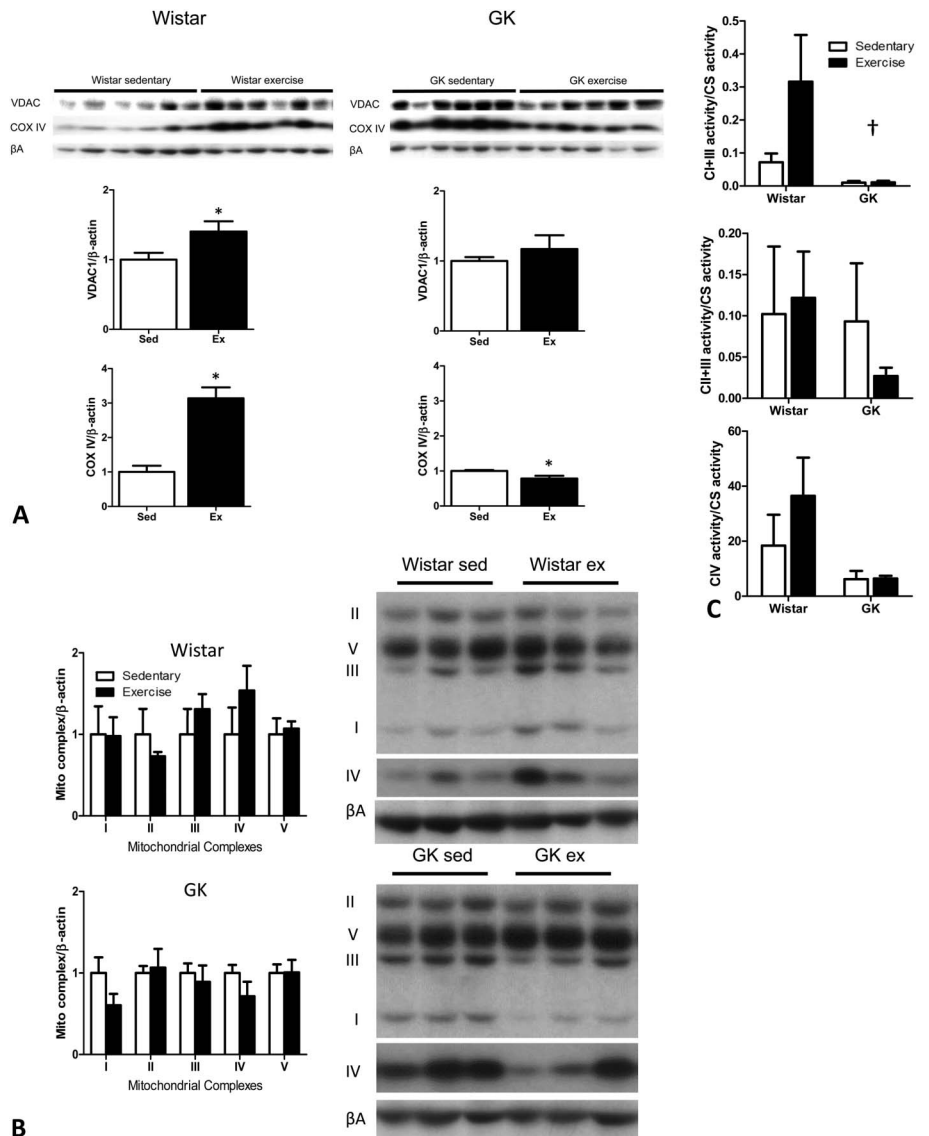
FIGURE 3. Changes in mitochondrial protein expression and activities in the aorta of sedentary (Sed) and exercised (Ex) Wistar and GK rats. A, VDAC1 (n = 6) and COX IV (n = 6) and (B) representative subunits of mitochondrial complexes I–V (n = 6). Data are normalized to β-actin (βA) and expressed as mean fold change from sedentary control group + SEM. *$P <$ 0.05, Student's *t* test. C, Mitochondrial complexes I + III (CI + III), II + III (CII + III), and IV (CIV) activities normalized to CS activity (n = 3–4); data are expressed as mean + SEM. *$P <$ 0.05 exercise effect; †$P <$ 0.05 model effect; ‡$P <$ 0.05 interaction effect; 2-way analysis of variance.
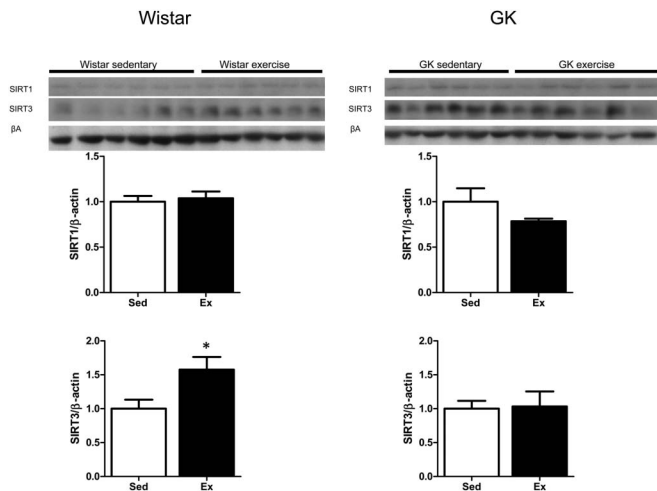
(Wistar exercise, GK sedentary and exercise, data not shown). DPP-4 activity was significantly elevated in the GK as compared with that in the Wistar ($P < 0.05$, Table 1). TBARS concentrations were significantly elevated in Wistar ($P < 0.05$, Table 1). Mitochondrial DNA to nuclear DNA ratios were higher in GK aortas compared with those in the Wistar ($P < 0.05$, Table 1), consistent with the protein profile in Figure 2A.

Following the 8-day exercise exposure, expression of VDAC1 protein was significantly increased in the Wistar rats but not in the GK rats ($P < 0.05$, Fig. 3A). COX IV protein was increased more than 3-fold in the Wistar rats after the exercise intervention ($P < 0.001$, Fig. 3A); conversely, a significant decrease in COX IV was observed in the GK ($P < 0.05$, Fig. 3A). CS protein content trended upward by 28% ± 10% in the Wistar rats after exercise intervention ($P = 0.056$) and was unchanged in the GK rats (data not shown). Taken together, this absence of vascular mitochondrial response in GK rats is consistent with our previous observations.[19] Representative subunits

of mitochondrial complexes III, IV, and V in the Wistar animals trended up after exercise ($P < 0.1$, Fig. 3B), whereas an opposite decreasing trend was seen in the mitochondrial complexes I, III, and IV of GK rats ($P < 0.1$, Fig. 3B).

To establish functional relevance of changes in mitochondrial protein expression, we investigated the activity of mitochondrial complexes I together with III (I + III), II together with III (II + III), and IV (Fig. 3C). All were normalized to CS activity. There was no exercise effect on any of the assayed activities. The activity of complexes I + III was significantly lower in the GK rat aortas compared with that in the Wistars ($P < 0.05$), and there was a trend toward lower complex IV activity in the GK compared with Wistars ($P = 0.075$).

AMPK and PGC-1α protein content was not changed by exercise in either the Wistar or GK rats in aortic lysate harvested 24 hours after final bout (data not shown). In addition, there was a trend of increased eNOS protein expression by

FIGURE 4. Changes in protein expression of SIRT1 and 3 in the aorta of sedentary (Sed) and exercised (Ex) Wistar and GK rats, n = 6. Data are normalized to β-actin (βA) and expressed as mean fold change from sedentary control group + SEM. *P < 0.05, Student's *t* test.

29% ± 7% (*P* = 0.084) after exercise in the Wistar rats only (data not shown). No changes in SIRT1 protein expression in response to exercise were observed (Fig. 4). However, mitochondrial SIRT3 protein significantly increased 24 hours after exercise in the Wistar rats but not in the GK (*P* < 0.05, Fig. 4).

MnSOD and mitochondrial UCP3, both critical to mitochondrial-specific antioxidant defense, were unchanged in response to exercise in either the Wistar or GK rat (data not shown). This conflicts with other studies using long-term exercise; these studies report an increase in aortic MnSOD as a result of training despite weight loss or blood glucose concentrations.[38,39] These endpoints have not been reported in response to a short-term exercise exposure.

Saxagliptin and 8-Day Exercise Intervention in GK Rat

Examination of the impact of saxagliptin in the context of hyperglycemia was the intent of this short-term exercise intervention in the GK rat instead of a full 8-week exercise training program. No significant differences were seen between treatment groups in body weight, glucose, or insulin with 8 days of exercise (Table 2). GLP-1 concentrations were below detectable limits of the assay (<0.9 pM) on the control diet

(Teklad Global Rodent Diet 2018) but were detectable with saxagliptin treatment (1.7 ± 0.1 and 1.4 ± 0.3 pM, sedentary and exercise, respectively). There was a significant saxagliptin effect on DPP-4 activity, showing elevated activity in animals without saxagliptin (*P* < 0.05, Table 2). TBARS were unchanged between groups (Table 2). These effects are overall indicative of appropriate saxagliptin dosage in our animal models. Of interest, DPP-4 levels were significantly increased in the GK before intervention (Table 2). These data are consistent with a previous study demonstrating high basal DPP-4 levels in the GK rats relative to the Wistar rats and detectable increases in GLP-1 levels with DPP-4 inhibition.[40]

In the GK animals, there was a significant saxagliptin effect on the mitochondrial complex IV representative subunit (*P* < 0.05, Fig. 5A). There was also a significant exercise, saxagliptin, and exercise plus saxagliptin interaction effect on cytochrome c protein expression (Fig. 5B). A significant increase in complex I + III activity/CS activity was also observed with saxagliptin treatment (Fig. 5C, *P* < 0.05).

Evaluation of exercise-mediated signaling to mitochondrial biogenesis 24 hours after the final bout in GK rats with or without saxagliptin consumption revealed a significant exercise, saxagliptin and interaction effect on AMPK expression (*P* < 0.05, Fig. 6A) without any significant differences seen in pAMPK. There was a significant saxagliptin effect and interaction trend for neuronal NOS expression (*P* < 0.05, *P* < 0.1, respectively), and the effects of exercise, saxagliptin and their interaction significantly increased eNOS (*P* < 0.05, Fig. 6B). Phosphorylated eNOS remained unchanged across groups 24 hours after bout (Fig. 6B). A significant interaction effect was observed in PGC-1α protein content (*P* < 0.05, Fig. 7A). Significant effects of saxagliptin, exercise, and the interaction of both interventions were observed in UCP3 expression (*P* < 0.05, Fig. 7A). Only a trend toward an interaction effect on SIRT3 expression was observed (Fig. 7B, *P* < 0.1) with no change in SIRT1 expression in GK rats.

Saxagliptin and Exercise Training in GK Rat

Running distance during an exhaustive exercise bout was measured in a second set of GK rats after training over 3 weeks (13 days of exercise in total) with or without saxagliptin. Exercise training significantly increased running distance (Fig. 8, *P* < 0.05), and there is also a significant interaction effect of exercise and saxagliptin on running distance (Fig. 8, *P* < 0.05).

TABLE 2. Metabolic Parameters of GK Rats

	Body Weight, g	Glucose, mg/dL	Insulin, ng/mL	DPP-4 Activity, pmole/min	TBARS, μM
	n = 11–12	n = 12	n = 12	n = 3–4	n = 4–5
Sedentary	389 ± 7	352 ± 39	2.22 ± 0.30	235 ± 25	6.7 ± 1.6
Exercise	374 ± 4	282 ± 17	1.89 ± 0.17	192 ± 4	6.0 ± 1.6
Sedentary + Saxagliptin	379 ± 13	305 ± 18	2.43 ± 0.28	35 ± 14*	6.5 ± 1.0
Exercise + Saxagliptin	366 ± 6	289 ± 29	1.98 ± 0.20	88 ± 21*†	6.4 ± 0.9

Values are expressed as mean ± SEM.
*P < 0.05 saxagliptin effect.
†P < 0.05 interaction effect, 2-way analysis or variance.
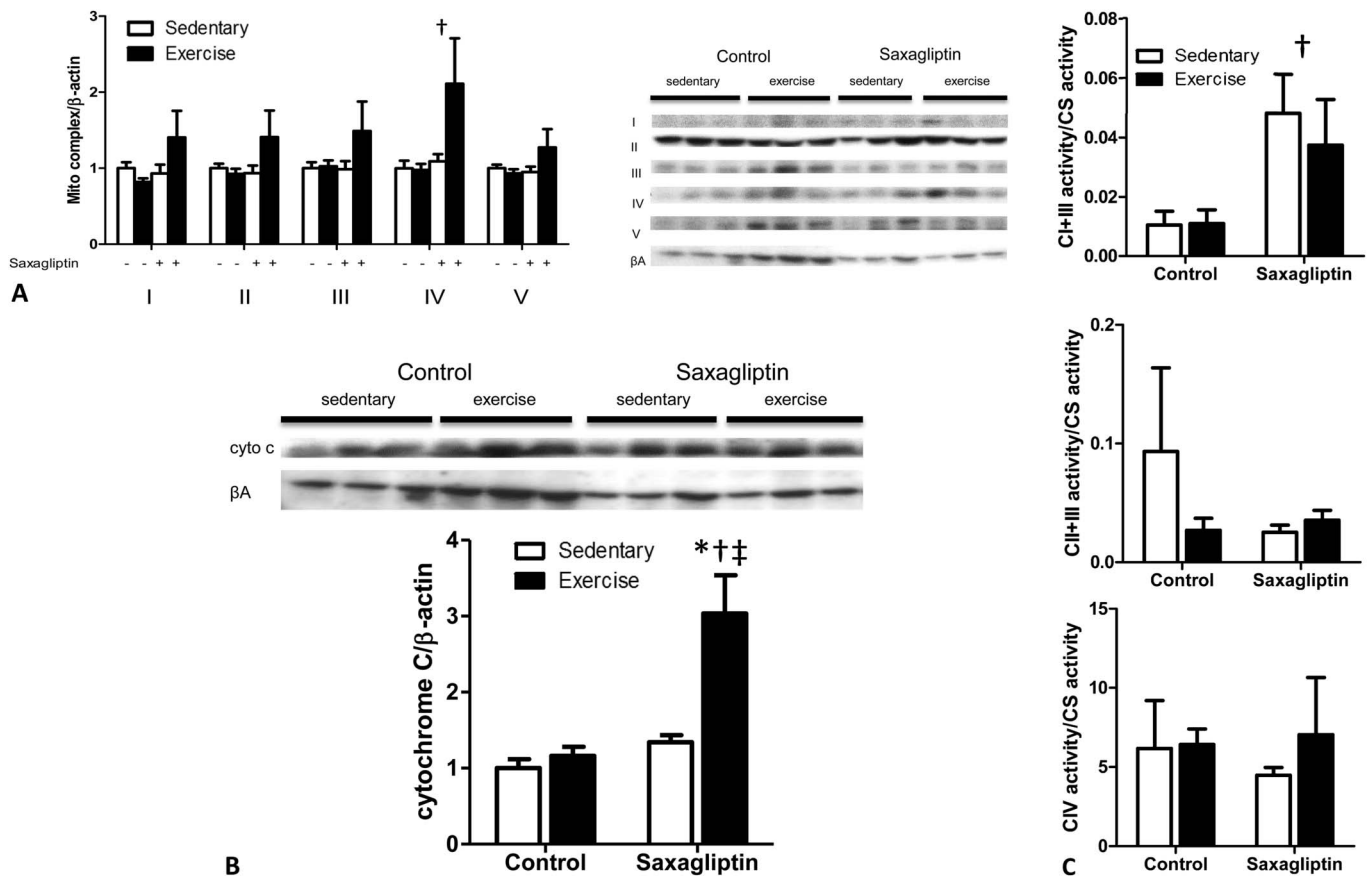
FIGURE 5. Changes in mitochondrial protein and activity in the aorta of GK rats with exercise alone or with saxagliptin. A, Representative subunits of mitochondrial complexes I–V (n = 12) and (B) cytochrome c (n = 6). Data are normalized to β-actin (βA) and expressed as mean fold change from sedentary control group + SEM. C, Mitochondrial complexes I + III (CI + III), II + III (CII + III), and IV (CIV) activities normalized to CS activity (n = 3–4); data are expressed as mean + SEM. *$P < 0.05$ exercise effect; †$P < 0.05$ saxagliptin effect; ‡$P < 0.05$ interaction effect; 2-way analysis of variance.

Saxagliptin Intervention in Wistar Rat

Wistar rats were treated with and without saxagliptin (n = 6) to characterize the effect of saxagliptin treatment on a "healthy" sedentary rat. No significant differences were seen in body weight (553 ± 23 g), insulin (2.82 ± 0.67 ng/mL), or glucose (153 ± 7 mg/dL) concentrations in Wistar rats treated with saxagliptin as compared with the sedentary or exercised groups reported in Table 1. Additionally, saxagliptin treatment did not result in significant changes in mitochondrial complex protein expression in sedentary rats; however, significant changes in AMPK (38.2% ± 2.9% of control), eNOS (63.0% ± 4.2% of control), and phosphorylated eNOS (57.9% ± 7.1% of control) content were observed with saxagliptin treatment ($P < 0.05$, data not shown).

DISCUSSION

People with T2DM have decreased functional exercise capacity that confers an excess risk of cardiovascular and all-cause mortality. Understanding the molecular changes in diabetes that contribute to defects in exercise adaptation may provide insights into therapies that will resolve this barrier. Mitochondria are a classic target of exercise in the skeletal muscle and heart and are functionally important in the vasculature for regulation of vascular tone, endothelial regulation of vasomotion and tight junctions, smooth muscle cell proliferation, and apoptosis.[41–44] We recently reported that the vasculature also responds to exercise with increased mitochondrial protein expression, and that this adaptive homeostatic response is absent in hypertensive only rats and obese, hypertensive T2DM rats with a leptin receptor mutation.[19] The GK model of diabetes appears to have more mitochondria in their aorta compared with Wistars as seen with the higher levels of mitochondrial proteins and mtDNA/nDNA. The respiration data suggest, however, that variability in substrate availability or processing may be occurring in our diabetes rat model. This is consistent with muscle and heart substrate preference in DM models.[45,46] The data from this study show that similar to the SHHF, the GK rat model of diabetes also fails to mount a vascular mitochondrial response to exercise, as evidenced by lack of change in mitochondrial
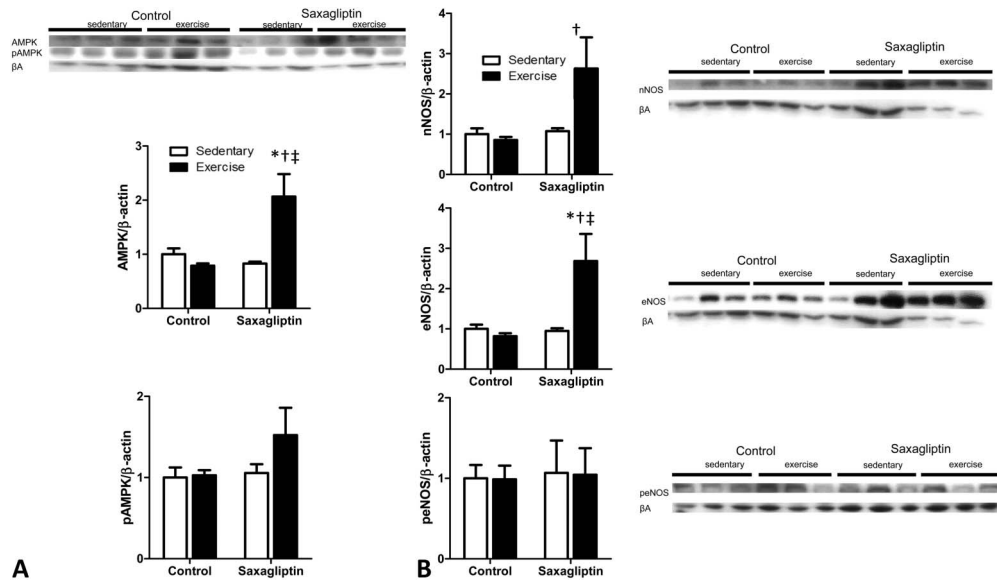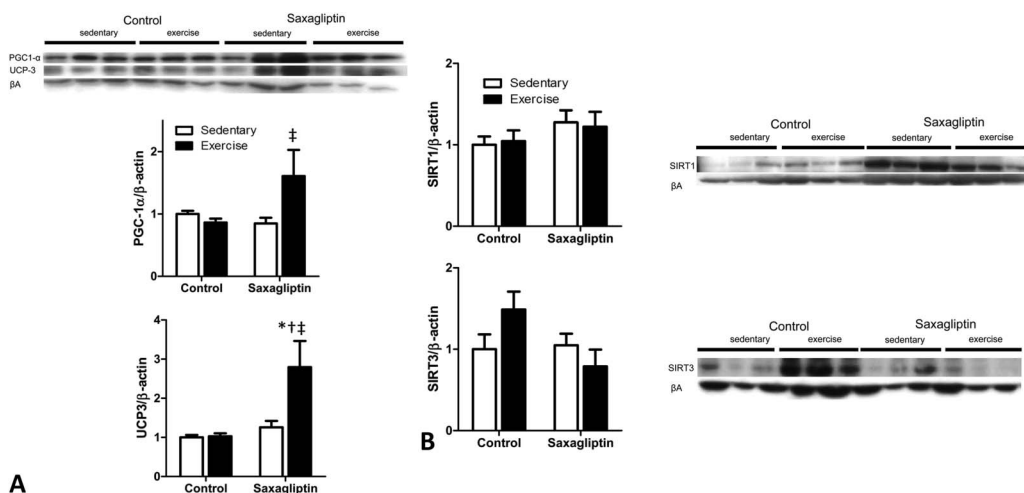
FIGURE 6. Changes in protein expression of (A) AMPK (n = 6), pAMPK (n = 12) and (B) nNOS (n = 6), eNOS (n = 6), and peNOS (n = 6) in the aorta of GK rats with exercise alone or with saxagliptin. Data are normalized to β-actin (βA) and expressed as mean fold change from sedentary control group + SEM. *$P < 0.05$ exercise effect; †$P < 0.05$ saxagliptin effect; ‡$P < 0.05$ interaction effect; 2-way analysis of variance.

protein profiles. Treatment with saxagliptin improved the vascular response to exercise intervention by increasing mitochondrial protein expression plus increased protein expression of upstream regulators of mitochondrial biogenesis. In addition, combined saxagliptin plus a separate exercise training intervention doubled the improvement in running distance from exercise training alone.

The goal of our investigation was to examine regulation of mitochondrial homeostatic adaptation to an acute exercise intervention. Analysis of mitochondrial "function" can be

assessed using a number of approaches including protein content, mitochondrial DNA content, respiration, and enzyme activities. To broaden our mitochondrial analysis, we used mitochondrial complex and CS enzyme activities. We used CS activity to normalize the complex activities to a mitochondrial measurement, because it has a strong association with mitochondrial content.[47] An 8-day exercise bout did not affect any of the measured complex activities, yet complex I + III activity was significantly lower in the aorta of GK animals compared with that in the Wistars. Of note, complex I + III

FIGURE 7. Changes in protein expression of (A) PGC-1α (n = 12), UCP3 (n = 6) and (B) SIRT1 (n = 12), SIRT3 (n = 6) in the aorta of GK rats with exercise alone or with saxagliptin. Data are expressed as mean fold change from sedentary control group + SEM, and all data are normalized to β-actin (βA). *$P < 0.05$ exercise effect; †$P < 0.05$ saxagliptin effect; ‡$P < 0.05$ interaction effect; 2-way analysis of variance.
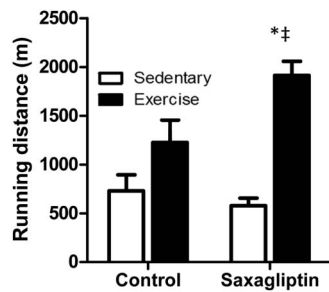
FIGURE 8. Running distance of GK rats with exercise alone or with saxagliptin. Data are expressed as mean + SEM. Control groups, n = 4; saxagliptin groups, n = 6. *$P < 0.05$ exercise effect; †$P < 0.05$ saxagliptin effect; ‡$P < 0.05$ interaction effect; 2-way analysis of variance.

activity was the only activity affected by saxagliptin treatment in the GK aorta. The change with saxagliptin led to complex I + III activity similar to sedentary Wistar levels. The findings suggest that there are saxagliptin effects on mitochondrial function; this will be measured directly in future studies.

To better understand the potential mechanism of failed adaptation to exercise in rodents with T2DM, we examined established signaling pathways for mitochondrial biogenesis. Exercise stimulates mitochondrial biogenesis through 2 interacting molecular pathways, both of which can be deleteriously affected by diabetes. The first pathway involves an upregulation of AMPK, which activates eNOS, cAMP response element-binding protein, and PGC-1α, in turn stimulating mitochondrial biogenesis.[23,48] Activation of eNOS is dynamically regulated in the vasculature in response to shear and metabolic stress. eNOS regulation of NO and cyclic guanosine monophosphate are potential modulators of mitochondrial biogenesis, and these targets are commonly dysregulated in vascular disease and diabetes.[22] We recently reported that eNOS/NOS regulates mitochondrial content and turnover in the aorta and that pharmacological blockade of NOS ablates exercise stimulation of mitochondrial dynamics.[21,22] The second pathway through which exercise modulates mitochondrial biogenesis is an upregulation of SIRT1 by nicotinamide adenine dinucleotide (NAD+).[48] SIRT1 is regulated independently from AMPK/eNOS in response to hemodynamic changes and has the potential to augment the activity of the AMPK/eNOS/PGC-1α pathway.[48] Targeting either of these pathways could present a therapeutic option for restoring impaired mitochondrial response to exercise in T2DM. Our observations suggest that induction of NOS protein expression and PGC-1α is affected by combined intervention with saxagliptin and exercise (Figs. 6, 7). However, we also observe no significant increase in SIRT1/3 along with a significant increase in acetylated lysine in a nonspecific acetylation profile (data not shown) with saxagliptin treatment, suggesting that SIRTs are not activated with this intervention. These data support a model wherein saxagliptin restores exercise-mediated mitochondrial adaptation through the AMPK/eNOS/PGC-1α pathway. This is in agreement with a previous study showing that saxagliptin stimulates eNOS coupling and increases NO concentrations in rats.[36]

We have not, in these experiments, directly tested the impact of our intervention on eNOS activity, SIRT1, SIRT3, or other deacetylase activity.

It has been recently reported that leptin sensitivity, in conjunction with eNOS regulation and REDOX balance, is important for maintaining vasculature response to signaling and contributes to exercise-mediated signaling in the vessel wall.[17] In the GK animals with intact genetic leptin signaling, we observe a diabetes-mediated failed adaptation consistent with general failed adaptation in diabetes and hypertension. These new findings support that the leptin receptor mutation in our previous report does not fully explain the mitochondrial phenotype we observed in the SHHF obese rat.[19] In a recent report, diet-induced defects in leptin responsiveness were reported to be restored by GLP-1/glucagon coagonism,[49] so it is possible that saxagliptin may also have an impact on exercise-mediated leptin signaling. We have not, in these experiments, ruled out that diabetes-induced defects in leptin signaling contribute to our observed lack of response to exercise intervention.

We observed that eNOS response to exercise was significantly decreased in the GK animals after an exercise intervention, consistent with our previous report.[19] Miller et al[21] demonstrated that NO and eNOS are essential for mitochondrial response to exercise. As stated earlier, GLP-1 improves muscle blood flow through a NOS-/NO-dependent mechanism.[31] This background informed our idea to repurpose a currently used agent for the management of diabetes to target eNOS for the potential restoration of vascular mitochondrial adaptation to exercise. GLP-1 activates NO production and phosphorylates NOS in endothelial cells through cAMP.[50] In addition, muscle microvascular blood flow is significantly increased in rats treated with GLP-1 through NOS regulation.[31,50] We speculate that the impact of saxagliptin in conduit vessels may also be observed in resistance and microvascular vessels based on the previously reported impact of GLP-1 on muscle blood flow. We did not detect a change in the active form of eNOS, peNOS. This could be due to our tissue harvest 24 hours after bout or changes in eNOS coupling. Direct measurements of NOS activity and NO in future studies will be valuable in fully describing the mechanism of action.

By using aortic lysates for many of our analyses, we are unable to separate vascular smooth muscle cells from endothelial cells. The majority of the proteins in aortic lysates are smooth muscle cell derived, thus our findings reflect mainly the smooth muscle contribution, which is the cell type that determines final vascular tone. The dynamic relationship between the endothelium and the medial smooth muscle cells is not directly assessed in this study, yet we observe a consistent failure of adaptive vascular mitochondrial regulation in the GK model that is, in part, restored with a short intervention with saxagliptin. Importantly, this effect is shown before significant improvement in DM endpoints of glucose and insulin. Finally, functional respiration data were performed in denuded aorta, closely reflecting smooth muscle cell–specific contributions to vascular response overall.

In human subjects with T1DM and T2DM, we have reported a defect in maximal and submaximal exercise capacity that correlates with decreased endothelial function as well as decreased stress-induced myocardial blood flow

and slowed skeletal muscle blood flow kinetics.[5–8,20,51] Therefore, in addition to our vascular targets, we also examined the impact of exercise training (3 weeks) plus saxagliptin on running distance. We tested this question based on our finding in human subjects with T2DM that defects in functional exercise capacity correlate with endothelial dysfunction and aortic stiffness,[52] and we predicted that GLP-1–mediated changes in skeletal muscle and heart perfusion could translate into a positive effect on exercise capacity. There was a significant effect of exercise in the GK rats resulting in an increase in running time; there was also a significant interaction effect of saxagliptin plus exercise training (Fig. 8). This finding suggests that combined pharmacological and behavioral therapy can impact exercise function. This finding is consistent with a previous report demonstrating that PPARγ ligands increase the impact of exercise training in a mouse.[53] We and others have observed an additive benefit of exercise and rosiglitazone in T2DM.[52,54] We do not see any change in running distance with saxagliptin alone, suggesting that exercise is necessary to gain the fitness benefit from saxagliptin. We are currently examining the impact of GLP-1 analogs in human subjects with uncomplicated T2DM (clintrial.gov: NCT01793909).

The improvement in running distance is intriguing, although they may not be a direct consequence of improved aortic vascular dynamics. More likely, these improvements are a reflection of improvements in skeletal muscle nutrient blood flow based on previous publications on GLP-1,[31,50] and further studies will examine effects of saxagliptin on vasomotion and skeletal muscle blood flow.

CONCLUSIONS

Exercise continues to impart benefits on the vasculature and overall health of those with T2DM. The data presented here indicate that vascular mitochondrial protein expression and enzyme activity do not respond to acute exercise exposure in a rat model of nonobese insulin resistance.[19] This report confirms that diabetes ablates vascular mitochondrial adaptation in a model without a leptin receptor mutation. We report that altered mitochondria function and biogenesis can be improved by a pharmaceutical intervention with saxagliptin, an agent currently used in the management of diabetes.

REFERENCES

1. Bloomgarden ZT. Cardiovascular disease and diabetes. *Diabetes Care.* 2003;26:230–237.
2. Gill JM, Malkova D. Physical activity, fitness and cardiovascular disease risk in adults: interactions with insulin resistance and obesity. *Clin Sci (Lond).* 2006;110:409–425.
3. Sundquist K, Qvist J, Johansson SE, et al. The long-term effect of physical activity on incidence of coronary heart disease: a 12-year follow-up study. *Prev Med.* 2005;41:219–225.
4. Wei M, Gibbons LW, Kampert JB, et al. Low cardiorespiratory fitness and physical inactivity as predictors of mortality in men with type 2 diabetes. *Ann Intern Med.* 2000;132:605–611.
5. Bauer TA, Reusch JE, Levi M, et al. Skeletal muscle deoxygenation after the onset of moderate exercise suggests slowed microvascular blood flow kinetics in type 2 diabetes. *Diabetes Care.* 2007;30:2880–2885.
6. Curtis JM, Horton ES, Bahnson J, et al. Prevalence and predictors of abnormal cardiovascular responses to exercise testing among individuals with type 2 diabetes: The Look Ahead (action for health in diabetes) Study. *Diabetes Care.* 2010;33:901–907.
7. Nadeau KJ, Zeitler PS, Bauer TA, et al. Insulin resistance in adolescents with type 2 diabetes is associated with impaired exercise capacity. *J Clin Endocrinol Metab.* 2009;94:3687–3695.
8. Regensteiner JG, Bauer TA, Reusch JE, et al. Abnormal oxygen uptake kinetic responses in women with type II diabetes mellitus. *J Appl Physiol (1985).* 1998;85:310–317.
9. Sward K, Dreja K, Lindqvist A, et al. Influence of mitochondrial inhibition on global and local [Ca(2+)](I) in rat tail artery. *Circ Res.* 2002;90:792–799.
10. Taggart MJ, Wray S. Hypoxia and smooth muscle function: key regulatory events during metabolic stress. *J Physiol.* 1998;509(pt 2):315–325.
11. Rocha M, Herance R, Rovira S, et al. Mitochondrial dysfunction and antioxidant therapy in sepsis. *Infect Disord Drug Targets.* 2012;12:161–178.
12. Rahman A, Ekman M, Shakirova Y, et al. Late onset vascular dysfunction in the R6/1 model of Huntington's disease. *Eur J Pharmacol.* 2013; 698:345–353.
13. McCarron JG, Olson ML, Chalmers S. Mitochondrial regulation of cytosolic Ca(2)(+) signals in smooth muscle. *Pflugers Arch.* 2012;464:51–62.
14. Galloway CA, Lee H, Nejjar S, et al. Transgenic control of mitochondrial fission induces mitochondrial uncoupling and relieves diabetic oxidative stress. *Diabetes.* 2012;61:2093–2104.
15. Shenouda SM, Widlansky ME, Chen K, et al. Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus. *Circulation.* 2011;124:444–453.
16. Wang H, Liu J, Wu L. Methylglyoxal-induced mitochondrial dysfunction in vascular smooth muscle cells. *Biochem Pharmacol.* 2009;77:1709–1716.
17. Park Y, Booth FW, Lee S, et al. Physical activity opposes coronary vascular dysfunction induced during high fat feeding in mice. *J Physiol.* 2012;590(pt 17):4255–4268.
18. Waypa GB, Marks JD, Guzy RD, et al. Superoxide generated at mitochondrial complex III triggers acute responses to hypoxia in the pulmonary circulation. *Am J Respir Crit Care Med.* 2013;187:424–432.
19. Knaub LA, McCune S, Chicco AJ, et al. Impaired response to exercise intervention in the vasculature in metabolic syndrome. *Diab Vasc Dis Res.* 2012;10:222–238.
20. Young CG, Knight CA, Vickers KC, et al. Differential effects of exercise on aortic mitochondria. *Am J Physiol Heart Circ Physiol.* 2005;288: H1683–H1689.
21. Miller MW, Knaub LA, Olivera-Fragoso LF, et al. Nitric oxide regulates vascular adaptive mitochondrial dynamics. *Am J Physiol Heart Circ Physiol.* 2013;304:H1624–H1633.
22. McConell GK, Rattigan S, Lee-Young RS, et al. Skeletal muscle nitric oxide signaling and exercise: a focus on glucose metabolism. *Am J Physiol Endocrinol Metab.* 2012;303:E301–E307.
23. Cacicedo JM, Gauthier MS, Lebrasseur NK, et al. Acute exercise activates Ampk and Enos in the mouse aorta. *Am J Physiol Heart Circ Physiol.* 2011;301:H1255–H1265.
24. Nisoli E, Clementi E, Paolucci C, et al. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science.* 2003;299:896–899.
25. Zhang QJ, McMillin SL, Tanner JM, et al. Endothelial nitric oxide synthase phosphorylation in treadmill-running mice: role of vascular signalling kinases. *J Physiol.* 2009;587(pt 15):3911–3920.
26. Momken I, Lechêne P, Ventura-Clapier R, et al. Voluntary physical activity alterations in endothelial nitric oxide synthase knockout mice. *Am J Physiol Heart Circ Physiol.* 2004;287:H914–H920.

27. Brown SB, Libonati JR, Selak MA, et al. Neonatal exendin-4 leads to protection from reperfusion injury and reduced rates of oxidative phosphorylation in the adult rat heart. *Cardiovasc Drugs Ther.* 2010;24:197–205.

28. Ding L, Zhang J. Glucagon-like peptide-1 activates endothelial nitric oxide synthase in human umbilical vein endothelial cells. *Acta Pharmacol Sin.* 2012;33:75–81.

29. Fan R, Li X, Gu X, et al. Exendin-4 protects pancreatic beta cells from human islet amyloid polypeptide-induced cell damage: potential involvement of Akt and mitochondria biogenesis. *Diabetes Obes Metab.* 2010; 12:815–824.

30. Green BD, Hand KV, Dougan JE, et al. Glp-1 and related peptides cause concentration-dependent relaxation of rat aorta through a pathway involving Katp and Camp. *Arch Biochem Biophys.* 2008;478:136–142.

31. Dong Z, Chai W, Wang W, et al. Protein kinase a mediates glucagon-like peptide 1-induced nitric oxide production and muscle microvascular recruitment. *Am J Physiol Endocrinol Metab.* 2013;304:E222–E228.

32. Kavazis AN, Smuder AJ, Min K, et al. Short-term exercise training protects against doxorubicin-induced cardiac mitochondrial damage independent of Hsp72. *Am J Physiol Heart Circ Physiol.* 2010;299: H1515–H1524.

33. Pauli JR, Ropelle ER, Cintra DE, et al. Acute physical exercise reverses S-nitrosation of the insulin receptor, insulin receptor substrate 1 and protein kinase B/Akt in diet-induced obese Wistar rats. *J Physiol.* 2008;586:659–671.

34. Liu Q, Anderson C, Broyde A, et al. Glucagon-like peptide-1 and the exenatide analogue Ac3174 improve cardiac function, cardiac remodeling, and survival in rats with chronic heart failure. *Cardiovasc Diabetol.* 2010;9:76.

35. Fura A, Khanna A, Vyas V, et al. Pharmacokinetics of the dipeptidyl peptidase 4 inhibitor saxagliptin in rats, dogs, and monkeys and clinical projections. *Drug Metab Dispos.* 2009;37:1164–1171.

36. Mason RP, Jacob RF, Kubant R, et al. Effect of enhanced glycemic control with saxagliptin on endothelial nitric oxide release and Cd40 levels in obese rats. *J Atheroscler Thromb.* 2011;18:774–783.

37. Spinazzi M, Casarin A, Pertegato V, et al. Optimization of respiratory chain enzymatic assays in muscle for the diagnosis of mitochondrial disorders. *Mitochondrion.* 2011;11:893–904.

38. Sun M, Shen W, Zhong M, et al. Nandrolone attenuates aortic adaptation to exercise in rats. *Cardiovasc Res.* 2013;97:686–695.

39. Moien-Afshari F, Ghosh S, Elmi S, et al. Exercise restores endothelial function independently of weight loss or hyperglycaemic status in Db/Db mice. *Diabetologia.* 2008;51:1327–1337.

40. Shigeta T, Aoyama M, Bando YK, et al. Dipeptidyl peptidase-4 modulates left ventricular dyfunction in chronic heart failure via angiogenesis-dependent and independent actions. *Circulation.* 2012;126:1838–1851.

41. Davidson SM. Endothelial mitochondria and heart disease. *Cardiovasc Res.* 2010;88:58–66.

42. Ungvari Z, Sonntag WE, Csiszar A. Mitochondria and aging in the vascular system. *J Mol Med (Berl).* 2010;88:1021–1027.

43. Giedt RJ, Pfeiffer DR, Matzavinos A, et al. Mitochondrial dynamics and motility inside living vascular endothelial cells: role of bioenergetics. *Ann Biomed Eng.* 2012;40:1903–1916.

44. Zhou RH, Vendrov AE, Tchivilev I, et al. Mitochondrial oxidative stress in aortic stiffening with age: the role of smooth muscle cell function. *Arterioscler Thromb Vasc Biol.* 2012;32:745–755.

45. Kelley DE, Goodpaster B, Wing RR, et al. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol.* 1999;277(6 pt 1):E1130–E1141.

46. Kota SK, Kota SK, Jammula S, et al. Effect of diabetes on alteration of metabolism in cardiac myocytes: therapeutic implications. *Diabetes Technol Ther.* 2011;13:1155–1160.

47. Larsen S, Nielsen J, Hansen CN, et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol.* 2012;590(pt 14):3349–3360.

48. Chen Z, Peng IC, Cui X, et al. Shear stress, Sirt1, and vascular homeostasis. *Proc Natl Acad Sci U S A.* 2010;107:10268–10273.

49. Clemmensen C, Chabenne J, Finan B, et al. Glp-1/glucagon coagonism restores leptin responsiveness in obese mice chronically maintained on an obesogenic diet. *Diabetes.* 2014;63:1422–1427.

50. Chai W, Dong Z, Wang N, et al. Glucagon-like peptide 1 recruits microvasculature and increases glucose use in muscle via a nitric oxide-dependent mechanism. *Diabetes.* 2012;61:888–896.

51. Regensteiner JG, Sippel J, McFarling ET, et al. Effects of non-insulin-dependent diabetes on oxygen consumption during treadmill exercise. *Med Sci Sports Exerc.* 1995;27:875–881.

52. Regensteiner JG, Bauer TA, Reusch JE. Rosiglitazone improves exercise capacity in individuals with type 2 diabetes. *Diabetes Care.* 2005;28: 2877–2883.

53. Narkar VA, Downes M, Yu RT, et al. Ampk and Ppardelta agonists are exercise mimetics. *Cell.* 2008;134:405–415.

54. Kadoglou NP, Iliadis F, Sailer N, et al. Exercise training ameliorates the effects of rosiglitazone on traditional and novel cardiovascular risk factors in patients with type 2 diabetes mellitus. *Metabolism.* 2010;59:599–607.