Supplemental Oxygen Improves In Vivo Mitochondrial Oxidative Phosphorylation Flux in Sedentary Obese Adults With Type 2 Diabetes

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Type 2 diabetes is associated with impaired exercise capacity. Alterations in both muscle perfusion and mitochondrial function can contribute to exercise impairment. We hypothesized that impaired muscle mitochondrial function in type 2 diabetes is mediated, in part, by decreased tissue oxygen delivery and would improve with oxygen supplementation. Ex vivo muscle mitochondrial content and respiration assessed from biopsy samples demonstrated expected differences in obese individuals with (*n* = 18) and without (*n* = 17) diabetes. Similarly, in vivo mitochondrial oxidative phosphorylation capacity measured in the gastrocnemius muscle via ^{31}P-MRS indicated an impairment in the rate of ADP depletion with rest (27 ± 6 s [diabetes], 21 ± 7 s [control subjects]; *P* = 0.008) and oxidative phosphorylation (*P* = 0.046) in type 2 diabetes after isometric calf exercise compared with control subjects. Importantly, the in vivo impairment in oxidative capacity resolved with oxygen supplementation in adults with diabetes (ADP depletion rate 5.0 s faster, *P* = 0.012; oxidative phosphorylation 0.046 ± 0.079 mmol/L/s faster, *P* = 0.027). Multiple in vivo mitochondrial measures related to HbA_{1c}. These data suggest that oxygen availability is rate limiting for in vivo mitochondrial oxidative exercise recovery measured with ^{31}P-MRS in individuals with uncomplicated diabetes. Targeting muscle oxygenation could improve exercise function in type 2 diabetes.

Type 2 diabetes is one of the most common diseases worldwide and is a prevalent cause of cardiovascular morbidity and mortality. Even individuals with uncomplicated diabetes have impaired maximal and submaximal exercise capacity (EC), yet the causes remain unclear. We and others have reported cardiac, vascular, and skeletal muscle abnormalities in people with diabetes associated with reduced EC, but these abnormalities do not completely account for the observed impairments in diabetes (1–6). Determining the causes of diabetes-related EC impairments is of great significance as reduced EC predicts shortened life span (7–9).

Muscle mitochondrial dysfunction is implicated as a potential contributor to impaired EC in diabetes based upon the role of mitochondrial energetics in exercise. Patti et al. (10) found that peroxisome proliferator–activated receptor γ coactivator 1-α (PGC1-α; the primary regulator of mitochondrial biogenesis) and nuclear respiratory factor 1 and their downstream target genes for oxidative metabolism

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were decreased in skeletal muscle from adults with diabetes. Scheuermann-Freestone et al. (11), using ^{31}P-MRS, reported that adults with diabetes who have normal cardiac function had impaired myocardial and skeletal muscle energy metabolism related to changes in circulating metabolic substrates (glucose and free fatty acids [FFAs]) before, during, and after exercise. In individuals with diabetes, exercise training improved in vivo mitochondrial oxidative phosphorylation flux to levels similar to those of untrained control subjects without diabetes in addition to improving insulin sensitivity and metabolic flexibility (12,13). Moreover, we reported that ^{31}P-MRS–based ADP time constant (TC), a blood flow–dependent mitochondrial function measure, was slowed and oxidative phosphorylation rates lower in the muscle of youth with diabetes after a brief contraction-induced metabolic stress, consistent with a link between mitochondrial dysfunction and blood flow in diabetes (14). In work by our group and others, muscle deoxygenation with exercise and muscle oxygen (O_2) extraction across the muscle suggests an impairment in muscle oxygenation and decreased arterial-venous O_2 difference with exercise in diabetes (15–18). However, the impact of manipulation of skeletal muscle oxygenation on in vivo mitochondrial dysfunction in diabetes has not been fully assessed.

The association between impaired blood flow and mitochondrial oxidative phosphorylation flux has been previously reported in the presence of decreased tissue O_2 delivery secondary to severe vascular disease. Specifically, individuals with symptomatic peripheral artery disease (PAD) have altered mitochondrial kinetics assessed by ^{31}P-MRS after exercise (19,20). Notably, the severity of the blood flow abnormality predicts the degree of decreased in vivo skeletal muscle mitochondrial function (21). Impairment in PAD-related mitochondrial function is at least partially reversible, as combined lifestyle and medical interventions that improve blood flow in PAD also improve mitochondrial function (22). Further, similar functional in vivo mitochondrial defects can be induced in healthy control subjects by experimental leg blood flow reduction with cuff occlusion (23). Thus, muscle blood flow and in vivo mitochondrial oxidative phosphorylation flux after exercise are associated and can be altered experimentally.

To date, the interaction between muscle oxygenation and in vivo muscle mitochondrial oxidative phosphorylation flux has not been directly tested in diabetes. We hypothesized that impaired in vivo muscle mitochondrial exercise recovery in diabetes is mediated by decreased muscle O_2 delivery and would be responsive to improved increased muscle oxygenation. We tested these hypotheses by measuring in vivo mitochondrial function using ^{31}P-MRS without and then with supplemental O_2 during and after calf exercise in BMI-similar sedentary overweight/obese adults with and without diabetes.

RESEARCH DESIGN AND METHODS

Thirty-five adults who were overweight or obese (BMI 25–40 kg/m^2) and sedentary with and without diabetes between the ages of 30 and 70 years were enrolled. Presence of diabetes was confirmed by medical chart review. Sedentary behavior in all participants was defined as not participating in a regular exercise program (less than one bout of exercise/week) and was further confirmed by use of a questionnaire (Low-Level Physical Activity Recall) (24). Participants were excluded if they had *1*) HbA_{1c} >9% (75 mmol/mol); *2*) insulin, thiazolidinedione, or oral steroid use; *3*) documented coronary or PAD, electrocardiography findings of cardiac ischemia or conduction system abnormalities with a stress exercise test, or use of β-blockers or other symptoms potentially limiting exercise function; *4*) uncontrolled hypertension (systolic blood pressure >150 mmHg and/or diastolic blood pressure >110 mmHg); *5*) obstructive pulmonary disease or asthma; *6*) peripheral neuropathy; *7*) current or past smoking within the last 2 years; *8*) anemia (Hb <10 mg/dL); *9*) autonomic dysfunction (e.g., fall in blood pressure >20 mmHg on standing without change in heart rate); or *10*) implanted metal (due to use of MRI). Overweight/obese control subjects (OC) could not have any health conditions other than excess weight and could not have first-degree relatives with diabetes. This study was approved by the University of Colorado Anschutz Medical Campus Institutional Review Board.

Participants underwent a screening visit to confirm eligibility and three additional study visits. Visit one included consent and a fasting blood sample for a lipid panel (FFAs and glycerol) and HbA_{1c}. Visit two included VO_{2peak} testing using a bicycle ergometer to establish peak exercise performance. Visit three included out-of-magnet leg exercise testing performed in combination with near infra-red spectroscopy (NIRS), which was followed by a biopsy of the dominant gastrocnemius. Visit four included MRI imaging of the leg for maximal cross-sectional area and in-MRI single leg exercise testing with ^{31}P-MRS to assess mitochondrial function. Visits two through four were 1–3 days apart.

Graded Exercise Test

VO_{2peak} was determined via graded exercise to exhaustion as previously described (1,3,6) using a stationary cycle ergometer (Lode Bike, Groningen, the Netherlands) and a metabolic cart (Medgraphics Ultima CPX; Medical Graphics Corp., St. Paul, MN). After the start of exercise, the work rate was increased in 10–20 W/min increments (depending on age and sex) in order to allow each participant to reach maximum within 7–12 min. VO_{2peak} was confirmed by a respiratory exchange ratio >1.1. During incremental exercise testing, the highest VO_2 and heart rate averaged over 20 s were defined as the maximum values.

MRI and MRS

Imaging and Spectroscopy Acquisition

All MRI and ^{31}P-MRS equipment and procedures have been previously described (14,25,26). In summary, imaging and MRS were performed on a General Electric 3T with HDx MRI running version 15M4 software and a Siemens 3T with a Skyra platform. Our scanners were also equipped with the

multinuclear spectroscopy hardware and research software upgrades and used a custom-built $^{1}H/^{31}P$ leg coil (Clinical MR Solutions, Brookfield, WI).

^{31}P-MRS Exercise Protocol

Strength testing of the dominant leg via single leg exercise was done on a custom-built MR-compatible plantar flexion device with force measurement capability, as previously described (14,26–28).

The ^{31}P-MRS exercise protocol consisted of measurements during rest for 60 s, isometric plantar flexion for 90 s at 70% maximal volitional contraction, and recovery for 8 min postexercise. We selected a 90-s isometric exercise bout as this perturbation has been extensively modeled and used for assessing both aerobic and anaerobic processes (14,25,27,29,30). Force was monitored and recorded in kilograms continuously throughout the exercise, with verbal and visual feedback to keep the force within the target goal. After this room air (RA) exercise bout, participants received 6–8 L of O_2 by face mask. After 5 min of O_2 saturation of 99–100%, a second identical exercise bout was performed.

Spectroscopy Analysis

Analysis of the spectroscopy was done as previously reported (14,25,27,30). In brief, peak positions and areas of interest (phosphocreatine [PCr], inorganic free phosphate [Pi], β-ATP [three peaks], α-ATP [two peaks], γ-ATP [two peaks], and phosphomonoester) were determined by time domain fitting using jMRUi (31) using AMARES (a method of accurate, robust, and efficient spectral fitting), a nonlinear least square–fitting algorithm with previously built prior knowledge files (32), as previously described (25,30). ADP concentrations were calculated using a Michaelis-Menten model of the creatine kinase reaction, as previously reported (30). All exercise spectra were corrected for saturation using the fully relaxed spectra for that day. Calculations used data from the end of exercise and during the immediate recovery period, and included the rates of oxidative phosphorylation (OxPhos) calculated as ΔPCr/time from the first 10 s after cessation of exercise, initial PCr synthesis (VPCr), and apparent maximum rate of oxidative ATP synthesis (QMAX), which is calculated with the initial rate of PCr resynthesis relative to end-exercise ADP concentration using an assumed Km = 30 μmol/L. TCs for ADP and PCr were calculated via regression analyses with Sigmaplot (Systat Software, Inc., San Jose, CA).

NIRS Data Acquisition and Analysis

Tissue total hemoglobin+myoglobin ([tHb]), deoxy [hemoglobin+myoglobin] ([HHb]), and oxy[hemoglobin+myoglobin] ([OHb]) were assessed by a frequency domain multidistance NIRS monitor (Optiplex TS; ISS, Champaign, IL), which was placed at the maximal circumference of the calf during the exercise bouts. The NIRS monitor emits two wavelengths (690 and 830 nm) and measures absorbance at distances of 2.0, 2.5, 3.0, and 3.5 cm. The NIRS data were sampled continuously and recorded at 50 Hz. Upon export, data were downsampled to 1 Hz using a running average of the higher-resolution 50-Hz data. The NIRS monitor was calibrated prior to each visit using a calibration phantom of known scattering and optical properties. Resting values of tissue [tHb], [HHb], and [OHb] were obtained by averaging the 30 s with and without supplemental oxygen.

Muscle Biopsy and Muscle Biopsy Analyses

Skeletal muscle biopsies were taken from the medial aspect of the gastrocnemius muscle from the same leg that underwent MRI. Biopsies were obtained at least 24 h after the last known exercise bout (either MRS or NIRS exercise) to ensure that the skeletal muscle was in a resting metabolic state. A modified Bergstrom needle technique was used to obtain 40–50 mg of skeletal muscle after local anesthesia with 2% lidocaine and a 1-cm skin incision (33). Samples were processed for muscle fiber respiration and tissue processed for Western analysis.

Mitochondrial Respiration

Mitochondrial respiration was measured using Oroboros Oxygraph-2k (O2k; Oroboros Instruments Corp., Innsbruck, Austria) according to modifications from previously described protocols (34–36). Immediately after biopsy, ~10–20 mg of skeletal muscle tissue was placed in ice-cold mitochondrial preservation buffer (BIOPS [10 mmol/L Ca-EGTA, 0.1 mmol/L free calcium, 20 mmol/L imidazole, 20 mmol/L taurine, 50 mmol/L K-MES, 0.5 mmol/L dithiothreitol, 6.56 mmol/L $MgCl_2$, 5.77 mmol/L ATP, 15 mmol/L PCr, pH 7.1]). Muscle fibers were separated mechanically (in BIOPS and on ice), partially teased apart by fine forceps, permeabilized by incubation with saponin in BIOPS on ice, and then washed in mitochondrial respiration buffer (MiR06 [0.5 mmol/L EGTA, 3 mmol/L magnesium chloride, 60 mmol/L K-lactobionate, 20 mmol/L taurine, 10 mmol/L potassium phosphate, 20 mmol/L HEPES, 110 mmol/L sucrose, 1 g/L BSA, 280 units/mL catalase, pH 7.1]). The 2–3 mg of fibers was added to prewarmed MiR06 + 25 μmol/L blebbistatin in the O2k. Oxygen in the MiR06 was started at 400 mmol/L and maintained at >250 mmol/L.

Two sets of substrates and inhibitors were added to assess respiration rates at several states. Rates for run 1 were measured after the addition of pyruvate (P) and malate (M) (state 2 PM); PM with ADP (state 3 PM); PM, ADP, and glutamate (G) and succinate (S) (state 3 PMGS); and PMGS, ADP, and oligomycin (state 4 PMGS). Rates for run 2 were measured after the addition of octanoylcarnitine and M (state 2 OCM); OCM with diphosphate (ADP) (state 3 OCM); OCM, ADP, G, and S (state 3 OCMGS); and OCMGS, ADP, and oligomycin (state 4 OCMGS). Cytochrome c was added to determine that mitochondrial membrane damage was minimal and the same between runs. Respiratory control ratios (RCRs) were calculated as a ratio of state 3 PM/state 2 PM (RCR_{PM}), state 3 PMGS/state 4 PMGS (RCR_{PMGS}), state 3 OCM/state 2 OCM (RCR_{OCM}), and state 3 OCMGS/state 4 OCMGS (RCR_{OCMGS}).

Biochemistry

Skeletal muscle (35–50 mg) was homogenized and then centrifuged (18,000*g* for 10 min at 4°C). The supernatant

Table 1—Participant characteristics and metabolic outcomes

	Control (n = 17)	Diabetes (n = 18)	P value
Sex (female/male)	9/8	11/7	0.099
Age (years)	56.7 (43.6, 64.6)	59.4 (53.2, 67.0)	0.276
Race, n (%)			0.793
White	10 (58.8)	11 (61.1)	
Black	3 (17.6)	4 (22.2)	
Asian	1 (5.8)	1 (5.5)	
Native American	2 (11.6)	0 (0.0)	
Latino	1 (5.8)	2 (11.0)	
Ethnicity, n (%)			
Hispanic	4 (23.5)	4 (22.2)	0.136
Non-Hispanic	13 (76.5)	14 (77.8)	
BMI (kg/m^2)	29.9 ± 3.5	31.5 ± 4.8	0.288
Body fat (%)	35.2 ± 8.4	37.7 ± 8.7	0.396
Diabetes-related variables			
Diabetes duration (years)	NA	9.5 ± 6.6	NA
Use of metformin, n (%)	NA	10 (77)	<0.001*
Use of statins, n (%)	2 (12)	7 (39)	<0.001*
Use of antihypertensive, n (%)	1 (8)	8 (44)	<0.001*
Diabetes markers			
HbA_{1c} (%)	5.4 (5.3, 5.5)	6.1 (5.6, 6.7)	<0.001*
HbA_{1c} (mmol/mol)	35.5 (34.5, 36.6)	43.2 (37.7, 59.7)	<0.001*
Adiponectin (mg/dL)	8.1 (6.9, 10.9)	7.5 (4.1, 10.7)	0.458
Fasting FFA (mmol/L)	388 (246, 542)	563 (467, 638)	0.038*
Fasting glycerol (nmol/L)	81 (59, 97)	93 (85, 121)	0.073
Serum lipids			
Total cholesterol (mg/dL)	179 ± 27	158 ± 33	0.052
Fasting triglycerides (mg/dL)	105 (71, 117)	114 (85, 192)	0.102
HDL (mg/dL)	52 ± 12	47 ± 12	0.217
LDL (mg/dL)	112 ± 27	91 ± 30	0.034*
Exercise-related variables			
VO_{2peak} (mL/kg/min)	21.6 (17.8, 28.0)	16.5 (13.9, 22.9)	0.012*
Absolute VO_{2peak} (mL/min)	2,068 ± 750	1,577 ± 498	0.028*

Data are median (25%, 75%) or mean ± SD, unless stated otherwise. Descriptive characteristics and metabolic descriptors of the participants are shown. P value from Mann-Whitney U testing. NA, not applicable. *$P < 0.01$.

was analyzed for protein concentration by Bradford assay. Citrate synthase enzyme activity was determined by previously described methods (34). Protein in Laemmli sample buffer was run on 4–20% Tris-HCl gels. Proteins were electrophoretically transferred to polyvinylidene fluoride membranes, and equivalence of protein loading was assessed by staining of membrane-bound proteins by Ponceau S stain. Blots were probed using antibodies against PGC1-α, voltage-dependent anion channel (VDAC), OxPhos complexes, and vinculin (1:1,000; overnight at 4°C) and followed by fluorescent secondary. Proteins were detected by Li-COR (Odyssey CLX) Western blot scanner, and densitometric analysis was performed using Image Studio v4.1. Data are normalized to vinculin protein expression.

Determination of Myosin Heavy Chain Fiber Type Distribution

A section of muscle from the biopsy was frozen in Tissue-Tek OCT (Sakura Finetek, Torrance, CA). Frozen muscle was equilibrated to the cryostat thermostat (23–24°C) prior to cutting 8-μm-thick sections from the mounted tissue onto charged slides. Slides were blocked in 5% BSA in PBS for 60 min and then incubated overnight at room temperature in primary antibodies: myosin heavy chain (MyHC) type I fibers: BA.D5 IgG2b supernatant (5 μg/mL in 5% BSA; Developmental Studies Hybridoma Bank [DHSB], Iowa City, IA), MyHC type IIa fibers: SC.71 IgG1 supernatant (5 μg/mL in 5% BSA; DHSB, SC-71 was deposited to the DSHB by S. Schiaffino), and MyHC type IIx fibers: 6H1 IgM supernatant (3.75 μg/mL in 5% BSA; DHSB). After the overnight incubation, slides were washed in PBS and blocked again in 5% BSA for 60 min. Slides were incubated in corresponding secondary antibodies for 90 min at room temperature (IgG2b: 1:2,000 [M32407], IgG1: 1:1,000 [A10551], IgM: 1:750 [A10677]; Thermo Fisher Scientific, Waltham, MA). Slides were washed in PBS, and 3,3′-diaminobenzidine tetrahydrochloride (DAB) was applied for 5 min. Slides were counterstained with hematoxylin for 4 min, dried in ethanol

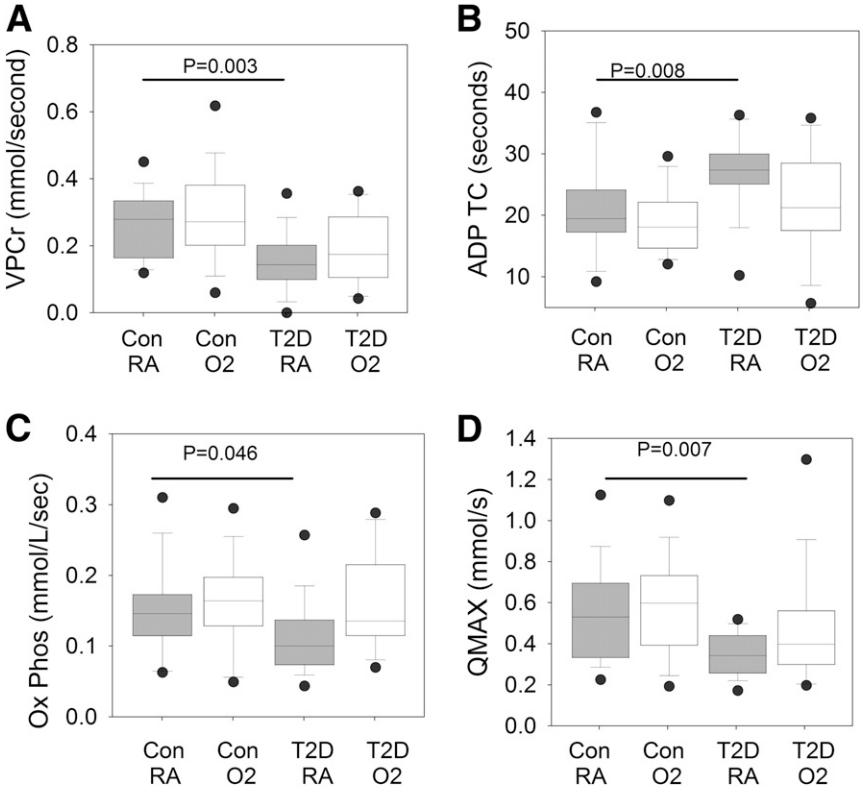
and xylene, and covered. Positive fiber identification was completed by an individual blinded to participant group.

Statistical Analysis

Adequate sample size was predicted to be 10 subjects in each group to demonstrate a difference in the a priori primary outcome determined in vivo mitochondrial outcomes, based on the variability seen in similar youth (14,25). The results are presented as mean ± SD, median (25th, 75th percentile), or proportions as appropriate. A general linear mixed model was fit to assess differences in four primary and eight control in vivo mitochondrial outcomes between interventions (RA and supplemental O_2) and disease status (diabetes and OC). The outcome was mitochondrial function, either in vivo or ex vivo. The predictors were supplemental O_2, disease status, and the two-way interaction between supplemental O_2 and disease status. The model was adjusted for sex and statin use. Df were adjusted using the Kenward-Roger method, and residual diagnostics were used to assess model assumptions. An F test was used to assess the significance of the two-way interaction, and contrasts were used to test for differences between disease groups and interventions. NIRS measures were analyzed using a Student t test. Pearson correlations were used to investigate relationships between HbA_{1c} and measures of in vivo and ex vivo mitochondrial function. Statistics were performed with SAS software, version 9.4 (Cary, NC).

RESULTS

A total of 35 participants were studied, 17 OC and 18 with diabetes. The participant characteristics, fasting laboratory, and exercise testing results are shown in Table 1. Groups were similar for BMI and age. As expected, those with diabetes had a higher HbA_{1c} than OC (6.1% [43 mmol/mol] vs. 5.3% [34 mmol/mol]). Average diabetes duration was 9.5 years, and all but three participants with diabetes were



	Control (OC)		Diabetes (T2D)	
	Room Air	Sup. Oxygen	Room Air	Sup. Oxygen
Oxygen Saturation during exercise (%)	95.5 ± 0.2	98.8 ± 0.6	94. 3 ± 0.2	98.7 ± 0.5
Muscle oxygen saturation StO2 (%)	68.9 ± 8.0	71.1 ± 7.7	72.3 ± 7.8	74.6 ± 7.7
Tissue total Hemoglobin and myoglobin [tHb] (uM)	56.3 ± 23.4	58.0 ± 23.7	66.2 ± 32.7	67.2 ± 33.3
Exercise force/area (kg/cm^2)	0.007 ± 0.002	0.007 ± 0.002	0.007 ± 0.002	0.007 ± 0.002
Change in ADP with exercise (mmol/L)	21 (16, 29)	25 (13, 32)	17 (10, 22)	18 (9, 26)

Figure 1—Spectroscopy-measured mitochondrial measurements and oxygen assessments at baseline and after O_2 supplementation. Data shown as mean, 25th to 75th in shaded box, then 5th and 95th with bars, and outliers as circles. *A*: VPCr after cessation of exercise is lower in diabetes on RA, but with supplemental O_2, the groups are not different. *B*: ADP TC after cessation of exercise tends to be longer at RA in diabetes, but with supplemental O_2, the groups are not different. *C*: Rate of OxPhos after cessation of exercise tends to be slower with RA in diabetes, but with supplemental O_2, the groups are not different. *D*: QMAX is lower in diabetes on RA, but with supplemental O_2, the groups are not different. Control measures for each bout are shown below the figure, and for hemoglobin measures include 12 control subjects and 13 subjects with diabetes. Con, control; T2D, type 2 diabetes.

taking at least 500 mg of metformin per day; although only two were taking 2,000 mg/day. Two OC and seven participants with diabetes were taking an ACE inhibitor or angiotensin receptor blocker for hypertension. By design, no subjects were on β-blockers. One OC and seven participants with diabetes were taking a statin for high cholesterol. Importantly, results did not differ in those taking or not takin statins (data not shown).

Oxygen saturation of hemoglobin (StO_2) at rest measured using NIRS did not differ between OC and participants with diabetes. Supplemental O_2 led to a significant increase in StO_2 that was not different between the groups (Fig. 1).

In Vivo Mitochondrial Measures

In vivo ^{31}P-MRS muscle mitochondrial end points are shown in Fig. 1 and raw metabolite curves in Supplementary Fig. 1. The groups had similar *1*) blood O_2 saturation in response to RA and supplemental O_2, *2*) work relative to their calf muscle size, and *3*) ADP generation during each calf exercise bout, as shown in the inset under the figure. After the cessation of exercise, VPCr was significantly slower in those with diabetes compared with OC in RA; however, VPCr was no longer different between the groups after treatment with supplemental O_2 (Fig. 1*A*). With RA, the ADP TC was significantly slower in diabetes compared with OC, and there was no difference between the groups with supplemental O_2 (Fig. 1*B*). The rate of OxPhos was significantly lower in those with diabetes in RA (Fig. 1*C*), and there was no difference between the groups with supplemental O_2. QMAX was significantly lower in participants with diabetes on RA; again this difference resolved during O_2 supplementation (Fig. 1*D*). The mean change with O_2 supplementation within each group is shown in Table 2, and comparisons between RA and O_2 measurements are shown in Fig. 1. In diabetes, the addition of supplemental O_2 led to faster ADP TC and OxPhos (P = 0.011 and P = 0.024, respectively). In contrast, there were no changes in any of the measured or calculated mitochondrial end points in OC with the addition of supplemental O_2.

Correlations Between HbA_{1c} and Mitochondrial End Points

Prior to the addition of supplemental O_2, positive correlations were found between HbA_{1c} with the PCr TC (r = 0.346, P = 0.03) and the ADP TC (r = 0.430, $P < 0.01$) (Fig. 2). In this condition, there was a negative relationship between HbA_{1c} and VPCr ($r = -0.370$, P = 0.02). The addition of supplemental O_2 changed the relationships between HbA_{1c} and these parameters. With supplemental O_2, there was no relation between HbA_{1c} and VPCr ($r = -0.124$, P = 0.5). Significant correlations with HbA_{1c} persisted with supplemental O_2 for the PCr TC (r = 0.458, P = 0.01) and the ADP TC (r = 0.527, $P < 0.01$). HbA_{1c} did not correlate with state 3 respiration measured ex vivo with either substrate suit ($P > 0.3$).

Ex Vivo Mitochondrial Measures

Gastrocnemius muscle lysates were analyzed for mitochondrial proteins and citrate synthase activity (Fig. 3*A*–*E*). Citrate synthase activity was lower in people with diabetes compared with OC (P = 0.012). There was no significant reduction in PGC1-α, VDAC-1, or any of the mitochondrial oxidative phosphorylation subunit proteins detected by the mitochondrial complex antibody.

There were no statistical differences between OC and participants with diabetes in O_2 consumption at a saturating oxygen level using either the carbohydrate substrates or in the oxidation of the lipid substrates, with the exception of uncoupled PMGS (Fig. 3*F*); calculated respiratory exchange ratio (a measurement of mitochondrial efficiency) was not different between groups.

MyHC Fiber Type Distribution

There were no differences in skeletal muscle fiber type distribution between the groups ($P > 0.2$). For control participants, 43.0 ± 15.8% stained positive for type I fibers, 33.9 ± 14.4% were type IIa, and 30.9 ± 15.9% were type IIb. For participants with type 2 diabetes, 38.8 ± 9.2% were type I fibers, 42.1 ± 12.1% were type IIa, and 33.6 ± 10.0% were type IIb.

DISCUSSION

Using a combination of in vivo and ex vivo methods, we found that after isolated isometric calf exercise, O_2 availability was limiting for in vivo mitochondrial oxidative phosphorylation flux in sedentary adults with type 2 diabetes compared with healthy adults of similar weight and activity level without diabetes. Our in vivo studies using ^{31}P-MRS confirm in adults with diabetes the significant

Table 2—Change in in vivo mitochondrial measures with supplemental oxygen

	Control		Diabetes	
	Difference	*P* value	Difference	*P* value
VPCr (mmol/s)	−0.036 ± 0.108	0.185	−0.0330 ± 0.101	0.159
ADP TC (s)	0.150 ± 6.263	0.923	−5.021 ± 7.528	0.012
OxPhos (mmol/L/s)	−0.0104 (−0.0553, 0.0345)	0.190	0.059 ± 0.094	0.016
QMAX (mmol/s)	0.05 (−0.09, 0.20)	0.452	0.14 (−0.01, 0.28)	0.068

Data are median (25%, 75%) or mean ± SD. The mean change in in vivo mitochondrial respiration measurements of diabetes and OC in response to supplemental oxygen are shown, and within each group, values with RA and with O_2 were compared and *P* values are presented. VPCr did not change with O_2 in OC but trended to increase in diabetes. ADP TC did not change with O_2 in OC but was faster in diabetes with O_2. OxPhos rates after cessation of exercise did not change with O_2 in OC but were faster in diabetes with O_2. QMAX did not change with O_2 in OC but trended to increase in diabetes. *P* value from Mann-Whitney *U* testing.

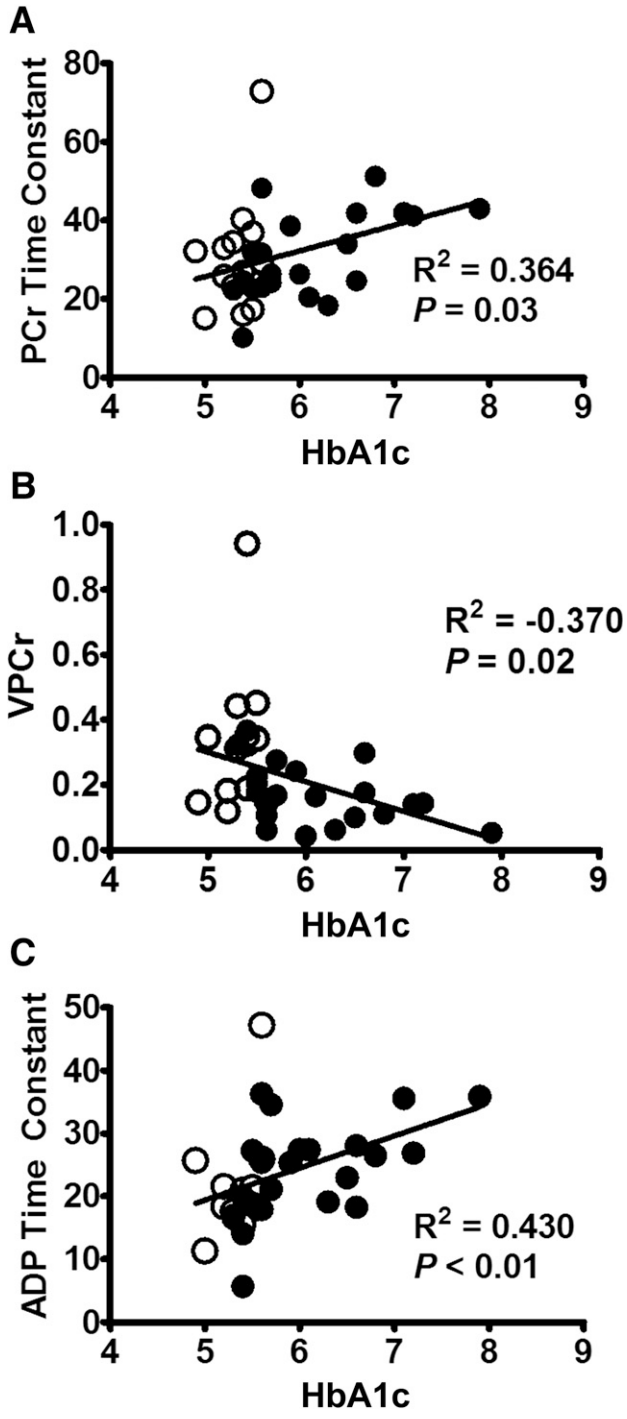
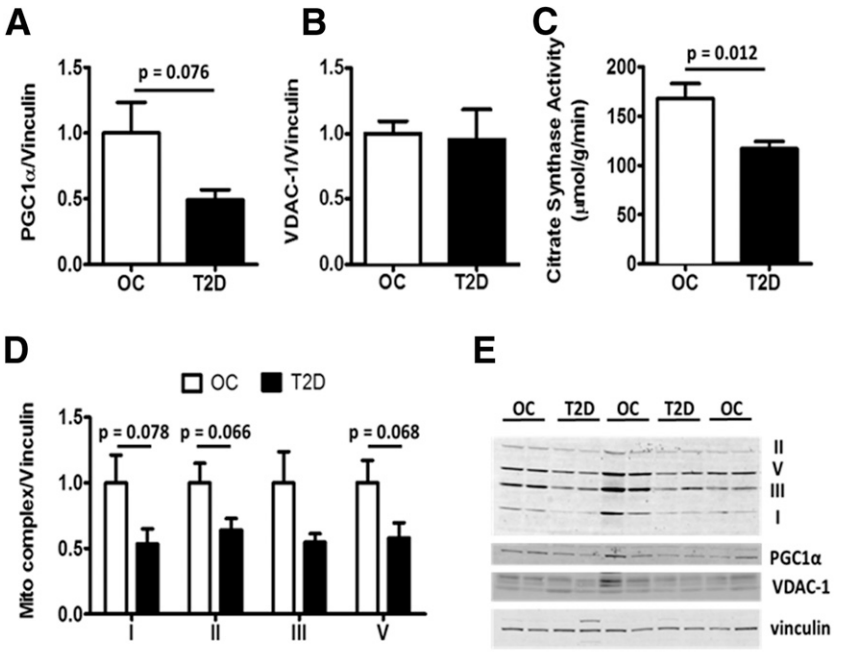


Figure 2—A higher HbA_{1c} relates to more impaired in vivo mitochondrial measures at RA. *A*: PCr TC during recovery from exercise. *B*: VPCr, i.e., initial PCr recovery rate during recovery from exercise. *C*: ADP TC during recovery from exercise. Open circles denote OC, and closed circles represent subjects with diabetes. R^2 is correlation coefficient.

mitochondrial functional impairment we reported in youth with diabetes (14), as measured by abnormal PCr recovery rate, oxidative phosphorylation rates, and ADP recovery and QMAX after calf exercise. We also observed the novel finding that the addition of supplemental O_2 significantly improved muscle StO_2 in overweight adults with and without diabetes. This increase in StO_2 only led to a measurably increased rate of oxidative phosphorylation and ADP TC in subjects with diabetes. In vivo PCr TC and ADP TC correlated with glucose control as measured by HbA_{1c}. Interestingly, supplemental O_2 resolved the difference in in vivo mitochondrial function between overweight sedentary adults with or without diabetes. In contrast, ex vivo biopsy measures demonstrate decreased citrate synthase and uncoupled mitochondrial oxygen consumption in skeletal muscle from subjects with type 2 diabetes compared with control subjects. Together, these findings suggest that muscle oxygenation is rate limiting for in vivo mitochondrial oxidative phosphorylation flux during exercise recovery in people with uncomplicated diabetes. We conclude that the in vivo postexercise oxidative metabolism impairment in overweight individuals with diabetes is related to limitations in muscle O_2 availability, rather than solely defects in the mitochondria itself.

Muscle insulin resistance leads to decreased glucose uptake, decreased glycogen stores, and decreased efficiency of ATP production (12,37–39). Insulin resistance in skeletal muscle of people with diabetes has been related to diminished activity of oxidative enzymes, the accumulation of muscle triglycerides and/or smaller lipid moieties, as well as a reduction in electron transport activity of intact mitochondria or reduced mitochondrial content (12). Mitochondrial impairment has been previously reported in youth and adults with diabetes based on both noninvasive ^{31}P-MRS analysis and muscle biopsy results (10–14). In this report, we observed a similar decrease in in vivo mitochondrial oxidative phosphorylation flux in adults with diabetes compared with OC and an inverse correlation with HbA_{1c}, as in previous reports (11). As could be predicted if O_2 is limiting oxidative function, supplemental O_2 resolved the diminished in vivo oxidative rates seen in individuals with diabetes such that there was no longer a difference between subjects with and without diabetes. The observation that there was no change in in vivo mitochondrial oxidative phosphorylation flux in the OC compared with the subjects with diabetes with O_2 supplementation is consistent with a specific O_2 availability limitation in diabetes.

Our prior work has demonstrated decreased cardiac and skeletal muscle perfusion using noninvasive methods in people with uncomplicated diabetes (2,5). Others have reported that skeletal muscle perfusion limitations in people with diabetes are due to decreased capillary density and capillary recruitment and are related to insulin resistance (40–43). There is additional evidence of capillary basement membrane thickening, muscle fiber abnormalities, and decreased capillary density in diabetes skeletal muscle (44). Butcher et al. (45) defined a contribution of perfusion to in vivo muscle oxygen consumption (muscle VO_2). We recently reported that the metabolic syndrome alters muscle blood flow patterns and decreases muscle O_2 extraction in a rat model of diabetes, largely due to changes in blood flow distribution pattern, i.e., perfusion heterogeneity (17).



F

(M/F)	State 3_{PM}	State 3_{PMGS}	Uncoupled$_{PMGS}$	State 3_{OCM}	State 3_{OCMGS}	Uncoupled$_{OCMGS}$
Overweight control	36.5±2.1 (5/8)	53.9±3.4 (7/9)	89.1±4.6 (7/9)	22.9±2.0 (7/8)	57.2±4.1 (7/8)	83.7±5.2 (7/8)
T2DM	31.2 ± 2.1 (4/10)	45.6 ± 3.0 (3/11)	71.3 ± 4.5* (3/11)	19.2 ± 1.3 (4/10)	49.2 ± 3.3 (3/10)	74.1 ± 3.3 (3/10)

*, p<0.05 vs overweight control; State 3 and uncoupled respiration, pmol $O_2 s^{-1} mg^{-1}$; P, pyruvate; M, malate; G, glutamate; S, succinate; OC, octanoyl carnitine

(M/F)	RCR_{PM}	RCR_{PMGS}	RCR_{OCM}	RCR_{OCMGS}
Overweight control	7.1±0.5 (5/7)	3.0±0.1 (7/9)	4.4±0.3 (7/8)	3.0±0.1 (7/8)
T2DM	7.9±1.2 (4/9)	2.9±0.2 (3/11)	4.7±0.4 (4/10)	2.9±0.2 (3/10)

RCR, respiratory control ratio; P, pyruvate; M, malate; G, glutamate; S, succinate; OC, octanoyl carnitine

Mean ± SE

Figure 3—Ex vivo assessment of gastrocnemius muscle of participants with diabetes and OC. *A*: PGC1-α protein content was not different between groups. *B*: VDAC protein content was not different between groups. *C*: Citrate synthase enzyme activity was significantly lower in diabetes compared with OC. *D*: OxPhos subunit (mitochondrial complexes I, II, III, and V) protein content was not different between groups. *E*: Representative gel. *F*: Mitochondrial respiration measurements performed on an Oroboros O2k from OC and subjects with diabetes. *Significant differences between groups ($P < 0.05$). *G*: Representative image of immunohistochemistry for fiber typing. Scale bars: 100 μm. *H–J*: Quantification of type I, type IIa, and type IIb fibers. There were no differences between groups. T2D, type 2 diabetes.

Changes in perfusion homogeneity can be modeled, and these simulations demonstrate that even modest changes in perfusion heterogeneity can result in mismatch between tissue supply and demand (46). Similar to this preclinical finding, Baldi et al. (18) reported reduced arterial-venous O_2 differences with exercise in people with type 2 diabetes. For the current study, to test whether O_2 availability was a limiting factor for postcontraction oxidative phosphorylation, we augmented muscle O_2 saturation with supplemental O_2. The observation that supplemental O_2 acutely improved in vivo muscle oxidative rates might be predicted by the simulations of Cano et al. (47), which reveal that perfusion heterogeneity can result in O_2 availability limiting muscle O_2 consumption. In a recent report in healthy participants, experimental generation of decreased muscle perfusion and effective tissue hypoxia led to decreased estimated muscle O_2 delivery and early muscle fatigue (48). In healthy control subjects, muscle deoxygenation correlates with EC; we recently reported a dissociation between skeletal muscle deoxygenation with exercise function in people with diabetes, consistent with uncoupled oxygen supply and demand (15). Improvement of in vivo mitochondrial oxidative phosphorylation flux in participants with diabetes with isolated O_2 supplementation is consistent with a mitochondrial reserve that can be harnessed when O_2 supply is not limiting (without changing the properties of muscle mitochondria). Moreover, the resolution of differences in in vivo oxidative function between people with and without diabetes in the setting of demonstrated impaired intrinsic skeletal muscle mitochondrial function indicates that augmentation

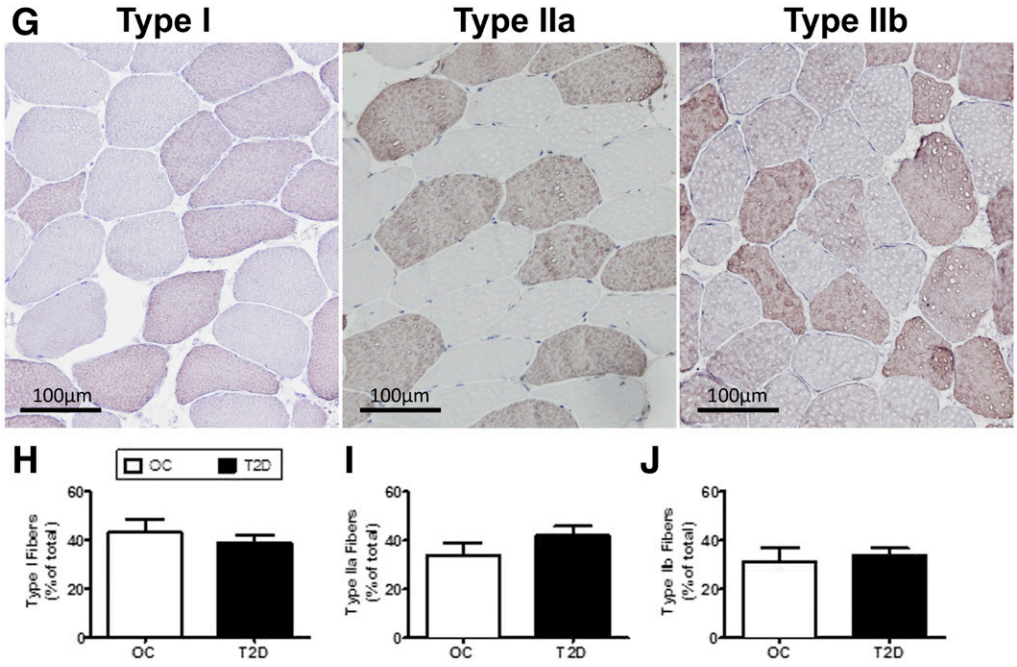


Figure 3—*Continued*.

of oxygen supply can compensate for the muscle mitochondrial defect in diabetes at this submaximal workload. These findings suggest that skeletal muscle mitochondrial dysfunction is not the sole determinant of decreased in vivo oxidative rate in diabetes. These findings do not demonstrate correction of the intrinsic muscle mitochondrial dysfunction in our subjects, nor do we have clarity on the details of how oxidative function is improved.

Abnormalities in muscle mitochondrial content and function have been documented for over 20 years in diabetes using both muscle biopsy and ^{31}P-MRS measurements (10–12,39,49). Using ^{31}P-MRS, we have documented similar abnormalities in youth with diabetes with <3 years of diabetes duration as compared with lean and obese youth without diabetes (14). In keeping with previous reports (10), our ex vivo biopsy results revealed decreased citrate synthase activity as well as trends toward decreased PGC1-α and mitochondrial oxidative phosphorylation subunit proteins in diabetes (Fig. 3). Mitochondrial respiration in permeabilized muscle fibers was significantly decreased in subjects with diabetes compared with control subjects for uncoupled mitochondrial oxygen consumption of PMGS. No difference in RCR is observed between groups. Studies examining mitochondrial respiration in diabetes have shown mixed results. Decreased, no change, or increased respiration have been reported in muscle biopsy samples; this variability may be due to participant selection, differences in muscle fiber type distribution, and study design (10,50,51). In our cohort, both the OC and the participants with diabetes were sedentary by study design and there was not a difference in fiber type distribution (Fig. 3). Seventy-seven percent of our subjects with type 2 diabetes were on metformin; this may have contributed to the decrease in mitochondrial oxygen consumption with PMGS observed in subjects with type 2 diabetes. Only subjects with type 2 diabetes improved in vivo mitochondrial oxidative phosphorylation flux with O_2 supplementation, despite skeletal muscle mitochondrial dysfunction in these subjects. Previous reports have primarily been conducted in the vastus lateralis rather than the gastrocnemius muscle in overweight sedentary participants; it is possible that the gastrocnemius muscle is used more in activities of daily living than the vastus lateralis given the central role of the gastrocnemius in walking. Future work will evaluate the adaptive changes in mitochondrial function with exercise training.

This was a physiological study designed specifically to test the impact of increasing muscle oxygenation with supplemental O_2 on muscle mitochondrial oxidative phosphorylation flux in vivo using ^{31}P-MRS. Limitations include small sample size and greater use of medications in the group with diabetes compared with the control group, and thus our findings may not be generalizable to all people with diabetes. The sample size was prespecified based on our publications in youth with type 1 and type 2 diabetes and polycystic ovarian disease (14,25). In terms of between-group differences in medications such as statins, metformin, or antihypertensives, participants were being treated with medications representing standard of care, and post hoc adjustment for medication status did not affect results. In people with insulin resistance, metformin has been reported to decrease VO_{2peak} (52,53). A strength of the study is that force output for each measure was recorded and is similar with and without O_2, and thus differences in work output do not confound results. In addition, we do not know precisely how the supplemental O_2 improves oxidative rates or why it was improved only in people with diabetes and not in OC, since

the increase in StO_2 did not differ between groups. Based on our recent simulated and experimentally confirmed data in rodents (15,17,46), we expect that O_2 conductance was improved by increasing muscle StO_2. Due to the altitude (5,280 feet above sea level) of Denver, StO_2 is lower at baseline and there is a 4–5% change with supplemental O_2, so it is also possible that O_2 supplementation at sea level would not produce similar results. However, Scheuermann-Freestone et al. (11) observed similar tissue hemoglobin deoxygenation by NIRS at sea level in people with diabetes as we have reported, consistent with our observation that the changes observed are being mediated by diabetes status.

In conclusion, established key markers of in vivo impairment in mitochondrial oxidative phosphorylation flux in people with diabetes are acutely resolved with supplemental oxygen. These data support that there is a targetable physiological limitation in in vivo mitochondrial oxidative phosphorylation flux due to limitation in O_2 availability, even in people with well-controlled, recently diagnosed, and clinically uncomplicated diabetes and intrinsic skeletal muscle mitochondrial dysfunction. This correctable defect may be explained by microvascular perfusion heterogeneity and may offer opportunities for targeted clinical intervention.

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Author Contributions. M.C.-G. designed the study, collected data, performed MRS analysis, and prepared the manuscript. R.L.S., K.H., and D.O. performed biopsy analysis and reviewed the manuscript. B.R.N. designed and analyzed the MRS study and reviewed the manuscript. I.E.S. designed the study, collected data, and reviewed the manuscript. A.G.H. and M.S.B. collected data. S.M. collected data and reviewed the manuscript. L.K. performed biopsy analysis. K.J.N. designed the study and reviewed the manuscript. P.M.M. analyzed data. T.A.B. designed the study. J.G.R. designed the study and prepared and reviewed the manuscript. J.E.B.R. designed the study, performed biopsy analysis, and prepared and reviewed the manuscript. J.E.B.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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