Muscle Oxygen Supply and Use in Type 1 Diabetes, From Ambient Air to the Mitochondrial Respiratory Chain: Is There a Limiting Step?

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Short running title: O_2 from lung to mitochondria in type 1 diabetes

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ABSTRACT

OBJECTIVE

Long before developing clinical complications of type 1 diabetes, oxygen supply and use can be altered during daily-life activities. We examined all steps of the oxygen pathway, from lungs to mitochondria, using an integrative *ex vivo* (muscle biopsies) and *in vivo* (during exercise) approach, in patients with uncomplicated type 1 diabetes.

RESEARCH DESIGN AND METHODS

Sixteen adults with type 1 diabetes (T1D) were compared with 16 strictly-matched healthy controls (CON). We assessed lung diffusion capacity for carbon monoxide (DL_{CO}) and nitric oxide (DL_{NO}), as well as exercise-induced changes in arterial O_2 content (SaO_2, PaO_2, Hb), muscle blood volume and O_2 extraction (near-infrared spectroscopy). Blood samples were analyzed for metabolic and hormonal vasoactive moieties and factors able to shift the O_2Hb dissociation curve. Mitochondrial oxidative capacities were assessed in *vastus lateralis* permeabilized muscle fibers.

RESULTS

Lung diffusion capacity and arterial O_2 transport were normal in T1D. However, they displayed blunted exercise-induced increase in muscle blood volume and O_2 extraction despite higher serum insulin and higher erythrocyte 2,3-diphosphoglycerate, respectively. While complexes I- and II-supported mitochondrial respiration were unaltered, complex IV relative (*vs.* complex I) capacity was impaired in T1D, and this was even more apparent in cases of longstanding diabetes and high HbA_{1c}. $\dot{V}O_{2max}$ was lower in T1D *vs.* CON.

CONCLUSIONS

Early defects in microvascular blood delivery to skeletal muscle and in mitochondrial respiratory chain complex IV capacity may negatively impact aerobic fitness. These findings are clinically relevant considering the major role of skeletal muscle oxidation in whole body glucose disposal.

MAIN TEXT

Large clinical trials in type 1 diabetes have underlined the important role of tissues' prolonged exposure to hyperglycemia in the pathogenesis of microvascular complications (1). Endothelial dysfunction can occur very early in the disease history, *i.e.*, before overt vascular complications (2), hence altering metabolites and oxygen (O_2) supply to major tissues. Hyperglycemia may also contribute to mitochondrial dysfunction leading to impairment of tissue energy production (3).

Long before developing overt clinical diabetes complications, oxygen supply and use can be challenged in daily-life situations such as aerobic exercise. Maximal oxygen consumption ($\dot{V}O_{2max}$), determined during exhaustive incremental exercise, reflects the highest achievable outcome of the integrated pathway. It relies on the serial steps of oxygen transfer from lungs to blood, delivery of oxygenated blood through the complicated branching networks of blood vessels, and its final use in skeletal muscle mitochondria. In type 1 diabetes, several studies have attempted to investigate some of these serial steps, albeit each of them in an isolated approach.

While a lower pulmonary diffusion capacity has been described in patients suffering from long-term diabetes complications (4), it does not appear as clearly in studies including uncomplicated patients (5; 6), possibly because of the wide range of patients' glycemic control (7). The lungs represent a suitable target for hyperglycemia-induced vessel dysfunction and non-enzymatic glycation of collagen proteins, because of their wide capillary network and their significant amount of connective tissue. Concerning the second step of the oxygen supply process, we have previously suggested normal arterial oxygenation in uncomplicated patients with poorly-controlled type 1 diabetes, but impaired exercise-induced muscle vasodilatation (8).

The ultimate step of O_2 utilization in the mitochondria has been only partially investigated in humans with type 1 diabetes. *In vivo* non-invasive approaches using either near-infrared spectroscopy (NIRS) (*i.e.*, muscle oxygen extraction) during aerobic exercise (8) or 31P-magnetic resonance spectroscopy (*i.e.*, calculated maximal rate of ATP oxidative resynthesis) following a local isometric exercise (9-11) indirectly opened up the possibility of an impaired muscle extraction and/or mitochondrial use of oxygen in comparison with non-diabetic controls, and more so in cases with a higher HbA_{1c} level. However, the exact causes for lower *in vivo* muscle oxygen extraction and/or use cannot be inferred from these indirect non-invasive approaches. Studies using muscle biopsies reported normal maximal oxidative enzymatic capacities in subjects with type 1 diabetes (10; 12-14).

Nevertheless, enzymatic assays of the individual steps of the Krebs cycle, beta oxidation, and respiratory chain complexes cannot reveal how well all enzymes interact with each other and may mask some mitochondrial defects. In contrast, *in situ* gold-standard experiments in permeabilized muscle fibers, using a specific substrate/inhibitor titration approach, provide a detailed characterization of functional intact mitochondria in their normal intracellular position and assembly, preserving essential interactions (15). Monaco *et al.* thus implemented this method to clarify mitochondrial (dys)function in type 1 diabetes and found a decreased complex II-supported respiration (16). However, the putative consequences of this *ex vivo* defect were not tested *in vivo*.

Therefore, by combining multiple *in vivo* (particularly during exercise) and *ex vivo* (in muscle biopsies) approaches in patients and their strictly matched healthy controls, the present study aims to gain a further in-depth insight into the impact of type 1 diabetes and glycemic control on all steps of the integrated pathway for oxygen, from the atmosphere to the mitochondrial respiratory chain in skeletal muscle.

RESEARCH DESIGN AND METHODS

This study was approved by the regional ethical committee (N°EudraCT:2009-A00746-51). Written consent was obtained before inclusion. Sixteen patients (18–40 years of age) with type 1 diabetes for at least 1 year and free from microvascular and macrovascular complications, were recruited (T1D group, Table 1). They were compared to 16 healthy subjects (CON group, normal glucose tolerance checked with OGTT, WHO criteria) selected (by verbal questioning) to strictly match each of the T1D patients according to gender and to pre-established ranges or values for age (± 7 years), body mass index (± 4 $kg.m^{-2}$), moderate-to-vigorous leisure time physical activity level (±1 $h{\cdot}wk^{-1}$ when the patients' physical activity category was 0 $h{\cdot}wk^{-1}$, ± 2 $h{\cdot}wk^{-1}$ for category 2–6 $h{\cdot}wk^{-1}$, ± 4 $h{\cdot}wk^{-1}$ for category > 6 $h{\cdot}wk^{-1}$; pairs of patient/control being in the same category), and tobacco use (grouped according to no smoking, < 10 cigarettes a day, and > 10 cigarettes a day). The matching of participants in terms of body composition and physical activity was further checked using dual-energy X-ray absorptiometry (HOLOGIC-Inc., USA),the validated Modified Activity Questionnaire (17) and accelerometry (GT1M;Actigraph) over 7 consecutive days.

Subjects came twice to the laboratory. They were requested to refrain from vigorous activity for 48 h prior to the visits and from using tobacco the morning of both visits.

During the first visit, patients received their usual morning insulin bolus and all subjects consumed a breakfast (mean ± SD: 8.1 ± 4.7% proteins, 43.3 ± 16.1% lipids, 48.6 ± 15.2% carbohydrates) based on their usual breakfast and previously verified by a dietitian. Afterwards, lung diffusion capacity for carbon monoxide (DL_{CO}) and nitric oxide (DL_{NO}) was assessed. An incremental maximal cycling exercise was performed 3.4 ± 0.5 h after breakfast, with concomitant measurements of respiratory gas exchanges, arterial O_2 transport, skeletal muscle perfusion, and O_2 extraction. After a 2-min resting period (baseline) sitting on the cycle ergometer (Excalibur-Sport, Lode, The Netherlands), the test started at 30W and was increased by 20W every 2 min until exhaustion (ambient temperature, 18−20°C).

Following an 8 h overnight fast, on the morning of the second visit, a muscle biopsy was taken from the *vastus lateralis* to assess *ex vivo* intrinsic mitochondrial respiratory capacity in permeabilized skinned muscle fibers.

Alveolar-capillary membrane diffusion capacity (DL_{CO})

DL_{CO} was assessed following the international guidelines with apnea maintained for at least 8 sec (Medisoft, Dinant, Belgium). To gain access to the determinants of DL_{CO}, *i.e.*, membrane transfer capacity (D_m) and capillary lung volume (V_c), DL_{NO} was further evaluated.

Cardiopulmonary response

The electrocardiogram and pulmonary gas exchanges were measured continuously throughout exercise (breath-by-breath system, Ergocard®). $\dot{V}O_{2max}$ (the highest 15-s average value at test termination) was obtained for all the subjects (Table 2). O_2 pulse (ratio $\dot{V}O_2$/heart rate) was used as a stroke volume indicator during exercise (18).

Muscle perfusion and O_2 extraction

Subjects were equipped with an NIRS probe (Oxymon MkIII, Artinis, The Netherlands) to monitor, at 10 Hz, light absorption (two continuous wavelengths, 780 and 850 nm) across *vastus lateralis* microvessels throughout exercise (8). Using the Beer-Lambert law and a normalization against the baseline period, we determined the changes in muscle oxygenation (oxyhemoglobin, ΔO_2Hb), deoxygenation due to O_2 extraction

(deoxyhemoglobin, ΔHHb), and blood volume (total hemoglobin, ΔTHb, as the arithmetical sum of ΔO_2Hb and ΔHHb) (19).

Blood analyses (details about assays in Table 2 legend)

Forearm venous (catheter) blood samples were taken at rest and at maximal exercise for measurement of plasma glucose, catecholamines, serum free insulin, free fatty acids, and glycerol. Likewise, at rest and at maximal exercise, microcapillary arterialized ear-lobe blood was collected for determination of erythrocyte 2,3-diphosphoglycerate, lactate, pH, K^+, $PaCO_2$ and components of arterial O_2 content (CaO_2) (*i.e.*, arterial O_2 saturation (SaO_2), PaO_2, Hb).

Mitochondrial respiratory capacity in muscle fibers

A sample of *vastus lateralis* muscle, obtained by the percutaneous Bergstrom technique after local anesthesia (lidocaine 2%), was immediately placed into an ice-cold solution mimicking the intracellular fluid (20). The muscle fibers were separated under a binocular microscope and permeabilized with saponin (50µg/mL) for 30 min (dissolving the sarcolemma but not the outer mitochondrial membrane). After being placed for 10 min in a respiration buffer (20) to wash out adenine nucleotides and creatine phosphate, skinned fibers were transferred in a 1 mL water-jacketed oxygraphic cell (Hansatech Instruments, UK) equipped with a Clark electrode. Oxygen consumption (flux) reflects the first time derivative of oxygen concentration in the respiration chambers, expressed as µmol $O_2 \cdot min^{-1} \cdot g$ dry $weight^{-1}$. Relative contributions of the respiratory complexes I, II, and IV (CI, CII, CIV), and of oxidation/phosphorylation were assessed using the following sequential additions of substrates/inhibitors: glutamate–malate (10:5 mM), generating NADH,H+ ($\dot{V}_{GM}$); the phosphate acceptor, ADP (2mM) ($\dot{V}_{GM\text{-}ADP}$); the CI inhibitor, rotenone (0.2 µM); the electron donor for CII, succinate (25 mM) ($\dot{V}_{Succ}$); the uncoupler, CCCP (carbonyl cyanide m-chloro phenyl hydrazone, 1µ M) ($\dot{V}_{Succ\text{-}CCCP}$); the CIII inhibitor, antimycin A (2.5 µM); and the artificial electron donor to cytochrom *c*, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD)–ascorbate (0.5:2 mM), assessing CIV capacity as an isolated step of the respiratory chain ($\dot{V}_{TMPD}$). The respiratory control ratio (RCR, $\dot{V}_{GM\text{-}ADP}/\dot{V}_{GM}$) was calculated as an index of coupling efficiency between oxidation (O_2 consumption) and phosphorylation (of ADP to ATP) with CI substrates. $\dot{V}_{Succ}/\dot{V}_{GM\text{-}ADP}$ and $\dot{V}_{TMPD}/\dot{V}_{GM\text{-}ADP}$ were calculated as internal normalizations to assess specific CII

and CIV relative capacities independently of mitochondrial content. Mitochondrial capacities to oxidize carbohydrates ($\dot{V}_{Pyr}$) and fatty acids ($\dot{V}_{PC}$) were assessed in separate samples in the respiration buffer, in the presence of ADP (2 mM) and malate (2 mM), using sequential additions of pyruvate (1 mM) and palmitoyl-carnitine (135 µM). Maximal citrate synthase activity (expressed per milligrams of protein) was assessed on muscle samples, immediately frozen in liquid nitrogen and preserved at −80°C, using spectrophotometric assay.

Statistical analyses

Statistical analyses were performed with IBM SPSS 19.0 software. Results are reported as means ± SD unless otherwise indicated. Non-repeated data were compared between groups with the Wilcoxon matched-pairs test. Repeated data (normally distributed according to Shapiro-Wilk) were compared between groups (fixed effect) and according to exercise (fixed effect: rest *vs.* peak exercise or, for NIRS, ventilatory expiratory flow [$\dot{V}_E$], and O_2 pulse outcomes, relative exercise intensities with a value every 10% $\dot{V}O_{2max}$ as well as absolute exercise intensities in Watts) using linear mixed models for repeated measurements. If significant main effects or interactions were observed, Bonferroni *post hoc* comparisons were applied. Correlations were tested using Spearman's rho. $P<0.05$ was considered statistically significant.

RESULTS

Demographic and anthropometric data did not differ between groups (Table 1). T1D had lower $\dot{V}O_{2max}$ (Table 2) than CON despite comparable levels of habitual physical activity (Table 1) as well as comparable heart rates at exhaustion. Plasma glucose increased during exercise in both groups, with overall higher values in T1D (Table 2). No hypoglycemia occured in T1D during exercise.

Alveolar-capillary diffusion (Table 2)

DL_{CO}, as well as its determinants D_m and V_c, did not differ between groups. V_c is influenced by the number of pulmonary capillaries in contact with ventilated alveoli, which increases during exercise because pulmonary blood flow and lung volumes increase. O_2 pulse, indirectly reflecting stroke volume, as well as $\dot{V}_E$ and its components (tidal volume, respiratory rate), increased throughout exercise without intergroup differences (data not shown, mixed models: Group and Interaction: NS).

Oxygen arterial transport (Table 2)

Although PaO_2 was slightly lower in T1D *vs.* CON, CaO_2 and correspondingly [Hb] were not impaired, and they increased even more during exercise in T1D *vs.* CON.

Muscle perfusion (ΔTHb) and O_2 extraction (ΔHHb) (Figure 1)

Despite higher serum insulin and normal catecholamine concentrations (Table 2), the levels and the increase in ΔTHb were lower in T1D *vs.* CON, especially at exercise intensities above 30% of $\dot{V}O_{2max}$. The levels and the increase in ΔHHb were lower in T1D *vs.* CON, particularly at exercise intensities above 50% of $\dot{V}O_{2max}$, and this, in spite of higher 2,3-diphosphoglycerate concentrations in T1D (Table 2).

Muscle O_2 mitochondrial use (Figure 2)

Citrate synthase activity was similar in T1D and CON (92 ± 47 and 85 ± 31 pmol·min^{-1}·mg protein^{-1}, respectively).

Oxygen fluxes with the different mitochondrial substrates, $\dot{V}_{GM\text{-}ADP}$ and $\dot{V}_{Pyr}$ (electrons through CI to CIII, CIV), as well as $\dot{V}_{Succ}$ (electrons through CII to CIII, CIV) and $\dot{V}_{PC}$ (electrons through CI and CII to CIII, CIV), did not differ between groups. The comparable $\dot{V}_{PC}$ and $\dot{V}_{Pyr}$, measured *in vitro* in muscle, were in accordance with *in vivo* whole-body lipid and carbohydrate estimated oxidation rates throughout exercise (*i.e.*, comparable $\dot{V}_{CO2}/\dot{V}_{O2}$, data not shown). Neither alterations in ATP synthase ($\dot{V}_{Succ\text{-}CCCP}$ minus $\dot{V}_{Succ}$, data not shown) nor in global electron transport system capacity (from CII: $\dot{V}_{Succ\text{-}CCCP}$), or oxidation/phosphorylation coupling efficiency (RCR) were noticeable in T1D. However, specific examination of the different mitochondrial chain complexes revealed impairment in CIV relative capacity ($\dot{V}_{TMPD}/\dot{V}_{GM\text{-}ADP}$) in T1D while CII relative capacity ($\dot{V}_{Succ}/\dot{V}_{GM\text{-}ADP}$) was unaltered.

It is noteworthy that in T1D, diabetes duration correlated negatively with CIV relative capacity ($\dot{V}_{TMPD}/\dot{V}_{GM\text{-}ADP}$, r = −0.59, P<0.05), and HbA_{1c} tended to negatively correlate with CIV capacity ($\dot{V}_{TMPD}$, r = −0.47, P<0.07). Moreover, longer diabetes duration and lower CIV relative capacity were predictors (covariates in mixed models) of a smaller ΔHHb rise with exercise intensity (interaction with exercise intensity, e = −0.004,

P<0.001 and e = +0.02, P<0.001, respectively). The other steps of O_2 transport from lung to mitochondria were not significantly associated with HbA_{1c} or diabetes duration.

In CON, $\dot{V}_{TMPD}$ correlated with $\dot{V}O_{2max}$ and maximal aerobic power (r>0.64, P<0.01).

CONCLUSIONS

The novelty of the present study resides in the examination of all steps of the pathway of oxygen from air to mitochondria by combining both *in vivo* and *ex vivo* approaches in patients with uncomplicated type 1 diabetes and strictly matched healthy controls (Supplemental Figure S1). We showed that alveolar-capillary membrane diffusion capacity and arterial O_2 transport were normal at this stage of the disease. However, we confirmed that these patients display blunted perfusion and oxygen extraction in active skeletal muscle microvessels at moderate-to-maximal exercise intensities. The defect in oxygen extraction occurred despite an overall normal intrinsic mitochondrial maximal respiratory capacity. The only detectable alteration in the mitochondrial chain appeared at the level of CIV, and all the more among patients with poorly-controlled longstanding diabetes.

Considering our results and the very few studies having matched their control population on physical activity levels (6; 21), it seems that DL_{CO}, D_m, and V_c are not impaired and do not correlate with glycemic control in patients with uncomplicated type 1 diabetes. Thus, the thickening of the pulmonary capillary basal lamina and pulmonary vasculature dysfunction are probably still absent, or without detectable consequences, when clinical complications are not overt. It is, however, still possible that some subtle alterations are already present in the T1D participants, as suggested by the reduced PaO_2. Notwithstanding, CaO_2 was adequately maintained throughout exercise in T1D. Subtle alterations in lung function might have been balanced by a higher affinity for O_2 when hemoglobin is glycated (22), leading to normal SaO_2. It could have also been the case in previous study in which patients with longstanding type 1 diabetes displayed alterations of DL_{CO} and D_m in a more demanding situation (intense exercise in a hypoxic environment) without any repercussion on SaO_2 (6).

While the first steps of oxygen supply occurring in the lungs and arteries resulted in a normal arterial O_2 transport throughout exercise in T1D, the subsequent steps in muscle microvessels appeared to be impaired. Very few studies have investigated exercise-induced muscle vasoreactivity in type 1 diabetes. Rissanen *et al.* reported reduced muscle blood flow in the active leg of adults with type 1 diabetes, at the peak intensity of an

incremental cycling exercise, using an indirect method based on deoxygenation patterns (%ΔHHb) and theoretical values of peripheral arterial-venous O_2 difference (23). In the current work and in our previous study (8), we as well highlighted an impaired exercise-induced increase in muscle blood volume in response to maximal incremental exercise.

Admittedly, cardiac output is one determinant of muscle perfusion (24). We used an indirect marker of stroke volume which appeared normal throughout exercise in T1D. The available literature related to cardiac output in uncomplicated patients with type 1 diabetes and physical activity-matched controls is conflicting, with one report highlighting lower cardiac output during submaximal exercise in adolescents (25), while others found no intergroup differences in adults at submaximal (23) or peak exercise (6; 10). Even if the involvement of central cardiovascular factors cannot be totally discarded in the understanding of lower muscle blood volume, the latter is probably also triggered by peripheral microvascular alterations. Supporting this hypothesis, Pichler *et al.* found an impaired increase in *brachioradialis* muscle blood flow in children with type 1 diabetes in the recovery period of a short rhythmic handgrip test, a local exercise involving a very small muscle mass where muscle blood flow is unlikely to be limited by cardiac output (26).

Following the few previous studies on exercise-induced muscle microvascular reactivity limits in type 1 diabetes (8; 23), we go here a step further into the understanding of putative underpinning mechanisms. Although their relative contribution remains controversial, it is well recognized that arterial, capillary, and venous compartments all participate in the muscle microvascular THb signal (19). Within muscle arterioles, while norepinephrine represents a vasoconstrictor devoted to blood pressure regulation, epinephrine, as well as insulin (27), can promote endothelial nitric oxide production and hence vasorelaxation. In our study, while plasma catecholamines were comparable, serum free insulin was markedly elevated in T1D *vs.* CON. The high insulin concentrations, concomitant with higher plasma glucose levels, reflect the well-described state of insulin resistance in type 1 diabetes, which could presumably also apply at the endothelial cell level, as already proven in obesity and type 2 diabetes (27). This might explain the impaired muscle vasoreactivity despite higher insulin concentrations observed in the T1D group. Of note, the higher plasma glucose levels observed in T1D throughout exercise can certainly not explain the concomitant lower increase in regional blood volume. Dye *et al.* (28) indeed found an inverse effect with an increased post-occlusive reactive hyperemia-induced vasodilatation in hyperglycemic (200 $mg.dL^{-1}$) compared to euglycemic conditions in patients with type 1

diabetes. The other vasoactive moieties that we considered ($PaCO_2$, K+, pH) did not significantly differ between the two groups. While muscle microvascular density is presumably normal in uncomplicated type 1 diabetes (10; 16), further studies are needed to fully determine the molecular mechanisms of the reduced exercise-induced muscle vasoreactivity. Although Fayh *et al.* (29) hypothesized that nitric oxide production is not involved in the lower post-exercise blood flow that they observed in young adults with type 1 diabetes, this result is worth a confirmation since they did not distinguish nitrates from nitrites in their measurements. Only nitrites are known to sensitively reflect acute changes in nitric oxide synthase activity (30).

Since muscle O_2 and high-energy phosphate stores are small, any sustained elevation in muscle ATP turnover in active skeletal muscle during exercise requires that the rate of O_2 delivery to muscle mitochondria precisely matches its O_2 requirements. In the current study, the last step of oxygen delivery to skeletal muscle before its utilization, *i.e.*, muscle O_2 extraction (ΔHHb), was significantly blunted in response to increased exercise intensity in T1D. This was more pronounced in the case of longstanding diabetes, and it occurred despite higher erythrocyte 2,3-diphosphoglycerate concentrations. As previously suggested (8), this result obtained in a sample with rather poorly-controlled diabetes could be partly explained by an impairment in oxyhemoglobin dissociation near active skeletal muscle, induced by the greater affinity of HbA_{1c} for O_2 compared to the non-glycated one (22). It is noteworthy that adjustments in 2,3-diphosphoglycerate concentrations compensatory to increased HbA_{1c} formation in type 1 diabetes may be insufficient to maintain the normal erythrocyte oxygen dissociation (31).

Besides the putative reduced oxyhemoglobin dissociation, the influence of impaired mitochondrial O_2 use on the blunted ΔHHb signal cannot be ruled out. To clarify the partition between both mechanisms, we combined, in an integrated approach, an *ex vivo* analysis in muscle biopsies with the *in vivo* exploration of O_2 extraction during exercise. In the T1D group, no major alteration in mitochondrial oxidative capacity appeared. Mitochondrial content (citrate synthase activity) was comparable in both groups as previously suggested (12; 13). We also found normal overall intrinsic mitochondrial maximal respiratory capacity with the different mitochondrial substrates. Of note, while *ex vivo* mitochondrial intrinsic capacity to oxidize palmitate as well as *in vivo* free fatty acid oxidation rate were normal in T1D, it remains that lipolysis might be blunted as suggested by the lower circulating glycerol at rest and at maximal exercise. The latter observation must be considered in

conjunction with the concomitant higher circulating insulin in T1D, since insulin is a potent inhibitor of lipolysis.

To date, only one other research group (16) provided insight into mitochondrial oxidative capacities in functional intact mitochondria under *in situ*-like conditions in type 1 diabetes. In line with our results, Monaco *et al.* did not observe any alteration in CI-supported mitochondrial respiration, regardless of the substrate used, in a smaller sample of subjects (11 patients *vs.* 8 healthy subjects) (16). However, in contrast to our results and to previous studies examining isolated maximal capacity of succinate dehydrogenase (12; 14), Monaco *et al.* observed a lower capacity of CII-supported respiration by succinate (16). In the latter study, Body Mass Index was higher in the patients with type 1 diabetes compared to the healthy controls. This intergroup difference might partly explain the discordance with our results about CII-supported respiration capacity. Diet-induced obesity in animals has indeed been shown to decrease the rate of CII substrate-driven ATP synthesis in cardiac muscle (32), and weight loss in obese humans is associated with improvement in adipose tissue CII activity (33). Accordingly, by further testing correlations between participants' characteristics and mitochondrial respiration among T1D patients, we found that fat mass percentage, as objectively measured by DEXA, inversely correlated with $\dot{V}_{Succ}$ ($r = -0.51$, $P<0.05$).

Complex IV of the electron transport chain was not specifically investigated in the study of Monaco *et al.*, although it represents a major site for mitochondrial diseases (34). Complex IV is the terminal component of the mitochondrial respiratory chain and is essential for mitochondrial energy transduction. It catalyzes electron transfer from cytochrome *c* to molecular oxygen, generating a proton gradient required for ATP synthesis. Strikingly, the CIV relative contribution was significantly reduced (by ~29%) in T1D patients involved in our study, and mostly in cases of longer diabetes duration and higher HbA_{1c} levels. The underlying mechanisms of such an impairment remain to be investigated, but chronic hyperglycemia-induced oxidative stress may be part of the picture. It is well known that excessive glucose provision to mitochondria elevates reactive oxygen species (3), and as demonstrated on bovine heart muscle, CIV represents an important target for oxidative damage (35), thereby contributing to mitochondrial dysfunction (36). Particularly in type 1 diabetes, the low insulin concentrations in the portal circulation due to the peripheral mode of insulin administration, shifts glucose metabolism into an excessive hepatic glucose production, while skeletal muscle is forced to accept the high glucose load in a context of high peripheral circulating insulin (37).

In studies closely mimicking the *in vivo* conditions by using saponin-permeabilized human muscle fibers, it has been demonstrated that CIV exerts a tight control on respiration, with only a low excess capacity of cytochrome oxidase. This is even more pronounced in cases of the lower, physiological oxygen concentrations (38), which can explain the pathological phenotype of mild cytochrome *c* oxidase deficiencies in mitochondrial myopathies (34). Consistently, in our study, the relative CIV capacity defect in T1D may have implications for aerobic fitness: the lower CIV capacity significantly predicted the blunted exercise-induced increase in muscle O_2 extraction in T1D, while higher CIV capacity was associated with higher aerobic fitness in CON.

Last, although changes in skeletal muscle have been intensively studied in rodent models of type 1 diabetes (39), further investigation in humans is required to supplement our mechanistic understanding of observed mitochondrial dysfunctions. Rodent models of diabetes are not directly transposable to humans because tight blood glucose control through multiple insulin injections is virtually impossible to achieve over long periods of time in animals. In particular, assessing complex IV supramolecular interactions with other complexes might be of great value. The structural and functional organization of the electron transport chain could indeed change from freely moving to assembled structures called supercomplexes, which are believed to increase the transport efficiency and limit the production of reactive oxygen species. In a mouse model of type 1 diabetes mellitus, overexpression of mitofilin, a protein that affect supercomplex assembly, was even able to restore mitochondrial function (40).

In summary, maximal aerobic exercise could represent a physiological way to identify possible subclinical defects in the serial steps responsible for appropriate adjustments of O_2 delivery and subsequent mitochondrial O_2 utilization. This investigation revealed that relatively young patients with type 1 diabetes display blunted muscle microvascular reactivity to exercise along with lower relative capacity of CIV in the mitochondrial respiratory chain. Early microvascular and muscle oxidative capacity dysregulations, in addition to negatively impacting aerobic fitness−a strong predictor of cardiovascular risk−could also have deep long-term consequences on the primary determinants of diabetic complications. Defects in blood and nutrient delivery to skeletal muscle, as well as altered subsequent mitochondrial oxidation, can indeed have a direct impact on glycemic and lipid profiles. Skeletal muscle is actually known to be responsible for most of the insulin-stimulated whole body glucose disposal and for roughly half of the non-insulin mediated glucose uptake in the

presence of hyperglycemia (41). It is also quantitatively the most dominant tissue with respect to lipid metabolism. In the face of these defects, implementing non-pharmacological interventions like specific exercise training programs might be of utmost clinical importance, especially since skeletal muscle is a highly malleable tissue with the capacity of metabolic adaptations in response to contractile activity. The challenge of future studies will be to ensure that these defects in peripheral tissues perfusion−observed even at light to moderate exercise intensities−could be improved by training and euglycemia.

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Author contributions. E. H. conceived the experiments and contributed to their performance, analyzed data, and wrote the manuscript. S. T. performed the experiments and contributed to the conception of the experiments, data analysis, and writing of the manuscript. F. D contributed to the performance of the

experiments, data analyses, and review of the manuscript. P. F. contributed to the conception of the experiments, patient recruitment, and review of the manuscript. V. W., R. C., P. B., J. A., E. L., A. C., and G. M., contributed to the performance of the experiments and review of the manuscript. R. M. and S. B. contributed to the conception of the experiments and review of the manuscript. A. D. contributed to patient recruitment and review of the manuscript. E. H. is the guarantor of this work and, as such, had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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TABLES

Table 1. Participants'characteristics.

	T1D	CON
Anthropometric and demographic data		
Total n (male/female)	16 (12/4)	16 (12/4)
Age (years)	28.5 ± 6.8	27.7 ± 6.6
BMI (kg/m2)	22.9 ± 2.2	23.1 ± 2.3
Smoking status (Smokers/Non-smokers)	4 / 12	4 / 12
Fat mass (%)	19.8 ± 6.4	18.4 ± 5.6
Fat mass right leg (%) †	20.7 ± 6.9	20.5 ± 7.8
HbA_{1c} (%)	8.3 ± 1.5**	5.2 ± 0.2
HbA_{1c} (mmol/mol)	67.0 ± 16.4**	33.0 ± 2.2
Diabetes duration (years)	8.5 ± 5.2	
Insulin delivery (MDI/CSII)	10 / 6	
Insulin dose ($units{\cdot}kg^{-1}{\cdot}day^{-1}$)	0.67 ± 0.18	
Physical activity		
Leisure activity ($h{\cdot}wk^{-1}$) (MAQ)	2.8 ± 3.3	3.0 ± 2.0
Leisure activity ($MET\text{-}h{\cdot}wk^{-1}$) (MAQ)	17.6 ± 16.7	21.6 ± 15.5
Total activity ($MET\text{-}h{\cdot}wk^{-1}$) (MAQ)	49.7 ± 93.7	45.0 ± 50.3
MVPA ($min.wk^{-1}$) (Accelerometry)	232.2 ± 204.5	264.5 ± 128.5
Sedentarity time ($h.day^{-1}$) (Accelerometry)	8.7 ± 2.2	10.9 ± 2.7
Usual daily macro nutrient intake		
Total caloric intake (TC) ($kcal{\cdot}day^{-1}$)	1992.6 ± 496.8	2291.2 ± 489.5
Protein (%TC)	16.1 ± 3.1	16.3 ± 3.2
Fat (% of TC)	33.5 ± 7.0	36.3 ± 3.7
Polyunsaturated / saturated fatty acids ratio	0.3 ± 0.4	0.3 ± 0.4
Cholesterol ($mg{\cdot}day^{-1}$)	292.7 ± 146.1	335.3 ± 145.2
Carbohydrate (% of TC)	50.4 ± 7.4	47.4 ± 5.2
High glycemic index carbohydrate (% of TC)	14.8 ± 5.3	17.1 ± 4.3
Fiber intake ($g{\cdot}day^{-1}$)	18.9 ± 4.8	19.0 ± 4.8

Legend. Values are means ± SD; T1D, patients with type 1 diabetes; CON, healthy control group; * Significantly different from CON group (Wilcoxon test) *P<0.05, **P<0.01, ***P<0.001.

MDI: under multiple daily insulin injections; CSII: under continuous subcutaneous insulin infusion; MAQ: Modifiable Activity Questionnaire; MVPA: Moderate-to-Vigorous physical activity; patients' HbA_{1c} levels ranged between 5.8% [40 mmol/mol] and 10.7% [93 mmol/mol]; patients were free from microvascular (retinopathy, nephropathy, neuropathy) and macrovascular (high blood pressure, coronary disease, peripheral arteriopathy) complications; fat mass was measured by dual energy X-ray absorptiometry; † we also checked that subcutaneous skinfold was <1.5cm at the *vastus lateralis* to ensure accuracy of NIRS measurements; HbA_{1c} was measured just before exercise in EDTA anticoagulated blood (VARIANT II TURBO System, Bio-Rad); usual daily macro nutrient intake was assessed using a 3-day diary (including 2 weekdays and 1 week end day), checked by a research trained dietitian during an appointment with the participant. Accelerometry (GT1M, Actigraph) data are displayed only

for 8 subjects per group because 1 healthy control and 4 patients with type 1 diabetes did not strictly follow our recommendations (mainly, wearing the accelerometer during all waking hours) and the accelerometer devices worn by 2 patients with type 1 diabetes were defective (no signal recorded at the end of the week). The *P*-values (Wilcoxon test) for leisure activity ($h.wk^{-1}$ and $MET\text{-}h.wk^{-1}$), total activity ($MET\text{-}h.wk^{-1}$) and moderate-to-vigorous physical activity (accelerometry, $min.wk^{-1}$) were 0.53, 0.43, 0.64 and 0.26, respectively.

Table 2. Aerobic fitness, alveolar-capillary exchanges, circulatory and metabolic data during incremental maximal exercise.

		T1D (n =16)	CON (n =16)	Mixed models main effects or Wilcoxon
Aerobic fitness				
$\dot{V}O_{2max}$ (mL·min^{-1}·kg^{-1})		34.9 ± 7.2	40.7 ± 6.7	P < 0.05
Maximal aerobic power (W)		207.5 ± 41.9	230.0 ± 45.6	P < 0.05
HRpeak (bpm)		186.5 ± 10.6	188.4 ± 11.1	NS
Oxygen pulse (mL/beat)	Rest	4.4 ± 1.5	5.2 ± 1.6	Exercise: P <0.001, Group: NS, Interaction: NS
	Peak	14.5 ± 3.0	15.9 ± 3.8	
RERpeak		1.2 ± 0.1	1.1 ± 0.1	NS
Blood lactate at peak (mM)		11.1 ± 3.0	11.4 ± 3.6	NS
Peak rate of perceived exertion		19.3 ± 0.9	18.7 ± 1.0	NS
Alveolar-capillary exchanges				
DL_{CO} (mL·min^{-1}·mmHg^{-1}) ‡		31.8 ± 6.1	32.3 ± 5.8	NS
DL_{NO} (mL·min^{-1}·mmHg^{-1})		166.8 ± 25.7	174.0 ± 36.3	NS
Dm (mL·min^{-1}·mmHg^{-1})		84.6 ± 13.0	88.3 ± 18.4	NS
Vc (mL)		92.6 ± 22.9	95.3 ± 21.0	NS
Arterial oxygen transport				
PaO_2 (mmHg)	Rest	93.8 ± 6.1	99.7 ± 10.7	Exercise: P<0.001, Group: P <0.01, Interaction: NS
	Peak	99.5 ± 6.7 †	110.9 ± 11.7 ††	
SaO_2 (%)	Rest	98.3 ± 1.1	98.5 ± 0.6	Exercise: P<0.01, Group: NS, Interaction: NS
	Peak	97.4 ± 0.6	98.1 ± 0.8	
Hb (g·dL^{-1})	Rest	14.9 ± 1.2	15.0 ± 1.3	Exercise: P<0.001, Group: NS, Interaction: P < 0.05
	Peak	16.7 ± 1.8 ††	15.5 ± 1.6	
CaO_2 (mL·100 mL^{-1})	Rest	20.4 ± 1.8	20.5 ± 1.7	Exercise: P<0.001, Group: NS, Interaction: P < 0.05
	Peak	22.7 ± 2.3 ††	21.2 ± 2.2	
Factors able to shift O_2Hb dissociation curve and vasoactive substances				
pH	Rest	7.41 ± 0.04	7.42 ± 0.01	Exercise: P<0.001, Group: NS, Interaction: NS
	Peak	7.26 ± 0.04	7.29 ± 0.08	
$PaCO_2$ (mmHg)	Rest	38.6 ± 2.4	37.5 ± 3.9	Exercise: P<0.001, Group: NS, Interaction: NS
	Peak	31.3 ± 3.2	29.6 ± 4.3	
2,3-DPG (mmol·mL-1 red blood cells)	Rest	3.97 ± 0.89	3.45 ± 0.68	Exercise: NS, Group: P < 0.05, Interaction: NS

	Peak	4.11 ± 0.88	3.71 ± 0.70	
Serum free insulin (pmol·L^{-1})	Rest	344.4 ± 361.6	76.0 ± 40.2	Exercise: NS, Group: P <0.001, Interaction: NS
	Peak	367.4 ± 442.0	73.2 ± 38.7	
Plasma epinephrine (pmol·L^{-1})	Rest	627.2 ± 425.4	561.8 ± 387.2	Exercise: P < 0.001, Group: NS, Interaction: NS
	Peak	1499.8 ± 1090.8	1478.0 ± 796.3	
Plasma norepinephrine (pmol·L^{-1})	Rest	2211.8 ± 922.6	2726.3 ± 2182.3	Exercise: P < 0.001, Group: NS, Interaction: NS
	Peak	11260.3 ± 5281.2	12519.9 ± 4743.0	
Arterial K+ (mmol·L^{-1})	Rest	4.9 ± 0.4	4.6 ± 0.5	Exercise: P < 0.001, Group: NS, Interaction: NS
	Peak	5.9 ± 1.0	5.4 ± 0.8	
Metabolic data				
Plasma glucose (mmol·L^{-1})	Rest	7.3 ± 2.9 *	5.1 ± 0.6	Exercise: P <0.05, Group: P < 0.001
	Peak	8.3 ± 2.1 *	6.4 ± 0.8 ††	Interaction: NS
Plasma free fatty acids (mmol·L^{-1})	Rest	0.297 ± 0.182	0.357 ± 0.190	Exercise: NS, Group: P < 0.05, Interaction: NS
	Peak	0.207 ± 0.104	0.350 ± 0.175	
Plasma glycerol (mg·L^{-1})	Rest	2.55 ± 1.37	2.64 ± 1.45	Exercise: P < 0.001, Group: P < 0.05, Interaction: NS
	Peak	5.44 ± 2.83 ††	7.86 ± 3.31 †††	

Legend. Values are means ± SD. Main effects from mixed models: Exercise, Exercise effect; Group, Group effect; Interaction, Exercise × group interaction. *Post hoc* analyses for group effect: significantly different from controls at * $P < 0.05$; *post hoc* analyses for time effect: significantly different from rest at † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$; NS: not significant.

Rest: at rest just before the exercise; peak: at exhaustion from the incremental exercise; T1D, patients with type 1 diabetes; CON: healthy control group; VO_2max: maximal oxygen uptake; HR: heart rate; RER: respiratory exchange ratio; DL_{CO} and DL_{NO}: lung diffusion capacity for carbon monoxide and for nitric oxide; ‡, corrected by individual hemoglobin concentrations; D_m: membrane transfer capacity; Vc: capillary lung volume; 2,3-DPG: 2,3-diphosphoglycerate; K^+: potassium.

Plasma (fluorinated) glucose was measured with hexokinase enzymatic assay on modular automatic analyzer; serum free insulin with noncompetitive radioimmunoassay (Cisbio), plasma (heparin, metabisulfite) catecholamines with HPLC, serum free fatty acids and glycerol with colorimetric assays (RANDOX reagents), arterialized (vasodilatory pomade applied 5 min before) erythrocyte 2,3-DPG using spectrophotometry (Sigma-Aldrich), and arterialized pH, K^+, $PaCO_2$ by potentiometry, SaO_2 and Hb by spectrophotometry, PaO_2 and lactate by amperometry on ABL800 FLEX.

FIGURE LEGENDS

Figure 1. Recordings from near-infrared spectroscopy in *vastus lateralis*

Values are means ± SE

Black squares, T1D, patients with type 1 diabetes; white squares, CON, healthy control group

Main effects from mixed models: Exercise, Exercise effect; Group, Group effect; Interaction, Exercise × group interaction. *Post hoc* analyses for group effect: significantly different from controls at *** $P<0.001$. *Post hoc* analyses for time effect: significantly different from rest at †† $P<0.01$, and ††† $P<0.001$.

Change in ΔTHb (**A**), change in ΔO_2Hb (**B**), change in ΔHHb (**C**).

The 40 mm-interspersed emitter-detector pair was placed on the belly of the right *vastus lateralis* muscle. NIRS outcomes changes are displayed according to exercise intensities expressed as relative values (% $\dot{V}O_{2max}$). Mixed models revealed almost identical results when studying NIRS according to absolute exercise intensities (*i.e.,* expressed as absolute work rates in Watts, data not shown).

Figure 2. Mitochondrial respiratory capacity in saponin skinned muscle fibers of *vastus lateralis*

Black bars, patients with type 1 diabetes; White bars, healthy controls.

Results are expressed as means ± SD. Significantly different from CON: **P*<0.05.

All respiratory measurements were performed simultaneously in duplicate, at 22°C under continuous stirring, in the presence of saturating concentrations of substrates at an initial oxygen concentration of ≈ 220 µM.

Figure 2A. Step-by-step analysis of various segments of the respiratory chain

$\dot{V}_{GM}$, *basal adenylate-free leak CI respiration* : oxygen flux with glutamate–malate (10:5 mM) to assess oxygen spent for maintenance of the membrane potential, with electron flow from malate dehydrogenase-produced NADH,H+, going though CI, CIII and CIV, *i.e.*, respiration due to protons leaking and slipping back into the mitochondrial matrix, cation cycling and, to a small extent, to reactive oxygen species production-induced electron leak (malate at 2 mM is used to initiate the Krebs cycle while redirecting 2-oxoglutarate outside of the mitochondria instead of producing succinate)

$\dot{V}_{GM\text{-}ADP}$, *oxidative phosphorylation (OXPHOS) capacity from CI (through CIII and CIV)*: oxygen flux after addition of the phosphate acceptor ADP at its saturating concentration (2 mM)

$\dot{V}_{Succ}$, *OXPHOS capacity from CII (through CIII and CIV):* oxygen flux after sequential addition of the complex I inhibitor, rotenone (0.2 µM) and then, of the electron donor for complex II, succinate (25 mM), for assessing mitochondrial respiration from $FADH_2$

$\dot{V}_{Succ\text{-}CCCP}$, *global electron transport system capacity from CII and capacity of succinate dehydrogenase*: oxygen flux after addition of the uncoupler CCCP (carbonyl cyanide m-chloro phenyl hydrazone, 1 µM) thereby bypassing the control of the phosphorylation system, for assessment of uncoupled O_2 flux with electron supply from $FADH_2$

$\dot{V}_{TMPD}$, *CIV capacity* : oxygen flux after the sequential addition of CIII inihibitor, antimycin A (2.5 µM) and then *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD)–ascorbate (0.5:2 mM as an artificial electron donor to cytochrom *c*

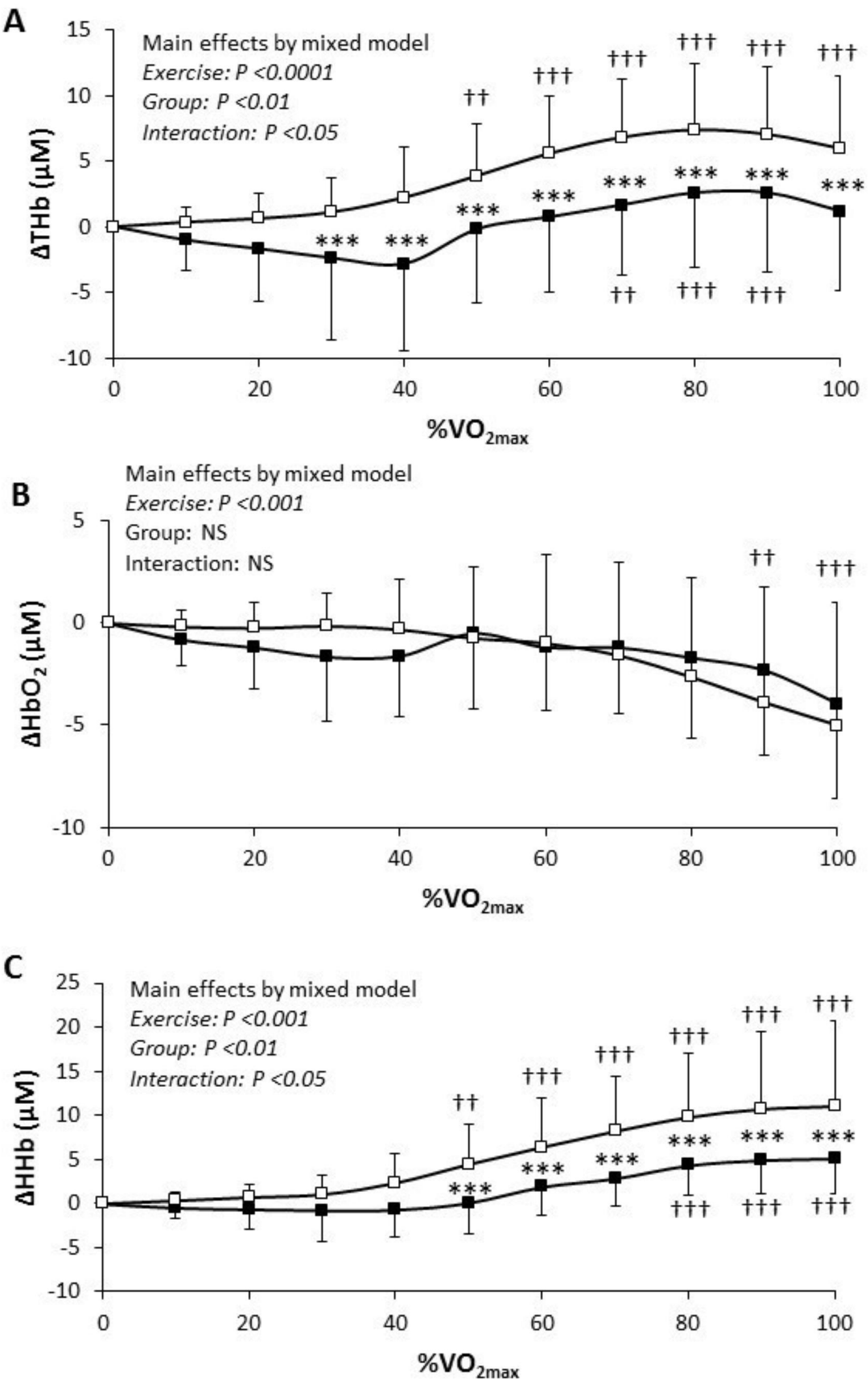
Figure 2B. Respiratory control ratios

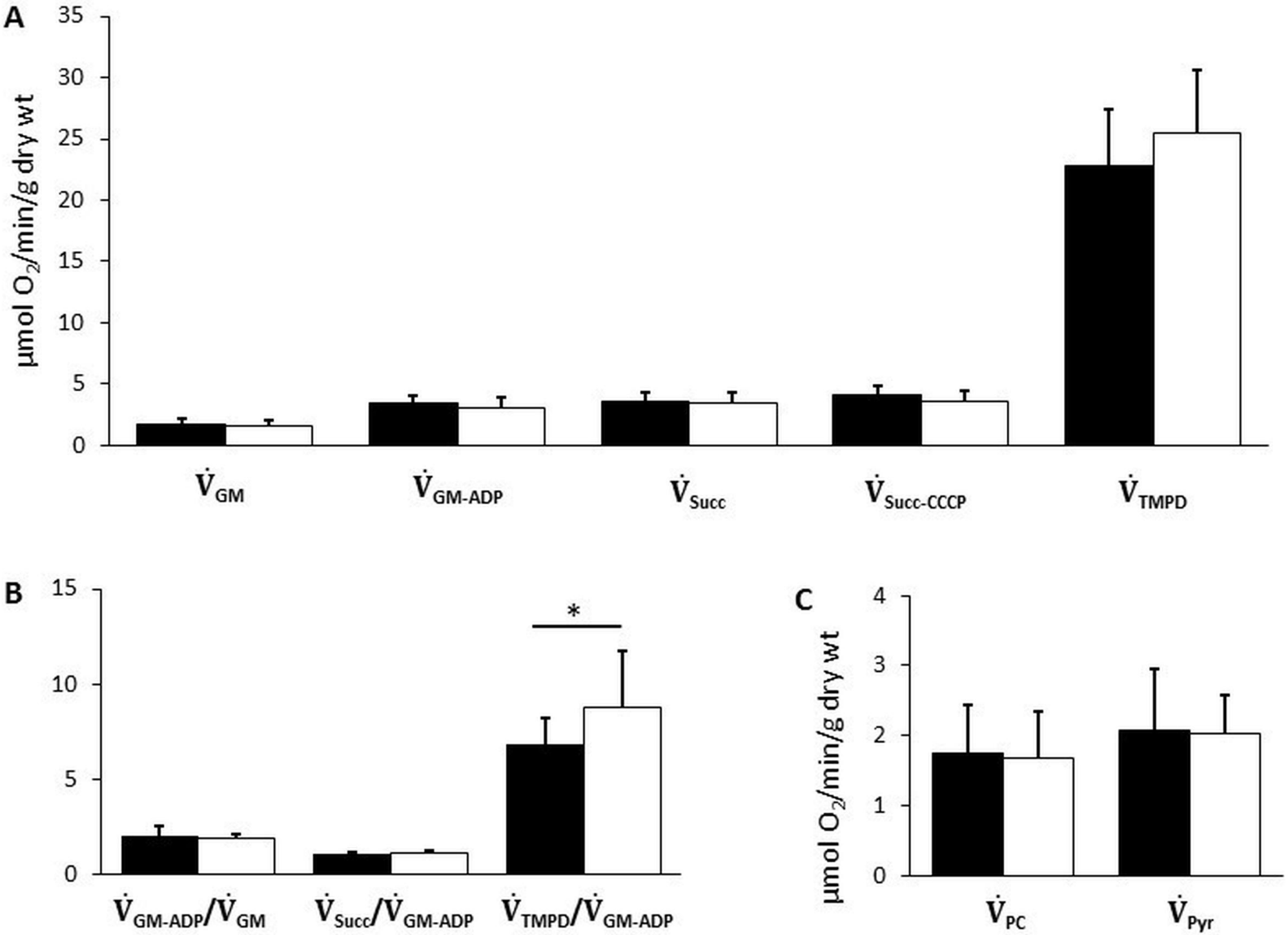
$\dot{V}_{GM\text{-}ADP}$ / $\dot{V}_{GM}$, *oxidation-phosphorylation coupling efficiency (RCR)*: ratio of respiration rate before and after the addition of the saturating concentration of ADP

$\dot{V}_{Succ}$ / $\dot{V}_{GM\text{-}ADP}$ and $\dot{V}_{TMPD}$ / $\dot{V}_{GM\text{-}ADP}$: specific CII and CIV relative capacities, respectively.

Figure 2C. Mitochondrial capacities to oxidize carbohydrates and fatty acids

$\dot{V}_{Pyr}$ *and* $\dot{V}_{PC}$*: mitochondrial capacities to oxidize carbohydrates and fatty acids, respectively:* assessed in separate samples in the respiration buffer, in the presence of ADP (2 mM) and malate (2 mM), using sequential additions of pyruvate (1 mM) (an index of glucose oxidation), and palmitoyl-carnitine (135 µM) (undergoing β-oxidation)


A
Main effects by mixed model
Exercise: P <0.0001
Group: P <0.01
Interaction: P <0.05
ΔTHb (μM)
%VO2max
B
Main effects by mixed model
Exercise: P <0.001
Group: NS
Interaction: NS
ΔHbO2 (μM)
%VO2max
C
Main effects by mixed model
Exercise: P <0.001
Group: P <0.01
Interaction: P <0.05
ΔHHb (μM)
%VO2max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V̇GM-ADP/V̇GM
V̇Succ/V̇GM-ADP
V̇TMPD/V̇GM-ADP
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