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Lactate oxidation in human skeletal muscle mitochondria

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Jacobs RA, Meinild AK, Nordsborg NB, Lundby C. Lactate oxidation in human skeletal muscle mitochondria. *Am J Physiol Endocrinol Metab* 304: E686–E694, 2013. First published February 5, 2013; doi:10.1152/ajpendo.00476.2012.—Lactate is an important intermediate metabolite in human bioenergetics and is oxidized in many different tissues including the heart, brain, kidney, adipose tissue, liver, and skeletal muscle. The mechanism(s) explaining the metabolism of lactate in these tissues, however, remains unclear. Here, we analyze the ability of skeletal muscle to respire lactate by using an in situ mitochondrial preparation that leaves the native tubular reticulum and subcellular interactions of the organelle unaltered. Skeletal muscle biopsies were obtained from vastus lateralis muscle in 16 human subjects. Samples were chemically permeabilized with saponin, which selectively perforates the sarcolemma and facilitates the loss of cytosolic content without altering mitochondrial membranes, structure, and subcellular interactions. High-resolution respirometry was performed on permeabilized muscle biopsy preparations. By use of four separate and specific substrate titration protocols, the respirometric analysis revealed that mitochondria were capable of oxidizing lactate in the absence of exogenous LDH. The titration of lactate and NAD^+ into the respiration medium stimulated respiration ($P \leq 0.003$). The addition of exogenous LDH failed to increase lactate-stimulated respiration ($P = 1.0$). The results further demonstrate that human skeletal muscle mitochondria cannot directly oxidize lactate within the mitochondrial matrix. Alternately, these data support previous claims that lactate is converted to pyruvate within the mitochondrial intermembrane space with the pyruvate subsequently taken into the mitochondrial matrix where it enters the TCA cycle and is ultimately oxidized.

lactate metabolism; mitochondrial function; lactate oxidation complex

OUR UNDERSTANDING OF LACTATE METABOLISM has evolved greatly over the past century (30, 32, 86). The omnipresent intermediate by-product of glycolysis is now recognized as an essential and common metabolite involved in human bioenergetics. It is ubiquitously utilized by different tissues throughout the body, including the heart, brain, kidney, adipose tissue, liver, and skeletal muscle (25, 59, 86, 87) and is produced even at rest despite a sufficient supply of oxygen to the tissue (7, 17, 34, 59). The lactate shuttle hypothesis (15) helped propel our current understanding of lactate metabolism. The premise for the cell-cell lactate shuttle (CCLS) is that lactate released from one cell could serve as a precursor carbon source for either oxidative phosphorylation or gluconeogenesis in other cells throughout the body (13, 15). The majority of this theory has been confirmed throughout the years (4, 8, 38, 56, 60, 87).

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The observations of high cytosolic ratios of lactate to pyruvate in skeletal muscle, especially apparent during exercise, along with the understanding that lactate can serve as a precursor for cellular respiration, led to the intracellular lactate shuttle (ILS) hypothesis. This hypothesis has transformed over the years from the initial speculation that lactate was transported into the mitochondrial matrix where it was subsequently oxidized (16, 18) into its current position (36, 37). It is now proposed that glycolytically produced lactate passively diffuses across the mitochondrial outer membrane (MOM) into the mitochondrial intermembrane space (MIS). An increase in lactate concentrations in the MIS facilitates conversion back into pyruvate catalyzed by an isoform of lactate dehydrogenase (LDH) located in the mitochondria (mLDH). Pyruvate is then shuttled across the mitochondrial inner membrane (MIM) into the matrix via a mitochondrial monocarboxylate transporter (mMCT), where it is oxidized (37), The MCTs most likely involved are MCT1 (36, 37) and/or the high-affinity pyruvate transporter MCT2, which has been reported to subsist in both subsarcolemmal and intermyofibrillar mitochondrial populations (95).

The specifics of lactate metabolism within skeletal muscle mitochondria are ardently debated (14, 18, 19, 31, 74, 75, 95). Previous investigations into the functional assessment of mitochondria-specific lactate oxidation have primarily utilized isolated mitochondrial preparations (18, 73–75, 95), for which mitochondria are extracted and purified by mechanical homogenization and differential centrifugation. Consequently, the dispute regarding mitochondrial lactate oxidation consists of challenging either the method used for mitochondrial isolation or the success achieved in purity and completeness of the mitochondrial isolations (14, 19, 74).

Mitochondria subsist in skeletal muscle as a reticular tubular network as opposed to discrete organelles (45). Mitochondrial isolation disrupts the native heterogeneous reticulum, producing somewhat artificially homogeneous spherical and discrete organelles (71, 82). Such disruption carries the risk of *1*) contaminating the mitochondrial fraction with proteins foreign to the mitochondria (21, 71), *2*) depleting contents specific to mitochondria (14, 19, 71), and *3*) altering mitochondrial function (51, 76, 80). Indeed, mitochondrial isolation has repeatedly been shown to affect mitochondrial respiratory function (5, 48, 61, 67, 70, 90).

Unlike previous studies, here we test the ability of mitochondria to oxidize lactate by means of high-resolution respirometry on preparations of human skeletal muscle biopsies. With this technique, the sarcolemma is chemically permeabilized, and soluble cytosolic proteins, including LDH, are lost without affecting native mitochondrial structure, allowing access to unaltered mitochondria for direct manipulation and investigation of respiratory control (49, 51, 80). The primary

aim of the current study is to challenge the hypothesis that mitochondria can directly oxidize lactate. Assuming that a lactate oxidation complex exists within skeletal muscle mitochondria (36), our hypothesis was that mitochondrial preparations of human skeletal muscle in situ would display the ability for lactate to facilitate mitochondrial respiration independent of any addition of exogenous LDH. Indeed, we demonstrate the ability of unaltered, intact mitochondria to utilize lactate as a substrate for respiration in human skeletal muscle. The additional titration of exogenous LDH fails to further stimulate respiration. Our results provide evidence that demonstrate the ability of human skeletal muscle mitochondria to utilize lactate as a substrate.

Glossary

C1	Complex I (NADH dehydrogenase)
C2	Complex II (succinate dehydrogenase)
CCLS	Cell-cell lactate shuttle
DTT	Dithiothreitol
ILS	Intracellular lactate shuttle
K-MES	2-(*N*-morpholino)ethanesulfonic acid hydrate
LDH	Lactate dehydrogenase
L_N	Leak respiration in absence of adenylates
MIM	MMitochondrial inner membrane
MiR05	Mitochondrial respiration medium
MiR06	MiR05 + catalase
MIS	Mitochondrial intermembrane space
mLDH	Mitochondria-specific LDH
mMCT	Mitochondrial-specific monocarboxylate transporters
MOM	Mitochondrial outer membrane
NAD	Nicotinamide adenine dinucleotide
P_{ETF}	Fatty acid oxidative capacity
P_{C1}	Submaximal respiratory state specific to complex I
P_{C2}	Submaximal respiratory state specific to complex II
P	Maximal state 3 respiration and oxidative phosphorylation capacity
ROX	Residual oxygen consumption
SIRT	Sirtuin

MATERIALS AND METHODS

Ethical Approval

Experimental protocols involving human subjects were approved by the ethics committees for the Eidgenössische Technische Hochschule in Zürich (EK 2011-N-51) and the Regional Ethics Committee of Region Hovedstaden in Denmark (H-1-2011-052), in accordance with the Declaration of Helsinki. Prior to the start of the experiments, informed oral and written consent was obtained from all participants.

Experimental Design

Sixteen young and healthy subjects (14 male and 2 female) voluntarily participated in this study. For logistic reasons, the subjects were divided into two groups of eight. The first eight subjects (*group 1*, 6 males and 2 females) and the second group of eight subjects (*group 2*, 8 males) had their skeletal muscle samples analyzed with separate substrate titration protocols. These protocols are explained in detail below.

Skeletal Muscle Sampling

Skeletal muscle biopsies were obtained from the vastus lateralis muscle under local anesthesia (1% Lidocaine) of the skin and superficial muscle fascia, using the Bergström technique (9) with a needle modified for suction. The biopsy was immediately dissected free of fat and connective tissue and divided into sections for measurements of mitochondrial respiration.

Skeletal Muscle Preparation

The biopsy was sectioned into parts to measure mitochondrial respiration. Each part was immediately placed in ice-cold biopsy preservation solution (BIOPS) containing 2.77 mM CaK_2EGTA buffer, 7.23 mM K_2EGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM 2-(*N*-morpholino)ethanesulfonic acid hydrate (K-MES), 0.5 mM dithiothreitol (DTT), 6.56 mM $MgCl_2{\cdot}6H_2O$, 5.77 mM ATP, and 15 mM phosphocreatine (pH 7.1). Muscle samples were then gently dissected with the tips of two 18-gauge needles, achieving a high degree of fiber separation verified microscopically, followed by chemical permeabilization via incubation in 2 ml of BIOPS with saponin (50 μg/ml) for 30 min at 4°C (50). Last, samples were washed with a mitochondrial respiration medium (MiR05) containing 0.5 mM EGTA, 3 mM $MgCl_2{\cdot}6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2P0_4, 20 mM HEPES, 110 mM sucrose, and 1 g/l bovine serum albumin (pH 7.1) for 10 min at 4°C. Cytosolic components of the skeletal muscle sample are lost during skeletal muscle preparation, including LDH (49, 53, 69, 78, 89).

Mitochondrial Respiration Measurements

Muscle bundles were blotted dry and measured for wet weight in a balance-controlled scale (XS205 DualRange Analytical Balance; Mettler-Toledo, Switzerland) maintaining constant relative humidity, providing hydration consistency as well as stability of weight measurements. Respiration measurements were performed in mitochondrial respiration medium 06 (MiR06; MiR05 + catalase 280 IU/ml). Measurements of oxygen consumption were performed at 37°C using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria) with all additions in each protocol added in series. The Oxygraph-2k is a two-chamber titration-injection respirometer with an oxygen detection limit of 0.5 $pmol{\cdot}s^{-1}{\cdot}ml^{-1}$. Standardized instrumental calibrations were performed to correct for back-diffusion of oxygen into the chamber from the various components, leak from the exterior, oxygen consumption by the chemical medium, and sensor oxygen consumption. Oxygen flux was resolved by software allowing nonlinear changes in the negative time derivative of the oxygen concentration signal (DatLab; Oroboros, Innsbruck, Austria). All experiments were carried out in a hyperoxygenated environment to prevent any potential oxygen diffusion limitation. This technique of permeabilization allows for the study of mitochondrial function in intact skeletal muscle samples in situ without altering the natural mitochondrial reticulum in small biopsy samples using high-resolution respirometry (41, 42, 51).

Respiratory Titration Protocols

Four different titration protocols were applied in the study, and they are illustrated in Table 1. Each protocol was specific to the examination of individual aspects of lactate-stimulated respiratory control through a sequence of coupling and substrate states induced via separate titrations. All titrations were added in series as presented.

LDH^-/NAD^- Titration Protocol

Leak respiration in absence of adenylates (L_N) was induced with the addition of malate (2 mM) and octanoyl carnitine (0.2 mM). This state represents the resting oxygen consumption of an unaltered and intact electron transport system in the absence of adenylates. In the L_N state, the chemiosmotic gradient is at maximum, specific to the substrates provided, and oxygen flux is at a minimum indicating proton leak, slip, cation cycling, and overall dyscoupling (33, 69). Fatty acid oxidative capacity and maximal electron transport through electron transferring flavoprotein (ETF) was determined following the

Table 1. *Experimental design of respirometric analysis*

	M	OC	ADP	Lac			Pyr	S	CytC	Rot	AmA
LDH^-/NAD^- *group 1* ($n = 8$)	2 mM	0.2 mM	5 mM	30 mM	X	X	5 mM	10 mM	10 μM	0.5 μM	2.5 μM
NAD^+/LDH^-/ *group 2* ($n = 8$)	2 mM	0.2 mM	5 mM	30 mM	X	NAD(2mM)	5 mM	10 mM	10 μM	0.5 μM	2.5 μM
NAD^+/LDH^+ *group 2* ($n = 8$)	2 mM	0.2 mM	5 mM	30 mM	NAD(2mM)	LDH(6IU/l)	5 mM	10 mM	10 μM	0.5 μM	2.5 μM
LDH^+/NAD^+ *group 1* ($n = 8$)	2 mM	0.2 mM	5 mM	30 mM	LDH(6IU/l)	NAD(2mM)	5 mM	10 mM	10 μM	0.5 μM	2.5 μM

Four different titration protocols were used in this study. They are indicated in the furthest *left* column, from *top* to *bottom*: *1*) LDH^-/NAD^-; *2*) NAD^+/LDH^-; *3*) NAD^+/LDH^+; *4*) LDH^+/NAD^+. Below the protocol is the subject group for which skeletal muscle specimens were analyzed with that specific protocol. T*op*: substrate or inhibitor added to the respiration medium. The sequence of the titrations into the chamber follows the order of substrates and inhibitors presented from *left* to *right*. Concentration of substrate or inhibitor (columns) added during respirometric analysis is indicated in the box located in the respective protocol of interest (row). M, malate; OC, octanoyl carnitine; ADP, adenosine diphosphate; Lac, lactate; NAD, nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase; Pyr, pyruvate; S, succinate; Cyt C, cytochrome *c*; Rot, rotenone; AmA, antimycin A.

addition of ADP (P_{ETF}, 5 mM). Here, the transfer of electrons requires the metabolism of CoA, hence the previous addition of malate, to allow β-oxidation to proceed. Following P_{ETF}, lactate (30 mM) was added to the chamber to measure the oxidation of lactate. Next, pyruvate (5 mM) was added. Pyruvate is a common substrate used to stimulate electron flow through complex I (C1; NADH dehydrogenase) providing a submaximal state 3 respiratory state specific to C1 (P_{C1}). Maximal state 3 respiration, oxidative phosphorylation capacity (P), was induced with the addition of succinate (10 mM). Maximal state 3 P represents respiration that is resultant to saturating concentrations of ADP as well as substrates specific for C1 and succinate dehydrogenase, complex II (C2) and demonstrates an intact electron transport system's capacity to catalyze a sequential set of redox reactions that are partially coupled to the production of ATP via ATP synthase. Convergent electron input to C1 and C2 provides higher respiratory values compared with the isolated respiration of either C1 (pyruvate/glutamate + malate) or C2 (succinate + rotenone) (33); accordingly, it is more representative and physiologically relevant to the study of mitochondrial function and necessary to establish confirmation of a complete and intact electron transport system (10). As an additional internal control for damaged mitochondria, the integrity of the outer mitochondrial membrane was assessed with the addition of cytochrome *c* (10 μM). Rotenone (0.5 μM) and antimycin A (2.5 μM) were finally added, in sequence, to terminate respiration by inhibiting C1, achieving state 3 respiration through C2 (P_{C2}), and complex III (cytochrome bc_1 complex), respectively. Inhibition of respiration allows for the determination and correction of residual oxygen consumption (ROX), indicative of nonmitochondrial oxygen consumption in the chamber. The concentrations of substrates and inhibitors used were based on prior experiments conducted for optimization of the titration protocols.

NAD^+/LDH^- Titration Protocol. The only difference in the NAD^+/LDH^- titration protocol was that NAD (2 mM) was titrated into the respiration medium following the addition of lactate and before the addition of pyruvate.

NAD^+/LDH^+ Titration Protocol. The NAD^+/LDH^+ titration protocol also titrated NAD (2 mM) following the addition of lactate. However, LDH (3 IU/ml) was then added following stabilization of NAD-stimulated respiration and prior to the titration of pyruvate. This concentration of LDH, which has been previously used (75, 95), is supraphysiological and not limited to lactate-stimulated respiration.

LDH^+/NAD^+ Titration Protocol. Finally, the LDH^+/NAD^+ titration protocol had LDH (3 IU/ml) titrated into the respiratory medium following the addition of lactate followed by the addition of NAD (2 mM) before the addition of pyruvate.

Control for Sirtuin Influence on Mitochondrial Respiration

To address any question of whether respiration in the presence of lactate is truly due to lactate or rather to an enhancement of fatty acid metabolism or electron transport chain activity in response to the NAD^+, we measured P_{EFT} and mitochondrial lactate respiration in 18 separate human subjects from another study. As opposed to the titration protocols detailed above in this study, we added NAD^+ (6 mM) prior to lactate (60 mM) in the titration protocol. We analyzed respirometric values using a one-way ANOVA on repeated measurements with a Bonferroni adjustment to specify location of differences (SPSS Statistics 17.0; SPSS, Chicago, IL).

Enzyme Activities

LDH activities were assayed in homogenates of the skeletal muscle samples used in respiration measurements: The contents of the Oxygraph-2k chambers (2 ml each) were removed after each respiration experiment and washed once with 2 ml of MiR05. One percent Triton X-100 and 2 μl of a protease inhibitor cocktail (Sigma Aldrich cat. 539134) were added to the combined solutions (content and wash) and then homogenized for 30 s with a T10 basic ULTRA-TURRAX homogenizer near maximum speed. The homogenate was then centrifuged for 15 min at 4°C, and the supernatant was removed, frozen in liquid nitrogen, and stored at −80°C. LDH activity was measured fluorometrically at 450 nm and 37°C according to the manufacturer (LDH-Cytotoxicity Assay Kit, BioVision).

Data Analysis

For all statistical evaluations, a P value of <0.05 was considered significant. Statistical analysis of changes to respiration within each respective titration protocol was analyzed by one-way ANOVA on repeated measurements (SPSS Statistics 17.0). Differences in percent changes among respiratory states across all titration protocols and LDH activities across samples were analyzed using a one-way ANOVA. When appropriate, a Bonferroni adjustment was used to specify location of differences.

RESULTS

Lactate Dehydrogenase

There was a significant difference in LDH between samples that did not have exogenous LDH added from those samples that did (Fig. 1, $P < 0.001$). The LDH^- group showed trace amounts of LDH (38.9 ± 13.4 mU/mg wet wt), whereas the LDH^+, as expected, presented with a concentration of LDH indicative of the amount added to the mitochondrial medium during respirometric analysis (2,850 ± 687 mU/ml).

Respirometric Analysis

Differential respiratory capacities across the mitochondrial respiratory system were made evident throughout each separate titration protocol (Fig. 2, *A–D*).

LDH^-/NAD^- respirometric analysis. Respirometric analysis of the skeletal muscle samples using the LDH^-/NAD^- titration protocol is illustrated in Fig. 2*A*. Malate and octanoyl carnitine induced leak respiration, L_N. Respiration significantly

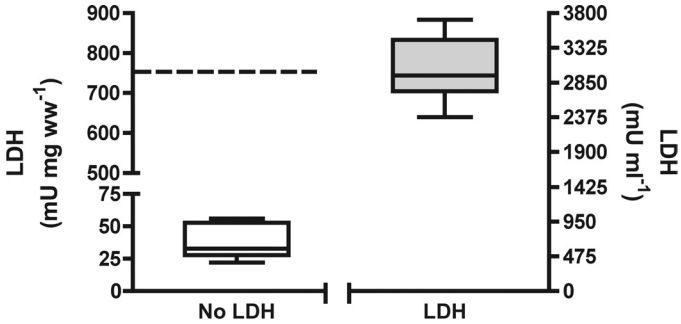

Fig. 1. Box-whisker plot representing lactate dehydrogenase (LDH) concentrations. Measurements of LDH activity (mU/mg wet wt) in permeabilized samples (*left y*-axis) that never had exogenous LDH added to the respiratory chamber and those that did have LDH activity (mU/ml) added to the respirometric chamber (*right y*-axis). LDH activity between the 2 sets of samples was significantly different ($P < 0.0001$). Black dashed line represents average skeletal muscle LDH activity (763 mU/mg wet wt) resported from active and inactive males and females (20, 43).

increased following the addition of ADP, demonstrating the capacity for fatty acid oxidation, P_{ETF} ($P < 0.001$). There was no increase from fat respiration following the addition of lactate (L). Respiration did, as expected, increase following pyruvate addition (P_{C1}, $P < 0.001$) and again following the titration of succinate ($P < 0.001$), the latter of which represents oxidative phosphorylation capacity, P. Finally, respiration diminished after inhibition of C1 with rotenone (P_{C2}, $P = 0.001$).

NAD^+/LDH^- respirometric analysis. Respirometric analysis of the skeletal muscle samples using the NAD^+/LDH^- protocol is illustrated in Fig. 2*B*. Malate and octanoyl carnitine induced L_N. Respiration significantly increased following the addition of ADP signifying P_{ETF} ($P < 0.001$). No increase was evident following the addition of lactate (L; 30 mM). Respiration did increase with the titration NAD ($P < 0.01$). There was another increase following the addition of pyruvate (P_{C1}, $P < 0.05$). Respiration again increased with the addition of succinate (P, $P \leq 0.001$). Finally, respiration diminished after inhibition of C1 with rotenone (P_{C2}, $P < 0.001$).

NAD^+/LDH^+ respirometric analysis. Respirometric analysis of the skeletal muscle samples using the NAD^+/LDH^+ protocol is illustrated in Fig. 2*C*. Malate and octanoyl carnitine induced L_N. Respiration significantly increased following the addition of ADP signifying P_{ETF} ($P \leq 0.001$). No increase was evident following the addition of lactate (L; 30 mM). Respiration did then increase with the titration NAD ($P \leq 0.001$). There was no further increase, however, with the addition of LDH. Respiration showed a trend to increase with pyruvate (P_{C1}, $P = 0.062$). Respiration did increase with the addition of succinate ($P < 0.001$). Finally, respiration diminished after inhibition of C1 with rotenone (P_{C2}, $P < 0.05$).

LDH^+/NAD^+ respirometric analysis. Respirometric analysis of the skeletal muscle samples using the LDH^+/NAD^+ protocol is illustrated in Fig. 2*D*. Malate and octanoyl carnitine induced L_N. Respiration significantly increased following the addition of ADP signifying P_{ETF} ($P < 0.001$). No increase was evident following the addition of lactate (L; 30 mM). Again, there was no increase in respiration following the titration of LDH. Respiration did then increase with the addition of NAD ($P \leq 0.01$). There was an increase following the addition of pyruvate (P_{C1}, $P < 0.01$). Respiration again increased with the addition of succinate (P, $P < 0.01$). Finally, respiration diminished after inhibition of C1 with rotenone (P_{C2}, $P < 0.01$).

Test of Mitochondrial Membrane Integrity

Across all titration protocols, cytochrome *c* had no additive effect on respiration. Respiration changed by −0.4% ($P = 0.962$), 3.1% ($P = 0.758$), −0.3% ($P = 0.971$), and −0.9% ($P = 0.846$) in groups LDH^-/NAD^-, NAD^+/LDH^-, NAD^+/LDH^+, or LDH^+/NAD^+, respectively (Figs. 2, *A–D*). The negligible changes in respiration from oxidative phosphorylation capacity, P, to the exogenous cytochrome *c*-stimulated respiration confirmed MOM intactness throughout all skeletal muscle samples.

Differences in Percent Changes Between Respiratory States Across Groups

Table 2 displays specific percent changes in respiration rates between different substrate-induced respiratory states across all groups. Only two significant differences were calculated, and both were between the NAD^+/LDH^+ and LDH^+/NAD^+ protocols. *1*) There was a difference in percent change from lactate-stimulated respiration and respirometric values obtained following the titration of LDH ($P < 0.001$); and *2*) There was a difference in percent change from the recorded LDH-stimulated respiration and respirometric values obtained following the titration of pyruvate ($P < 0.001$). The only difference between these two protocols is the order in which LDH and NAD were titrated into the respiratory chambers during analysis. As such, the former protocol, NAD^+/LDH^+, contained 2 mM NAD in the LDH-induced respiratory state, whereas the latter protocol, LDH^+/NAD^+, did not.

DISCUSSION

The current study was conducted to test whether human skeletal muscle mitochondria can oxidize lactate. The results *1*) confirm that lactate cannot be taken up directly by human skeletal muscle mitochondria into the mitochondrial matrix and oxidized; *2*) suggest that lactate is converted to pyruvate within the mitochondrial intermembrane space and that the pyruvate is then taken into the mitochondrial matrix where it consequently enters the TCA cycle and is ultimately oxidized; and *3*) is consistent with the existence of a functional lactate oxidation complex in human skeletal muscle mitochondria. The novelty of this study is the mitochondrial preparation and multiple substrate titrations utilized to examine the mechanism by which lactate is oxidized. In contrast to mitochondrial fractions from isolated preparations, the mitochondrial preparation applied in the current study preserved both structural integrity and functional interactions with cellular components (Figs. 2).

Lactate is undoubtedly a metabolic precursor for oxidative phosphorylation in human skeletal muscle (3, 56, 59, 60, 62, 87) as well as other tissues such as the brain (88). Hitherto, the primary disagreement in regard to the metabolism of lactate is whether mitochondria, independently of cytosolic enzymes and pathways, are capable of lactate metabolism and how, specifically, lactate is used as a substrate for cellular respiration.

It was reported that, in mitochondrial preparations isolated from rat hindlimb skeletal muscle, "malate+lactate"-stimulated respiration was similar to "malate+pyruvate"-stimulated respiration (18). Moreover, malate+lactate-stimulated respiration was greatly diminished when oxamate, a competitive

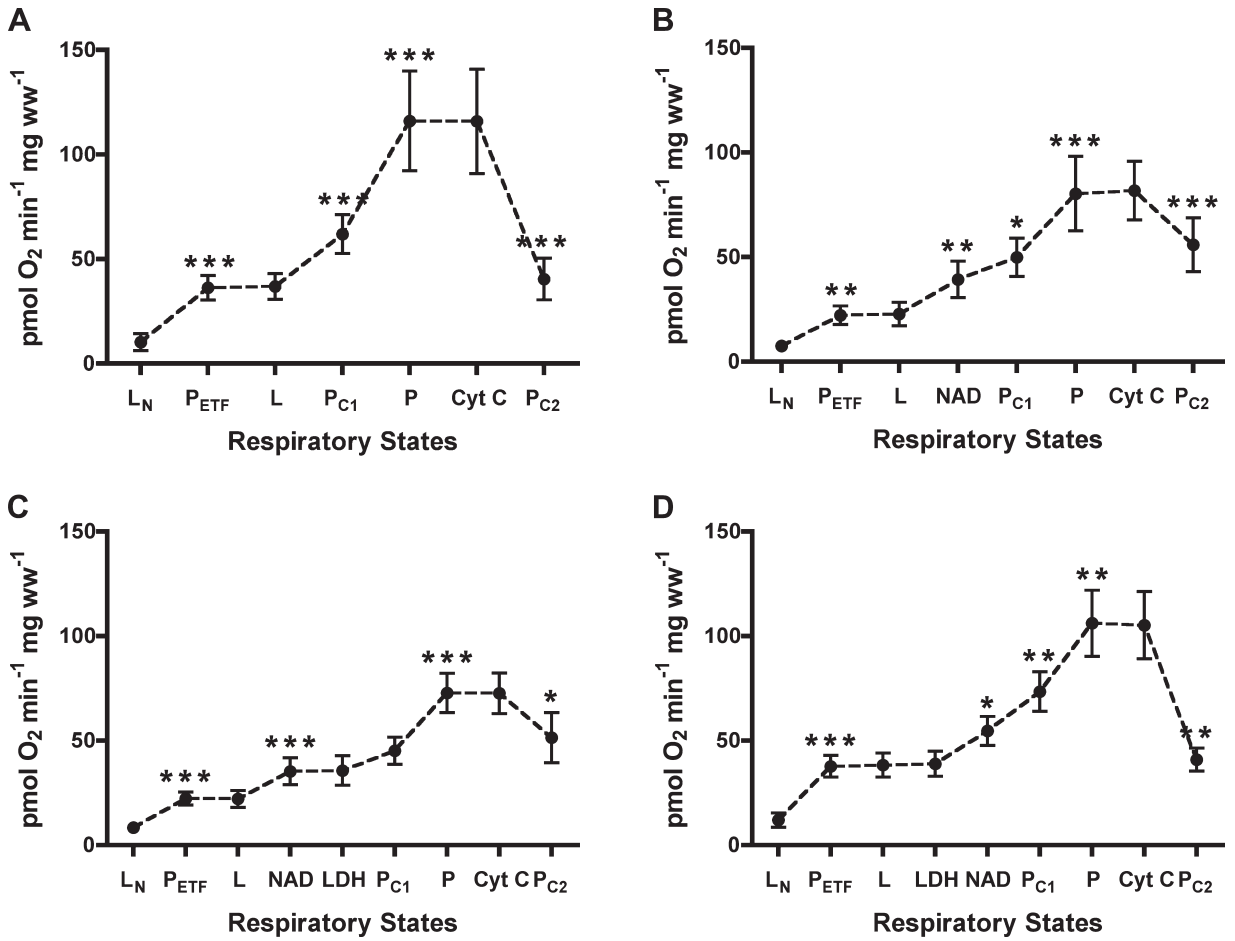

Fig. 2. Mass-specific respirometric analysis. Titration protocols for skeletal muscle samples with all titrations, presented from *left* to *right*, added in series. *A*: protocol without addition of LDH or NAD (LDH^-/NAD^-). *B*: protocol with NAD added but without LDH (NAD^+/LDH^-). *C*: protocol with NAD and LDH both added to respiration medium in successive order (NAD^+/LDH^+). *D*: protocol with LDH and NAD both added to respiration medium in successive order (LDH^+/NAD^+). L_N, respiration in absence of adenylates; P_{ETF}, capacity for fatty acid oxidation; L, lactate; P_{CI}, submaximal state 3 respiration through complex I stimulated with pyruvate; P, maximal state 3 respiration minus oxidative phosphorylation capacity; Cyt C, cytochrome *c*; P_{C2}, submaximal state 3 respiration through complex II following addition of rotenone. Differential respiratory capacities across the mitochondrial respiratory system were made evident throughout the titration protocol utilized, validating our mitochondrial preparation. Values are presented as means ± SD. *, **, and ***, difference in the specific respiration state between groups; $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively.

inhibitor of LDH (58), was included in the respirometric analysis. The presence of LDH was reported in the intermembrane space, inner membrane, and matrix fractions of the skeletal muscle (18). Mitochondria isolated from skeletal muscle, however, are often so fraught with cytosolic LDH contamination that the inclusion of an LDH inhibitor, such as oxamate, is recommended to improve enzymatic analyses in mitochondrial preparations (21).

Several studies (73–75, 94, 95) across different laboratories have reported evidence in opposition to these findings (18), challenging the capacity for lactate oxidation in skeletal muscle mitochondria. These studies (73–75, 94, 95) reported the inability of various skeletal muscles across different species, including humans, to directly oxidize lactate. These studies were also unable to verify the presence of LDH in the mitochondrial fraction of skeletal muscle homogenates, further challenging the existence of a lactate oxidation complex in skeletal muscle mitochondria (74, 75, 95). All respirometric measurements collected in these studies, however, were obtained using isolated mitochondrial preparations. Accordingly,

Table 2. *Percent changes in respiration across all groups*

	L_N to FAT	FAT to Lac	Lac to NAD	Lac to LDH	NAD to Pyr	NAD to LDH	LDH to NAD	LDH to Pyr	Lac to Pyr
LDH^-/NAD^- *group 1* ($n = 8$)	307 ± 71	2 ± 4							69 ± 14*
NAD^+/LDH^- *group 2* ($n = 8$)	233 ± 69	2 ± 6	75.2 ± 28.1		29 ± 18				126 ± 52*
NAD^+/LDH^+ *group 2* ($n = 8$)	182 ± 87	1 ± 9	60.7 ± 20.9	62 ± 19*	30 ± 20	1 ± 5		29 ± 19*	108 ± 34
LDH^+/NAD^+ *group 1* ($n = 8$)	226 ± 66	2 ± 4	50.9 ± 10.3	2 ± 2*	35 ± 14		41.7 ± 17.2	90 ± 20*	93 ± 18

Values are presented as means ± SD. Percent change from one substrate-induced respiratory state to another (columns) is presented across all titration protocols (rows) used throughout the study. Respiration in absence of adenylates (L_N), capacity for fatty acid oxidation (P_{ETF}), lactate (Lac), LDH, and pyruvate (Pyr). *Significant difference in specific respiration state between groups, $P < 0.05$.

they have been theoretically rebutted due to the possibility of mLDH depletion during the isolation procedure (14, 19).

Mitochondrial isolation techniques disrupt the native mitochondrial reticular networks in skeletal muscle, producing unnaturally discrete and spherical organelles through unknown and unregulated means (6, 71, 82). This is often associated with an alteration to natural mitochondrial characteristics (14, 19, 21, 48, 61, 67, 70, 90) such as the loss of mitochondrial membrane integrity (5, 93) and the ability to oxidize fatty acids (67). Crude skeletal muscle homogenizations often result in mitochondrial damage but are required for high fractional yield. Maintaining integrity of mitochondrial membranes during isolation using a more temperate homogenization comes at the expense of a lower mitochondrial yield (52). Consequently, the isolation procedure often limits mitochondrial yield, preventing examination of the total mitochondrial population. As such, conclusive deductions regarding mitochondrial utilization of lactate through respirometric investigations using isolated mitochondria are difficult to establish.

Eukaryotic cells represent an integrated multicompartmental system, making it difficult to isolate and study mitochondrial function in living cells. Mitochondrial structure and function are intricately associated (76). Techniques using selective permeabilization in skeletal muscle samples, as applied in the current study, allow direct access to skeletal muscle mitochondria unaffected by the preparation and remain intact within their natural cytoarchitectural environment (33, 51, 53, 69, 71, 77, 80). They also preserve subcellular interactions with the nucleus, endoplasmic reticulum (51), and sarcoplasmic reticulum (71, 89) in addition to the cytoskeleton (53, 61, 80). Metabolic channeling and the intracellular transfer of energy are dependent on these interactions (33, 44, 51, 80).

The current study used saponin treatment to permeabilize the skeletal muscle samples. Saponin preferentially interacts with membranes rich in cholesterol such as the sarcolemmal membrane, which has a greater cholesterol concentration than both the MOM and MIM (24, 47). This preferential perforation of the sarcolemma initiates the loss of soluble cytosolic metabolites, cofactors, enzymes, and coenzymes (49, 53, 78, 89), allowing rapid equilibration with the respiration medium and the ability to manipulate mitochondrial respiration (Fig. 2). The samples in the current study showed LDH concentrations that were ~5% of previously reported LDH activities from the vastus lateralis muscle in both males and females (20, 43), which is similar to the volume density of mitochondria in skeletal muscle (29, 40, 91). The presence of LDH within mitochondria has repeatedly been demonstrated in different tissues and cell types across several species (1, 2, 11, 12, 18, 22, 26–28, 37, 46, 54, 55, 57, 63, 65, 68, 83–85, 92), and there is also evidence that LDH has a genetic tag for mitochondrial allocation (39); yet opposing reports maintain skepticism as to its mitochondrial subsistence (74, 75, 95). The most likely explanation to this discrepancy is the location of LDH in the mitochondria, which appears to reside in the MIS on the outside layer of the MIM (2, 37), making this soluble enzyme easily lost with the disruption of mitochondrial membrane integrity (68). Another possibility that cannot be fully discounted is the tethering of LDH to the outside of the MOM, opposed to within the MIS. The coprecipitation of LDH and mitochondrial respiratory complex IV cytochrome *c* oxidase, however, supports the existence of LDH to be within the MIS (37). The results of the current study closely reflect those presented from rat liver mitochondria that also identified the location of mitochondrial LDH in the intermembrane space (84). While we cannot definitively exclude the possibility that lactate- and NAD^+-stimulated respiration was facilitated by remnants of cytosolic LDH, our data, specifically the required addition of NAD+ in combination with the negligible response to exogenous LDH titration and successful control of mitochondrial membrane integrity, suggest a successful preparation in which cytosolic LDH is routinely lost (49, 51, 80) and the existence of LDH is within the MIS but not the matrix.

Despite the loss of cytosolic LDH concomitant to the perforation of the sarcolemma, the integrity of the mitochondrial outer and inner membranes was confirmed, as exogenous cytochrome *c* had no effect on respiration across all groups (respiration LDH^-/NAD^-, NAD^+/LDH^-, NAD^+/LDH^+, and LDH^+/NAD^+ groups changed by −0.4%, 3.1%, −0.3%, and −0.9%, respectively; Fig. 2). Endogenous cytochrome *c* is lost during skeletal muscle preparations that corrupt mitochondrial membrane integrity. Titration of exogenous cytochrome *c* confirms damage by stimulating respiration (51, 79). Acceptable elevation in respiration following exogenous cytochrome *c* that substantiates membrane integrity is typically between 5 and 15% (51). In addition to our control of mitochondrial membrane integrity, we also show a functional capacity for fatty acid oxidation, which can be altered with mitochondrial isolation (67), as well as discernible responses in respiration to all substrates and inhibitors alike (Fig. 2), all of which demonstrate a complete, intact, and functional respiratory system.

The results presented in this study directly conflict with those reported in a prior study using similar mitochondrial preparation techniques. They reported the inability of rat skeletal muscle to directly oxidize lactate (72). These results were later challenged with the suggestion that mLDH was lost during skeletal muscle preparation (37). While the MOM is rather permeable to molecules up to ~4 kDa that lack a net charge and strong dipoles (6, 23), it is impermeable to large molecules, such as cytochrome *c* (5). Cytochrome *c* has a molecular mass of ~12 kDa, whereas LDH has a molecular mass of ~140 kDa. The loss of mLDH is highly unlikely if much smaller molecules, such as cytochrome *c*, are not lost during mitochondrial preparation (Fig. 2). Unfortunately, Ponsot et al. (72) failed to confirm the integrity of the MOM.

A more likely explanation for the inability of rat skeletal muscle to oxidize lactate in the study by Ponsot et al. (72) is the loss of cytosolic reducing agents during the skeletal muscle preparation. Assuming that a lactate oxidation complex exists in skeletal muscle mitochondria as proposed (37), then the presence of both lactate and NAD^+ are necessary to facilitate the conversion to pyruvate and NADH in the MIS via mLDH. Small molecules in the MIS such as NAD^+ equilibrate with cytosolic concentrations and would be lost during skeletal muscle preparation. We demonstrate that "lactate-stimulated" respiration does increase above P_{EFT} with the addition of NAD^+ (Fig. 2, *B* and *C*); however, lactate-stimulated respiration was not sufficient to support P_{C1}.

The failure of lactate to stimulate respiration independently from exogenous titrations of NAD^+ brought into question our interpretation of the results. Certain mitochondrial proteins, such as sirtuins (SIRT), are bioenergetic cellular sensors that are dependent on NAD^+ concentrations (64). Specifically,

SIRT3, in skeletal muscle mitochondria (66), modifies functional expression of mitochondrial proteins involved in β-oxidation and electron transport by deacetylation (35, 81). Posttranslational modification of these enzymes via SIRT3 are reported to alter mitochondrial fatty acid oxidation and oxidative phosphorylation (64). However, we found no difference ($P = 0.958$) between P_{ETF} (with mean ± SD of 20.4 ± 3.5 pmol $O_2 \cdot min^{-1} \cdot mg\ wet\ wt^{-1}$) and respiration following NAD^+ (6 mM) titration into the respiration medium (20.1 ± 3.0 pmol $O_2 \cdot min^{-1} \cdot mg\ wet\ wt^{-1}$). In agreement with the current study's findings, we did observe an increase ($P < 0.001$) when lactate was then added into the respiration medium (46.5 ± 9.5 pmol $O_2 \cdot min^{-1} \cdot mg\ wet\ wt^{-1}$; data not shown).

In conclusion, we have demonstrated the ability of skeletal muscle mitochondria to utilize lactate as a substrate for respiration, which is consistent with the existence of a lactate oxidation complex in human skeletal muscle mitochondria. The data suggest that the mitochondrial utilization of lactate as a substrate requires conversion of lactate to pyruvate prior to entry into the mitochondrial matrix and that this conversion occurs independently of cytosolic LDH. These findings were obtained using a mitochondrial preparation that prevents mitochondrial disruption and provides direct access to intact mitochondria in their natural reticular network with functional subcellular interactions. This allows for data interpretation to be autonomous of possible contamination of nonmitochondrial proteins or loss of mitochondrial-specific proteins.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.A.J. conception and design of research; R.A.J. and A.-K.M. performed experiments; R.A.J. and A.-K.M. analyzed data; R.A.J., A.-K.M., N.B.N., and C.L. interpreted results of experiments; R.A.J. prepared figures; R.A.J. drafted manuscript; R.A.J., A.-K.M., N.B.N., and C.L. edited and revised manuscript; R.A.J., A.-K.M., N.B.N., and C.L. approved final version of manuscript.

REFERENCES

1. **Agostoni A, Vergani C, Villa L.** Intracellular distribution of the different forms of lactic dehydrogenase. *Nature* 209: 1024–1025, 1966.
2. **Baba N, Sharma HM.** Histochemistry of lactic dehydrogenase in heart and pectoralis muscles of rat. *J Cell Biol* 51: 621–635, 1971.
3. **Baldwin KM, Hooker AM, Herrick RE.** Lactate oxidative capacity in different types of muscle. *Biochem Biophys Res Commun* 83: 151–157, 1978.
4. **Bangsbo J, Gollnick PD, Graham TE, Saltin B.** Substrates for muscle glycogen synthesis in recovery from intense exercise in man. *J Physiol* 434: 423–440, 1991.
5. **Benz R.** Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins. *Biochim Biophys Acta* 1197: 167–196, 1994.
6. **Benz R.** Porin from bacterial and mitochondrial outer membranes. *CRC Crit Rev Biochem* 19: 145–190, 1985.
7. **Bergman BC, Horning MA, Casazza GA, Wolfel EE, Butterfield GE, Brooks GA.** Endurance training increases gluconeogenesis during rest and exercise in men. *Am J Physiol Endocrinol Metab* 278: E244–E251, 2000.
8. **Bergman BC, Wolfel EE, Butterfield GE, Lopaschuk GD, Casazza GA, Horning MA, Brooks GA.** Active muscle and whole body lactate kinetics after endurance training in men. *J Appl Physiol* 87: 1684–1696, 1999.
9. **Bergström J.** Muscle electrolytes in man. *Scand J Clin Lab Invest* 68: 1–110, 1962.
10. **Brand MD, Nicholls DG.** Assessing mitochondrial dysfunction in cells. *Biochem J* 435: 297–312, 2011.
11. **Brandt RB, Laux JE, Spainhour SE, Kline ES.** Lactate dehydrogenase in rat mitochondria. *Arch Biochem Biophys* 259: 412–422, 1987.
12. **Brody IA, Engel WK.** Isozyme histochemistry: the display of selective lactate dehydrogenase isozymes in sections of skeletal muscle. *J Histochem Cytochem* 12: 689–695, 1964.
13. **Brooks GA.** Cell-cell and intracellular lactate shuttles. *J Physiol* 587: 5591–5600, 2009.
14. **Brooks GA.** Lactate shuttle—between but not within cells? *J Physiol* 541: 333–334, 2002.
15. **Brooks GA.** Lactate: Glycolytic end product and oxidative substrate during sustained exercise in mammals—the "lactate shuttle". In: *Circulation, Respiration, and Metabolism: Current Comparative Approaches*, edited by Gilles R. Berlin: Springer-Verlag, 1985, p. 208–218.
16. **Brooks GA.** Mammalian fuel utilization during sustained exercise. *Comp Biochem Physiol B Biochem Mol Biol* 120: 89–107, 1998.
17. **Brooks GA, Butterfield GE, Wolfe RR, Groves BM, Mazzeo RS, Sutton JR, Wolfel EE, Reeves JT.** Decreased reliance on lactate during exercise after acclimatization to 4,300 m. *J Appl Physiol* 71: 333–341, 1991.
18. **Brooks GA, Dubouchaud H, Brown M, Sicurello JP, Butz CE.** Role of mitochondrial lactate dehydrogenase and lactate oxidation in the intracellular lactate shuttle. *Proc Natl Acad Sci USA* 96: 1129–1134, 1999.
19. **Brooks GA, Hashimoto T.** Investigation of the lactate shuttle in skeletal muscle mitochondria. *J Physiol* 584: 705–706; author reply 707–708, 2007.
20. **Burke ER, Cerny F, Costill D, Fink W.** Characteristics of skeletal muscle in competitive cyclists. *Med Sci Sports* 9: 109–112, 1977.
21. **Chretien D, Pourrier M, Bourgeron T, Sene M, Rotig A, Munnich A, Rustin P.** An improved spectrophotometric assay of pyruvate dehydrogenase in lactate dehydrogenase contaminated mitochondrial preparations from human skeletal muscle. *Clin Chim Acta* 240: 129–136, 1995.
22. **Coleman RA, Ramp WK, Toverud SU, Hanker JS.** Electron microscopic localization of lactate dehydrogenase in osteoclasts of chick embryo tibia. *Histochem J* 8: 543–558, 1976.
23. **Colombini M.** VDAC structure, selectivity, dynamics. *Biochim Biophys Acta* 1818: 1457–1465, 2012.
24. **Comte J, Maisterrena B, Gautheron DC.** Lipid composition and protein profiles of outer and inner membranes from pig heart mitochondria. Comparison with microsomes. *Biochim Biophys Acta* 419: 271–284, 1976.
25. **Donovan CM, Pagliassotti MJ.** Quantitative assessment of pathways for lactate disposal in skeletal muscle fiber types. *Med Sci Sports Exerc* 32: 772–777, 2000.
26. **Dubouchaud H, Butterfield GE, Wolfel EE, Bergman BC, Brooks GA.** Endurance training, expression, and physiology of LDH, MCT1, and MCT4 in human skeletal muscle. *Am J Physiol Endocrinol Metab* 278: E571–E579, 2000.
27. **Fahimi HD, Amarasingham CR.** Cytochemical localization of lactic dehydrogenase in white skeletal muscle. *J Cell Biol* 22: 29–48, 1964.
28. **Fahimi HD, Karnovsky MJ.** Cytochemical localization of two glycolytic dehydrogenases in white skeletal muscle. *J Cell Biol* 29: 113–128, 1966.
29. **Ferretti G, Antonutto G, Denis C, Hoppeler H, Minetti AE, Narici MV, Desplanches D.** The interplay of central and peripheral factors in limiting maximal O2 consumption in man after prolonged bed rest. *J Physiol* 501: 677–686, 1997.
30. **Gladden LB.** 200th anniversary of lactate research in muscle. *Exerc Sport Sci Rev* 36: 109–115, 2008.
31. **Gladden LB.** Is there an intracellular lactate shuttle in skeletal muscle? *J Physiol* 582: 899, 2007.
32. **Gladden LB.** Lactate metabolism: a new paradigm for the third millennium. *J Physiol* 558: 5–30, 2004.
33. **Gnaiger E.** Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. *Int J Biochem Cell Biol* 41: 1837–1845, 2009.
34. **Graham TE, Saltin B.** Estimation of the mitochondrial redox state in human skeletal muscle during exercise. *J Appl Physiol* 66: 561–566, 1989.

35. **Hallows WC, Lee S, Denu JM.** Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. *Proc Natl Acad Sci USA* 103: 10230–10235, 2006.
36. **Hashimoto T, Brooks GA.** Mitochondrial lactate oxidation complex and an adaptive role for lactate production. *Med Sci Sports Exerc* 40: 486–494, 2008.
37. **Hashimoto T, Hussien R, Brooks GA.** Colocalization of MCT1, CD147, and LDH in mitochondrial inner membrane of L6 muscle cells: evidence of a mitochondrial lactate oxidation complex. *Am J Physiol Endocrinol Metab* 290: E1237–E1244, 2006.
38. **Henderson GC, Horning MA, Lehman SL, Wolfel EE, Bergman BC, Brooks GA.** Pyruvate shuttling during rest and exercise before and after endurance training in men. *J Appl Physiol* 97: 317–325, 2004.
39. **Holmes RS, Goldberg E.** Computational analyses of mammalian lactate dehydrogenases: human, mouse, opossum and platypus LDHs. *Comput Biol Chem* 33: 379–385, 2009.
40. **Hoppeler H, Howald H, Conley K, Lindstedt SL, Claassen H, Vock P, Weibel ER.** Endurance training in humans: aerobic capacity and structure of skeletal muscle. *J Appl Physiol* 59: 320–327, 1985.
41. **Jacobs RA, Boushel R, Wright-Paradis C, Calbet JA, Robach P, Gnaiger E, Lundby C.** Mitochondrial function in human skeletal muscle following high altitude exposure. *Exp Physiol* doi:10.1113/expphysiol.2012.066092:2012.
42. **Jacobs RA, Rasmussen P, Siebenmann C, Diaz V, Gassmann M, Pesta D, Gnaiger E, Nordsborg NB, Robach P, Lundby C.** Determinants of time trial performance and maximal incremental exercise in highly trained endurance athletes. *J Appl Physiol* 111: 1422–1430, 2011.
43. **Karlsson J, Diamant B, Saltin B.** Lactate dehydrogenase activity in muscle after prolonged severe exercise in man. *J Appl Physiol* 25: 88–91, 1968.
44. **Kay L, Nicolay K, Wieringa B, Saks V, Wallimann T.** Direct evidence for the control of mitochondrial respiration by mitochondrial creatine kinase in oxidative muscle cells in situ. *J Biol Chem* 275: 6937–6944, 2000.
45. **Kirkwood SP, Munn EA, Brooks GA.** Mitochondrial reticulum in limb skeletal muscle. *Am J Physiol Cell Physiol* 251: C395–C402, 1986.
46. **Kline ES, Brandt RB, Laux JE, Spainhour SE, Higgins ES, Rogers KS, Tinsley SB, Waters MG.** Localization of L-lactate dehydrogenase in mitochondria. *Arch Biochem Biophys* 246: 673–680, 1986.
47. **Korn ED.** Cell membranes: structure and synthesis. *Annu Rev Biochem* 38: 263–288, 1969.
48. **Kunz WS, Kudin A, Vielhaber S, Elger CE, Attardi G, Villani G.** Flux control of cytochrome c oxidase in human skeletal muscle. *J Biol Chem* 275: 27741–27745, 2000.
49. **Kunz WS, Kuznetsov AV, Schulze W, Eichhorn K, Schild L, Striggow F, Bohnensack R, Neuhof S, Grasshoff H, Neumann HW, Gellerich FN.** Functional characterization of mitochondrial oxidative phosphorylation in saponin-skinned human muscle fibers. *Biochim Biophys Acta* 1144: 46–53, 1993.
50. **Kuznetsov AV, Schneeberger S, Seiler R, Brandacher G, Mark W, Steurer W, Saks V, Usson Y, Margreiter R, Gnaiger E.** Mitochondrial defects and heterogeneous cytochrome *c* release after cardiac cold ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 286: H1633–H1641, 2004.
51. **Kuznetsov AV, Veksler V, Gellerich FN, Saks V, Margreiter R, Kunz WS.** Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat Protoc* 3: 965–976, 2008.
52. **Lanza IR, Nair KS.** Functional assessment of isolated mitochondria in vitro. *Methods Enzymol* 457: 349–372, 2009.
53. **Lin A, Krockmalnic G, Penman S.** Imaging cytoskeleton–mitochondrial membrane attachments by embedment-free electron microscopy of saponin-extracted cells. *Proc Natl Acad Sci USA* 87: 8565–8569, 1990.
54. **Machado de Domenech E, Domenech CE, Aoki A, Blanco A.** Association of the testicular lactate dehydrogenase isozyme with a special type of mitochondria. *Biol Reprod* 6: 136–147, 1972.
55. **Mattisson AG, Johansson RG, Bostrom SL.** The cellular localization of lactate dehydrogenase in skeletal muscle of eel (*Anguilla anguilla*). *Comp Biochem Physiol B* 41: 475–482, 1972.
56. **Mazzeo RS, Brooks GA, Schoeller DA, Budinger TF.** Disposal of blood [1-^{13}C]lactate in humans during rest and exercise. *J Appl Physiol* 60: 232–241, 1986.
57. **McClelland GB, Brooks GA.** Changes in MCT 1, MCT 4, and LDH expression are tissue specific in rats after long-term hypobaric hypoxia. *J Appl Physiol* 92: 1573–1584, 2002.
58. **McPherson A Jr.** Binding of oxamate to the apoenzyme of dogfish M4 lactate dehydrogenase. *J Mol Biol* 76: 528–531, 1973.
59. **Miller BF, Fattor JA, Jacobs KA, Horning MA, Navazio F, Lindinger MI, Brooks GA.** Lactate and glucose interactions during rest and exercise in men: effect of exogenous lactate infusion. *J Physiol* 544: 963–975, 2002.
60. **Miller BF, Fattor JA, Jacobs KA, Horning MA, Suh SH, Navazio F, Brooks GA.** Metabolic and cardiorespiratory responses to "the lactate clamp". *Am J Physiol Endocrinol Metab* 283: E889–E898, 2002.
61. **Milner DJ, Mavroidis M, Weisleder N, Capetanaki Y.** Desmin cytoskeleton linked to muscle mitochondrial distribution and respiratory function. *J Cell Biol* 150: 1283–1298, 2000.
62. **Mole PA, Van Handel PJ, Sandel WR.** Extra O2 consumption attributable to NADH2 during maximum lactate oxidation in the heart. *Biochem Biophys Res Commun* 85: 1143–1149, 1978.
63. **Nakae Y, Stoward PJ, Shono M, Matsuzaki T.** Localisation and quantification of dehydrogenase activities in single muscle fibers of mdx gastrocnemius. *Histochem Cell Biol* 112: 427–436, 1999.
64. **Nogueiras R, Habegger KM, Chaudhary N, Finan B, Banks AS, Dietrich MO, Horvath TL, Sinclair DA, Pfluger PT, Tschop MH.** Sirtuin 1 and sirtuin 3: physiological modulators of metabolism. *Physiol Rev* 92: 1479–1514, 2012.
65. **Novikoff AB, Masek B.** Survival of lactic dehydrogenase and DPNH-diaphorase activities after formol-calcium fixation. *J Histochem Cytochem* 6: 217, 1958.
66. **Onyango P, Celic I, McCaffery JM, Boeke JD, Feinberg AP.** SIRT3, a human SIR2 homologue, is an NAD-dependent deacetylase localized to mitochondria. *Proc Natl Acad Sci USA* 99: 13653–13658, 2002.
67. **Pande SV, Blanchaer MC.** Preferential loss of ATP-dependent long-chain fatty acid activating enzyme in mitochondria prepared using Nagarse. *Biochim Biophys Acta* 202: 43–48, 1970.
68. **Passarella S, de Bari L, Valenti D, Pizzuto R, Paventi G, Atlante A.** Mitochondria and L-lactate metabolism. *FEBS Lett* 582: 3569–3576, 2008.
69. **Pesta D, Gnaiger E.** High-resolution respirometry. OXPHOS protocols for human cell cultures and permeabilized fibres from small biopsies of human muscle. *Mitochondrial Bioenergetics: Methods and Protocols* 810: 25–58, 2011.
70. **Picard M, Ritchie D, Wright KJ, Romestaing C, Thomas MM, Rowan SL, Taivassalo T, Hepple RT.** Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared with permeabilized myofibers. *Aging Cell* 9: 1032–1046, 2010.
71. **Picard M, Taivassalo T, Gouspillou G, Hepple RT.** Mitochondria: isolation, structure and function. *J Physiol* 589: 4413–4421, 2011.
72. **Ponsot E, Zoll J, N'Guessan B, Ribera F, Lampert E, Richard R, Veksler V, Ventura-Clapier R, Mettauer B.** Mitochondrial tissue specificity of substrates utilization in rat cardiac and skeletal muscles. *J Cell Physiol* 203: 479–486, 2005.
73. **Popinigis J, Antosiewicz J, Crimi M, Lenaz G, Wakabayashi T.** Human skeletal muscle: participation of different metabolic activities in oxidation of L-lactate. *Acta Biochim Pol* 38: 169–175, 1991.
74. **Rasmussen HN, van Hall G, Rasmussen UF.** Lactate dehydrogenase is not a mitochondrial enzyme in human and mouse vastus lateralis muscle. *J Physiol* 541: 575–580, 2002.
75. **Sahlin K, Fernstrom M, Svensson M, Tonkonogi M.** No evidence of an intracellular lactate shuttle in rat skeletal muscle. *J Physiol* 541: 569–574, 2002.
76. **Saks V, Guzun R, Timohhina N, Tepp K, Varikmaa M, Monge C, Beraud N, Kaambre T, Kuznetsov A, Kadaja L, Eimre M, Seppet E.** Structure-function relationships in feedback regulation of energy fluxes in vivo in health and disease: mitochondrial interactosome. *Biochim Biophys Acta* 1797: 678–697, 2010.
77. **Saks VA, Belikova YO, Kuznetsov AV.** In vivo regulation of mitochondrial respiration in cardiomyocytes: specific restrictions for intracellular diffusion of ADP. *Biochim Biophys Acta* 1074: 302–311, 1991.
78. **Saks VA, Kaambre T, Sikk P, Eimre M, Orlova E, Paju K, Piirsoo A, Appaix F, Kay L, Regitz-Zagrosek V, Fleck E, Seppet E.** Intracellular energetic units in red muscle cells. *Biochem J* 356: 643–657, 2001.
79. **Saks VA, Vasil'eva E, Belikova Yu O, Kuznetsov AV, Lyapina S, Petrova L, Perov NA.** Retarded diffusion of ADP in cardiomyocytes: possible role of mitochondrial outer membrane and creatine kinase in cellular regulation of oxidative phosphorylation. *Biochim Biophys Acta* 1144: 134–148, 1993.
80. **Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, Olivares J, Winkler K, Wiedemann F, Kunz WS.** Permeabilized cell

and skinned fiber techniques in studies of mitochondrial function in vivo. *Mol Cell Biochem* 184: 81–100, 1998.

81. **Schwer B, Bunkenborg J, Verdin RO, Andersen JS, Verdin E.** Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2. *Proc Natl Acad Sci USA* 103: 10224–10229, 2006.
82. **Schwerzmann K, Hoppeler H, Kayar SR, Weibel ER.** Oxidative capacity of muscle and mitochondria: correlation of physiological, biochemical, and morphometric characteristics. *Proc Natl Acad Sci USA* 86: 1583–1587, 1989.
83. **Sjodin B.** Lactate dehydrogenase in human skeletal muscle. *Acta Physiol Scand* Suppl 436: 1–32, 1976.
84. **Skilleter DN, Kun E.** The oxidation of L-lactate by liver mitochondria. *Arch Biochem Biophys* 152: 92–104, 1972.
85. **Szczesna-Kaczmarek A.** Regulating effect of mitochondrial lactate dehydrogenase on oxidation of cytoplasmic NADH via an "external" pathway in skeletal muscle mitochondria. *Int J Biochem* 24: 657–661, 1992.
86. **van Hall G.** Lactate kinetics in human tissues at rest and during exercise. *Acta Physiol (Oxf)* 199: 499–508, 2010.
87. **Van Hall G, Jensen-Urstad M, Rosdahl H, Holmberg HC, Saltin B, Calbet JA.** Leg and arm lactate and substrate kinetics during exercise. *Am J Physiol Endocrinol Metab* 284: E193–E205, 2003.
88. **van Hall G, Stromstad M, Rasmussen P, Jans O, Zaar M, Gam C, Quistorff B, Secher NH, Nielsen HB.** Blood lactate is an important energy source for the human brain. *J Cereb Blood Flow Metab* 29: 1121–1129, 2009.
89. **Veksler VI, Kuznetsov AV, Sharov VG, Kapelko VI, Saks VA.** Mitochondrial respiratory parameters in cardiac tissue: a novel method of assessment by using saponin-skinned fibers. *Biochim Biophys Acta* 892: 191–196, 1987.
90. **Villani G, Greco M, Papa S, Attardi G.** Low reserve of cytochrome c oxidase capacity in vivo in the respiratory chain of a variety of human cell types. *J Biol Chem* 273: 31829–31836, 1998.
91. **Vogt M, Puntschart A, Geiser J, Zuleger C, Billeter R, Hoppeler H.** Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. *J Appl Physiol* 91: 173–182, 2001.
92. **Walker DG, Seligman AM.** The use of formalin fixation in the cytochemical demonstration of succinic and DPN- and TPN-dependent dehydrogenases in mitochondria. *J Cell Biol* 16: 455–469, 1963.
93. **Wicker U, Bucheler K, Gellerich FN, Wagner M, Kapischke M, Brdiczka D.** Effect of macromolecules on the structure of the mitochondrial inter-membrane space and the regulation of hexokinase. *Biochim Biophys Acta* 1142: 228–239, 1993.
94. **Willis WT, Thompson A, Messer JI, Thresher JS.** Vmax of mitochondrial electron shuttles in rat skeletal muscle and liver. *Med Sci Sports Exerc* 35: S396, 2003.
95. **Yoshida Y, Holloway GP, Ljubicic V, Hatta H, Spriet LL, Hood DA, Bonen A.** Negligible direct lactate oxidation in subsarcolemmal and intermyofibrillar mitochondria obtained from red and white rat skeletal muscle. *J Physiol* 582: 1317–1335, 2007.