Control of human energy expenditure by cytochrome c oxidase subunit IV-2

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Schiffer TA, Peleli M, Sundqvist ML, Ekblom B, Lundberg JO, Weitzberg E, Larsen FJ. Control of human energy expenditure by cytochrome c oxidase subunit IV-2. Am J Physiol Cell Physiol 311: C452–C461, 2016. First published July 27, 2016; doi:10.1152/ajpcell.00099.2016.—Resting metabolic rate (RMR) in humans shows pronounced individual variations, but the underlying molecular mechanism remains elusive. Cytochrome c oxidase (COX) plays a key role in control of metabolic rate, and recent studies of the subunit IV-2 isoform (COX IV-2) indicate involvement in the cellular response to hypoxia and oxidative stress. We evaluated whether the COX subunit IV-2 isoform composition may explain the pronounced individual variations in resting metabolic rate (RMR). RMR was determined in healthy humans by indirect calorimetry and correlated to RMR of COX IV-2 and COX IV-1 in vastus lateralis. Overexpression and knock down of the COX IV isoforms were performed in primary myotubes followed by evaluation of the cell respiration and production of reactive oxygen species. Here we show that COX IV-2 protein is constitutively expressed in human skeletal muscle and strongly correlated to RMR. Primary human myotubes overexpressing COX IV-2 displayed markedly (>60%) lower respiration, reduced (>50%) cellular H2O2 production, higher resistance toward both oxidative stress, and severe hypoxia compared with control cells. These results suggest an important role of isoform COX IV-2 in the control of energy expenditure, hypoxic tolerance, and mitochondrial ROS homeostasis in humans.

resting metabolic rate; mitochondria; high-resolution respirometry

The ongoing worldwide obesity epidemic contributes to a great interest in exploring the underlying mechanisms of weight regulation. On a simplistic level weight gain occurs when energy intake exceeds energy expenditure and the excess energy is stored as adipose tissue. However, the inclination toward weight gain seems to show dramatic interindividual variations even when caloric intake is held constant. In parallel, the resting metabolic rate (RMR) shows similar large interindividual variations that could explain the propensity toward weight gain. Indeed, when a large group of individuals were followed for three years, it was found that those subjects with the lowest RMR at baseline showed the largest weight gain at follow-up (29). The underlying cause behind these variations is still largely unknown even if differences in fat-free mass, thyroid hormones (15), and brown adipose tissue (33) may be of some importance. In cross-sectional studies RMR has been shown to be associated with the maximum aerobic power (VO2 max) (27), but exercise training interventions have no or only trivial effects on RMR. In addition there is an apparent lack of a causal link between RMR and VO2 max although a higher mitochondrial density and higher mitochondrial uncoupling in well-trained subjects has been proposed as a possible cause of their higher RMR.

From an evolutionary perspective, having a low metabolic rate means that the demand for substrate and oxygen is low, a capability that can be advantageous in times of food shortage or in hypoxic environments. However, the evolution of energetically efficient structures that decreases resting metabolic rate is often at the expense of maximum power production. In the animal kingdom this is exemplified by the slow movements of the three-toed sloth (Bradypus) with one of the lowest metabolic rates of mammalian species that allows them to thrive while consuming only ~60 g of tree leaves per day. In contrast, while flying, the hummingbird has the highest known metabolic rate among vertebrates but needs to consume more than its own body weight in nectar each day to survive. Cellular structures that allow for rapid switching between efficient and powerful structures would therefore be of great evolutionary advantage regardless of species.

As the majority of energy transformations in the body are of mitochondrial origin, differences in mitochondrial efficiency may be reflected as variations in RMR. The main factors influencing mitochondrial efficiency are proton leak across the inner membrane and electron leak, where electrons may exit the respiratory chain generating superoxide and hydrogen peroxide (28). Proton leak has been estimated to account for a substantial portion of the in vivo resting metabolic rate (6).

Cytochrome c oxidase (COX) is the terminal enzyme in the electron transport system (ETS) and it consists of 13 subunits of which several have multiple isoforms. A growing number of studies lend support to the idea that COX is central in metabolic regulation and control of oxidative phosphorylation and reactive oxygen species (ROS) formation. Proton slip occurs at COX, allowing electrons to reduce oxygen without concomitant pumping of protons to the intermembrane space thereby lowering mitochondrial efficiency (8, 16). In 2001 a second isoform of the COX subunit IV was discovered (12). COX IV-1 is ubiquitously expressed and is responsible for allosteric regulation of COX activity via binding sites for both ADP and ATP, where the ATP/ADP ratio regulates its activity (4). COX IV-2 is far more scarcely expressed, found mainly in lung tissue, fetal skeletal muscle, and neurons (12). An increased expression of COX IV-2 seems to contribute to a more unregulated respiration due to loss of ATP inhibition (11). This may have impact on the mitochondrial ROS production due to a possible alteration of the membrane potential.

The COX IV-2 isoform has been suggested to improve COX efficiency during hypoxia (9). Controversial findings surround the signaling pathways involved in the induction of COX IV-2. Several groups have reported that COX IV-2 is a downstream...
target in the HIF-1α pathway (9, 11, 37). Indeed, COX IV-2 expression is increased after hypoxic exposure and chemical hypoxia in astrocytes (32). However, a recent study assessed the extent of COX IV-2 induction by hypoxia and found that a majority of a wide variety of animal models and cell lines were unresponsive to hypoxia (17).

An important aspect of the functionality of COX is to keep the mitochondrial affinity for oxygen at a high enough level to allow efficient oxygen extraction in low oxygen environment such as in tissues with high metabolic demand. We have previously found a strong negative correlation between human skeletal muscle mitochondrial oxygen affinity (p50_mito) and RMR (20) and we suggested that p50_mito may be a sensitive variable reflecting mitochondrial efficiency.

In the present study, we aimed to explore the presence of COX IV isoforms in human skeletal muscle and hypothesized that COX IV-2 is a critical regulator of energy expenditure and mitochondrial function. Moreover, we hypothesized that the COX IV isoforms were important determinants of cellular ROS homeostasis and that the subunit composition may be involved in the regulation of defense against oxidative insults and hypoxia.

MATERIALS AND METHODS

Ethical approval. This study was approved by the Regional Ethics Committee in Stockholm, Sweden. The study participants gave written informed consent before initiation of the study, and all procedures were performed according to the latest revision of the Declaration of Helsinki.

Subjects. Sixteen healthy medium- to well-trained male subjects were recruited with a mean age of 34 ± 8 yr, weight 79 ± 9 kg, and height 183 ± 5 cm.

Skeletal muscle biopsies from human subjects. After a small incision through the skin and fascia, Blakesley’s conchotome was used to extract skeletal muscle biopsies from the subjects’ vastus lateralis (100–150 mg) under local anesthesia (lidocaine without epinephrine). The biopsy was partitioned in three where one part was snap frozen in liquid nitrogen for biochemical analysis, one was used for immediate isolation of mitochondria and the last piece was placed in ice-cold sterile phosphate-buffered saline PBS for the extraction of myogenic satellite cells within 24 h.

Resting metabolic rate. The subjects arrived to the lab after an overnight fast and were instructed to refrain from exercise at least 36 h before the indirect calorimetry measurements. The resting metabolic rate was assessed by indirect calorimetry (OxyconPro, Jaeger, Hoechberg, Germany). RMR was calculated as the lowest mean energy expenditure recorded for a period of 10 min.

Maximal oxygen consumption. A maximal exercise test was performed where the subjects mounted a stationary cycle ergometer (SRM GmbH, Jülich, Germany). The subjects started at a workload corresponding to an estimated 30% of VO2_max and was incremented by 15–30 W every 30 s until volitional exhaustion. The highest average oxygen consumption recorded over 45 s was used to determine the subject’s VO2_max.

Verification of COX IV-2 in skeletal muscle. Homogenates from control cells and cells overexpressing COX IV-2 together with homogenates from vastus lateralis were loaded on the same polyacrylamide gel for verification of the presence of COX IV-2 in human skeletal muscle (Fig. 1A).

Fig. 1. Cytochrome 3 oxidase (COX) subunit expression in muscle tissue and human primary myotubes. A: Western blot analysis of protein extracted from vastus lateralis biopsies. First an antibody specific for COX IV-2 (~20 kDa) was used, the same membrane was then washed with a stripping buffer removing all antibodies and then an antibody for COX IV-1 (~20 kDa) was added. Finally an antibody specific for the mitochondrial marker citrate synthase (CS) was used (n = 16). B: protein was extracted from untreated primary human myotubes, COX IV-1/2 myotubes and skeletal muscle biopsies from vastus lateralis from two healthy human subjects. An antibody specific for COX IV-2 was used. C: protein expression of COX IV-2 was plotted against protein expression of COX IV-1 to determine if there was a significant cross-reactivity of the antibodies. D: correlation analysis between the transcript ratio of COX IV-2/COX IV-1 in human vastus lateralis (RT-PCR) and whole body resting metabolic rate measured with indirect calorimetry.
Isolation of human myogenic satellite cells and cell culture procedures. Myogenic satellite cells were isolated from biopsies extracted from the subject’s vastus lateralis. After overnight storage in PBS at 4°C, extraction of myogenic satellite cells from the biopsies was performed as follows. Samples were washed and cleaned from visible connective tissue and thereafter minced with a pair of scissors in trypsin (0.25%) and 1 mM EDTA followed by enzymatic dissociation in a small beaker with 5 ml 0.25% trypsin, 1 mM EDTA under gentle agitation in 37°C, 5% CO2 for 20 min. The sample was allowed to sediment for 5 min and the supernatant was collected in growth medium [50% Dulbecco’s modified Eagle’s medium (DMEM-F-12) and 50 U/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml amphotericin containing 20% fetal bovine serum (FBS)] followed by 5 min centrifugation at 1,500 rpm (Sigma, 1A), and was then resuspended in growth medium and plated on a Petri dish for 30 min to allow separation of adherent cells. Cells in suspension were then replated in T25 flasks (Sarstedt, Nümbrecht, Germany) and transferred to T75 flasks upon confluence.

Cell culture procedures. Cells were cultured in 37°C in a humidified atmosphere of 5% CO2 using T75 S Cell Culture Surface Flask (Sarstedt, Nümbrecht, Germany). The cell culture medium consisted of F12 (Gibco) and Dulbecco’s modified Eagle’s medium (DMEM, Gibco, low glucose) at a ratio of 1:1 with addition of 20% FBS, 50 U/ml streptomyacin, 50 U/ml penicillin and 1.25 μg/ml amphotericin. Upon differentiation, FBS was reduced to 2%.

Cell respirometry. After treatment with trypsin, the cells were resuspended in cell medium and spun down at 200 g during 5 min. Cells were then washed in PBS, resuspended, and resuspended in cell respiration medium [EGTA (0.5 mM), MgCl2 (3 mM), K-lactobionate (60 mM), taurine (20 mM), KH2PO4 (10 mM), HEPES (20 mM), sucrose (110 mM), BSA (1 g/l), dithiothreitol (0.3 mM)]. Cell viability was determined by staining with Trypan blue 0.4% in respiration medium 1:1 and counted manually with a hemocytometer. Cells here then added into the oxygraph and the chamber was closed. After stabilization of the oxygen signal, measurement of basal respiration was made in intact cells. Thereafter cells were permeabilized with digitonin and cellular respiration was measured by using high-resolution respirometry (O2-K Oroboros, Innsbruck, Austria) at 37°C as previously described (26). Saturating levels of pyruvate, malate, and succinate were used as substrates (Sigma-Aldrich) and leak respiration was assessed. Subsequently, saturating amounts of ADP was added to induce state 3 respiration. In cellular experiments using transfected cells, the respiration media including the cells were recovered, cells were centrifuged at 1,500 rpm, 10 min, and the successful transfection was confirmed by Western blotting.

In isolated mitochondria, two different protocols were used. In a first protocol leak and state 3 respiration were assessed as above, followed by state 4 respiration as the added ADP was phosphorylated into ATP. Actryloside was added to inhibit the adenine nucleotide transporter (ANT) and titration of FCCP was used to uncouple respiration from phosphorylation to assess uncoupled respiratory flux. In a separate protocol mitochondrial P/O ratio was assessed using pyruvate and malate as respiratory substrates, 2 mM ATP was added and ADP was then infused at around 50% of mitochondrial Vmax through a feedback controlled infusion pump (Tip2K, Oroboros, Austria). P/O ratio was calculated as the rate of ADP infusion divided by the respiratory rate.

Respirometric analysis of mitochondria. Respirometric analysis on isolated mitochondria was performed by high-resolution respirometry in a respiration medium containing EGTA (0.5 mM), MgCl2 (3 mM), K-lactobionate (60 mM), taurine (20 mM), KH2PO4 (10 mM), HEPES (20 mM), sucrose (110 mM), and BSA (1 g/l). Pyruvate (5 mM), malate (1 mM), and ADP (2.5 mM) were used as substrates for the determination of complex I-mediated respiration, and succinate (10 mM) was added for complex I+II-mediated respiration.

COX activity in cell homogenates and isolated mitochondria. COX activity was measured spectrophotometrically by using a commercial kit (CYTOCOX1, Sigma-Aldrich) according to the manufacturer’s instructions. COX activity was measured in assay buffer containing 10 mM Tris-HCl, 120 mM KCl, 1 U/ml pyrophosphatase, 0.5 μM unlabeled phosphatase inhibitor cocktail 2 (Sigma-Aldrich).

Transfection protocol. To achieve COX IV-2 overexpression and COX IV-1 knock down (COX IV-1 /2 ), cells were seeded in T75 flasks until reaching ~80% confluence. After differentiation of the cells by changing to cell medium containing 2% FBS during 5 days, the cells were cotransfected as follows to achieve overexpression of COX IV-2 and knock down of COX IV-1. DharmaFECT Duo Transfection Reagent (Thermo Fisher Scientific) was used for all cotransfections following the instructions of the manufacturer. The untagged 4.7-kb circular cloning expression vector human cDNA Clone SC126284, NM_026209.2, vector pCMV6XL5, and promoter T7 (15 μg/T75 flask) (Oregene Technologies, Rockville, MD) was used together with siRNA (1.5 nmol/T75 flask), ON-TARGETplus siRNA, L-011625-01 (Dharmacon). To effectively knock down COX IV-1, the transfection protocol was repeated after 3 days. Only the transfection protocol for the siRNA was used during the first 3-day period followed by dual transfection the last 2-day period. Cells were harvested at the 6th day of transfection. Control cells were treated identically: Plasmid, CMV6XL5 empty vector. Oregene; siRNA: ON-TARGET plus Non-targeting Control siRNA no. D-001810-01, (Dharmacon). Successful transfections were verified after the respirometric experiments by Western blotting.

Cell harvesting for immunoblot analysis. Cells were put on ice and washed with ice cold PBS twice. PBS (1.5 ml) was added to the T75 flasks and cells were collected with a cell scraper followed by centrifugation at 3,000 rpm for 5 min, 4°C. The pellet was resuspended in 75 μl RIPA buffer [50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 μg/ml protease inhibitor cocktail (P-8340, Sigma-Aldrich)]. Samples were allowed to stand on ice for 30 min, vortexed every 10 min, and then sonicated during 3 s (MSE, Soniprep 150), followed by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was collected and kept at −80°C until analyzed.

Protein concentration. The BCA method was used for protein concentration determination (Micro BCA protein assay kit, Thermo Scientific) and spectrophotometrically analyzed (SPECTRAmax Plus384 Microplate reader, Molecular Devices).

Immunoblot analysis. Muscle samples were freeze-dried, cleaned and homogenized as previously described (7). Protein concentration was determined in the muscle and cell homogenates before adjusting the samples to the same final protein concentration by diluting them in RIPA buffer followed by further dilution in Laemmli sample buffer [125 mM Tris-HCl, 4% weight per volume SDS, 0.004% bromophenol blue, 20% glycerol beta-mercaptoethanol (40 μM/l) (pH 6.8)]. After heating at 95°C for 5 min, samples were separated by SDS-PAGE (Criterion cell gradient gels, 4–20% acrylamide, Bio-Rad Laboratories). Gels were equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine, and 15% methanol) for ~15 min before proteins were transferred to polyvinylidenedifluoride membranes (Bio-Rad Laboratories). Membranes were blocked in Tris-buffered saline (TBS; 20 mM Tris base, 137 mM NaCl, pH 7.6, 5% nonfat dry milk), 1 h at room temperature. Membranes were incubated with primary antibodies diluted 1:1,000 in TBS, 0.1% Tween-20, 2.5% nonfat dry milk (TBSTM) either overnight or 1.5 h at room temperature. Membranes were washed with TBSTM and incubated by the appropriate secondary antibody diluted 1:10,000 for 1 h at room temperature. After serial washing, the membranes were visualized by chemiluminescent detection using SuperSignal West Femto Chemiluminescent Substrate, Thermo Scientific. The bands were visualized using a Fujifilm LAS-1000 CH luminescent image analyzer, and quantification of intensities was done using Image Gauge software, version 3.46, FUJI PHOTO Film Co. A commercial buffer was used for stripping the membranes (Restore Plus Western Blot Stripping Buffer, Thermo Scientific). Primary antibodies were used as follows:
COX IV isoforms and resting metabolic rate. We hypothesized that the COX IV-2 protein is a structure that suppresses metabolic rate in humans. We therefore wanted to test if there was a relationship between COX IV isoforms and whole body RMR measured in the same individuals. Indeed, a Pearson’s r correlation analysis revealed a strong negative correlation between COX IV-2/IV-1 transcript ratio and resting metabolic rate ($R^2 = 0.639$, $P < 0.001$) (Fig. 2A). In addition, a significant correlation was also found between COX IV-2 normalized to citrate synthase and RMR ($R^2 = 0.39$, $P < 0.01$, Fig. 2B), indicative of COX IV-2 being the driving factor influencing energy expenditure. By contrast, COX IV-1 normalized to citrate synthase did not show any correlation to RMR (Fig. 2C).

We have previously shown that RMR is strongly related to mitochondrial oxygen affinity ($p50_{mito}$) where low mitochondrial oxygen affinity (high $p50_{mito}$) is coupled to low RMR (20). We performed the same analysis in the present study by measuring $p50_{mito}$ in isolated mitochondria and again found a significant negative correlation (Pearson’s $r$) between $p50_{mito}$ and RMR ($R^2 = 0.45$, $P < 0.05$, see Fig. 2D).

**COX subunit composition is positively associated with maximum aerobic power but not mitochondrial density.** We performed correlation analysis between $V_{O2\ max}$, RMR and COX IV isoform composition to assess the interdependence between these variables and found significant associations (Fig. 3, A and
We then analyzed several parameters of mitochondrial density, mitochondrial respiration, and efficiency and related them to both RMR and \( \dot{V}O_2 \max \) (see Table 1). Surprisingly, none of the traditional markers of mitochondrial density, respiration, or efficiency correlated with RMR except COX activity.

**COX IV-2 protein is absent in cultured human primary myotubes.** To investigate the causality between the COX IV-2/COX IV-1 ratio and resting metabolic rate, we cultured isolated primary myoblasts harvested from the skeletal muscle biopsies. Interestingly, following differentiation the myotubes expressed only COX IV-1 and no detectable levels of COX IV-2 (see Fig. 1B). Earlier reports indicate that COX IV-2 can be induced by hypoxic exposure in several cell types \((3, 9, 11, 13)\). To investigate the possibility that the atmospheric oxygen tension \((\sim 20 \text{ kPa})\) in the cell incubator prevented expression of COX IV-2 in the primary myoblasts that are normally exposed to \(\sim 4 \text{ kPa}\) in resting skeletal muscles \((31)\), we repeatedly exposed cultured myoblasts in a hypoxic chamber at different degrees of hypoxia \((0.5–4 \% \text{ oxygen})\) and at incubation times between 12 and 72 h. However, we failed to detect any significant levels of COX IV-2 protein (data not shown).

**Concomitant overexpression of COX IV-2 and knockdown of COX IV-1 reduces cellular oxygen consumption in primary human myotubes.** To be able to assess the functional characteristics of different COX IV isoform compositions we overexpressed COX IV-2 along with concomitant knockdown of COX IV-1 in cultured intact myoblasts (COX IV-1 \(^{-/2+}\)). This procedure lowered basal cell respiration in intact myotubes by 66\% compared with control cells treated with empty vectors (Fig. 4A). Similar treatment of HEK293 cells (COX IV-1 \(^{-/2+}\)) lowered basal cell respiration with 34\% (Fig. 4B).

To assess if the lower respiration was an effect of a more efficient oxidative phosphorylation or if it was due to a generalized impairment of ETS capacity, cells were permeabilized with digitonin to allow direct stimulation of mitochondria with respiratory substrates. Leak respiration with pyruvate, malate, and succinate as substrates was also reduced compared with control cells \((-54\%, \text{ Fig. 4C})\). However, during ADP stimulated respiration there was no significant difference between COX IV-1 \(^{-/2+}\) and control cells (Fig. 4D), demonstrating a preserved ETS capacity but higher mitochondrial efficiency.

To confirm that the ETS capacity was maintained in COX IV-1 \(^{-/2+}\) cells we also analyzed COX activity by spectropho-

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**Fig. 2.** COX subunit composition and mitochondrial oxygen affinity predicts RMR. A: a strong correlation was found when the ratio of COX IV-2/COX IV-1 protein in vastus lateralis (Western blotting) was plotted against RMR in the same individual (indirect calorimetry). A significant correlation was found when COX IV-2 normalized to the mitochondrial density marker CS (B) but not when COX IV-1 normalized to CS was plotted against RMR (C). D: a significant correlation was found when p50\(_{\text{mito}}\) of isolated mitochondria was correlated to RMR in the same subjects.

**Fig. 3.** Aerobic power predicts RMR and COX subunit composition. A significant correlation was found when aerobic power \(\dot{V}O_2 \max \) measured during incremental exercise to exhaustion was plotted against RMR (A) and COX IV-2/COX IV-1 ratio (B) in vastus lateralis.
Table 1. Correlation analysis between resting metabolic rate (RMR) and $V_{O_{2}}$max and variables of mitochondrial density, function, and efficiency

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RMR, kcal 24 h$^{-1}$ kg lean body mass$^{-1}$</th>
<th>$V_{O_{2}}$max, ml min$^{-1}$ kg body $^{\text{atm}}$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mitochondrial density</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>0.32</td>
<td>0.53*</td>
<td>16</td>
</tr>
<tr>
<td>TFAM</td>
<td>−0.10</td>
<td>−0.23</td>
<td>16</td>
</tr>
<tr>
<td>UCP-3</td>
<td>0.09</td>
<td>−0.12</td>
<td>16</td>
</tr>
<tr>
<td>ANT</td>
<td>−0.49</td>
<td>−0.11</td>
<td>14</td>
</tr>
<tr>
<td>COX I</td>
<td>0.39</td>
<td>0.41</td>
<td>16</td>
</tr>
<tr>
<td>COX activity</td>
<td>0.55*</td>
<td>0.42</td>
<td>16</td>
</tr>
<tr>
<td><strong>Mitochondrial function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEAK respiration</td>
<td>0.34</td>
<td>−0.32</td>
<td>12</td>
</tr>
<tr>
<td>State 3 respiration</td>
<td>0.23</td>
<td>−0.26</td>
<td>14</td>
</tr>
<tr>
<td>State 4 respiration</td>
<td>0.25</td>
<td>−0.03</td>
<td>14</td>
</tr>
<tr>
<td>State 4 + Atractyl</td>
<td>0.25</td>
<td>−0.13</td>
<td>12</td>
</tr>
<tr>
<td>Uncoupled respiration</td>
<td>0.11</td>
<td>−0.26</td>
<td>14</td>
</tr>
<tr>
<td><strong>Mitochondrial efficiency</strong></td>
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<td></td>
<td></td>
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<tr>
<td>P/O ratio</td>
<td>−0.26</td>
<td>0.16</td>
<td>14</td>
</tr>
<tr>
<td>RCR</td>
<td>0.05</td>
<td>−0.26</td>
<td>14</td>
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</table>

RMR was measured by indirect calorimetry after an overnight fast. $V_{O_{2}}$max was measured during incremental cycle ergometer exercise. Both these parameters were correlated against protein levels of five established mitochondrial density markers, measured with Western blotting. COX activity in vastus lateralis was measured spectrophotometrically. Intrinsic mitochondrial function was measured by high resolution respirometry in the presence of respiratory substrates and absence of ADP (LEAK) or in the presence of 2 mM ADP (state 3) and during state 4 when all ADP had been phosphorylated into ATP. Maximal electron transport system capacity was measured by titration of the protonophore FCCP. Mitochondrial efficiency was measured by infusing submaximal concentrations of ADP by an infusion pump in the presence of 2 mM ATP (P/O ratio). The mitochondrial concentrations during submaximal infusion of ADP were adjusted to reflect approximately half maximal respiration rates (state 3). Respiratory control ratio (RCR) was calculated by dividing state 3 with LEAK respiration. The values represent the obtained Pearson’s correlation coefficient ($r$). *$P < 0.05$.

There is growing evidence that ATP inhibits COX activity at least under certain conditions (2, 5, 23, 30) but the physiological relevance of ATP inhibition remains controversial. We therefore set out to test the influence of ATP in regard to COX IV subunit isoform composition in permeabilized cells and mitochondria. During high oxygen tensions in the oxygraph (~15–20 kPa), the addition of 5 mM ATP did not cause any inhibition of respiration in either control or treated cells (Fig. 4F). Intriguingly, when oxygen tension was reduced to 5 kPa, closer to the value found in resting skeletal muscle (31), in the presence of 5 mM ATP respiration was 30% lower in the control cells compared with the COX IV-1+/−2− cells (Fig. 4G). This respiratory inhibition was the opposite of what was found in the absence of ATP in isolated mitochondria where we found a significant positive correlation between COX IV-2/COX IV-1 ratio and p50mito ($R^2 = 0.487, P < 0.05$, see Fig. 4H).

Since the ATP/ADP ratio is high at rest but decreases with increasing metabolic demand, the control of ATP over metabolic rate should be relieved during exercise. To test this hypothesis in vivo we measured metabolic rate during standardized submaximal cycle ergometer exercise and found no association between metabolic rate and COX IV-2/COX IV-1 ratio (Fig. 4I), indicating that the COX IV isoform setup loses its control over metabolic rate as ATP/ADP ratio decreases and metabolic rate increases.

**ROS production.** To investigate the influence of COX IV-2 on ROS production, we simultaneously measured respiration and $H_2O_2$ generation in the COX IV-1/−2+ myotubes. The $H_2O_2$ generation in relation to cell number was significantly reduced in the treated cells compared with control cells during several respiratory states including ADP stimulated respiration (Fig. 5, A–D).

$H_2O_2$ and hypoxic challenge. To determine the resistance to a ROS challenge we exposed COX IV-1/−2+ myotubes and control cells to increasing concentrations of $H_2O_2$ and subsequently measured viability. We found that COX IV-1/−2+ cells were significantly more resistant to high concentrations of $H_2O_2$ compared with control cells (Fig. 5E). Based on the previously published indications of improved respiratory efficiency during low oxygen tensions (9), we also exposed HEK-cells to hypoxia (1%) for 60 h. Indeed, after the hypoxic exposure, viability was significantly higher in the COX IV-1/−2+ cells compared with control cells (Fig. 5F).

Next, we set out to determine if this increased cellular resilience in COX IV-1/−2+ cells was secondary to an increase in proteins involved in antioxidant defense or other mitochondrial alterations induced by changing the isoform composition of COX IV. As can be seen in Fig. 6, protein levels of all assessed antioxidative and mitochondrial proteins were similar in COX IV-1/−2+ cells and cells treated with empty vectors. Collectively these results imply that expression of COX IV-2 renders cells more resistant to hypoxia and oxidative stress, possibly because of a lower baseline mitochondrial ROS production in this cell type.

**DISCUSSION**

Here we show that the cytochrome c oxidase subunit COX IV-2 is expressed in human skeletal muscle, and the levels of COX IV-2 correlate negatively with whole body resting energy expenditure. These findings are further supported by respirometric experiments in cultured myotubes, overexpressing COX IV-2 with a simultaneous knockdown of COX IV-1. These cells show markedly reduced oxygen consumption coupled with lower ROS generation and increased resistance to both hypoxia and to a $H_2O_2$ challenge that suggests a central role of COX IV-2 in physiological regulation of energy expenditure and cellular resilience in humans.

Since the COX IV-2 protein has not been found in rodent skeletal muscles, and rodents have around 7-fold higher metabolic rate per unit mass (39), we hypothesized that COX IV-2 may contribute to suppress the resting metabolic rate. The observed strong relationship between RMR and the COX IV-2/COX IV-1 ratio in humans indeed indicated that COX subunit composition could explain the wide individual variation in RMR. An interesting finding in the present study is that we find no associations between RMR and classical mitochondrial parameters including mitochondrial density, UCP-3 expression or mitochondrial efficiency. This could in part be explained by our rather small population size or the fact that COX subunit composition overrides more subtle differences in other mitochondrial parameters in this study.
Hüttemann and colleagues (13, 14) showed that COX IV-2 contributes to an increased COX activity in purified enzyme, whereas Oliva and colleagues (25) showed the opposite in glioma cells that express COX IV-2 compared with glioma cells that predominantly express COX IV-1. The present study support those results showing that COX IV-1/-2 expression had both markedly reduced basal respiration rates and upon permeabilization, lower LEAK respiration together with unchanged ADP stimulated respiration. In addition, the measured COX activity after the treatment was unchanged. This strongly suggests that COX IV-2 is involved in the control of energy expenditure in vivo and offers a possible explanation for the lower RMR observed in the subjects with a higher expression of this subunit isoform.

Several previous studies have presented mRNA expression levels of COX IV-2 in different tissues of humans and mice (11, 12), but the actual protein levels have been poorly investigated. COX IV-2 mRNA has been shown to be highly expressed in adult human and rat lung and also in fetal skeletal muscle (12) while lower or undetectable levels have been observed in other adult tissues such as abdominal skeletal muscle and brain neurons (11, 12). Despite the presence of COX IV-2 protein in skeletal muscle biopsies in this study, cultured myotubes derived from the same subjects did no longer express this protein (Fig. 1B). Previous findings have shown that hypoxia increases the transcription of COX IV-2 (9, 11). Indeed, Hüttemann et al. (13) identified a 13-bp sequence in the proximal promoter that functions as an oxygen responsive element (ORE), and recently Aras and colleagues (3) identified three transcription factors that can bind to this ORE that is maximally active at 4% oxygen. However, upon exposing the myotubes to either hypoxia at different doses (0.5–4% for 12–72 h) or chemical hypoxia with CoCl₂, we were never able to detect measurable levels of COX IV-2 protein. This is in accordance with recently published results failing to detect any COX IV-2 transcripts in human myoblasts, adenocarcinoma cells or several other animal tissues (17). In fact, Kocha and colleagues (17) found little evidence supporting a hypoxia-induced switch from COX IV-1 to COX IV-2 in their models. In addition, COX IV-2 is paradoxically most markedly expressed in the lung, a tissue that experience particularly high oxygen tensions (12). This indicates the presence of alternative signaling pathways involved in the induction of the COX IV-2 expression. Therefore, COX IV-2 in human vastus lateralis is likely regulated by other signaling pathways yet to be explored in future studies.

Future studies may reveal whether the lower cell respiration could be explained by a COX IV-2-mediated increased COX-efficiency through a decreased proton slip which is known to occur with isoform COX IV-1 under certain circumstances (24). In addition, recent evidence shows that respiratory complexes within mitochondria are organized into supercomplexes and able to adjust to external stimuli by assembly into structures to match the metabolic demands (18). The switch from COX IV-1 to COX IV-2 may affect the formation of these supercomplexes and thereby activity and efficiency of the mitochondria (1) which will not show when analyzing activity in purified respiratory complexes.

Recently, we found a strong correlation between mitochondrial oxygen affinity and resting metabolic rate (20). Here we confirm these findings again showing the relation between mitochondrial oxygen affinity and resting metabolic rate where a high oxygen affinity (lower \( p_{50_{mito}} \)) predicts a higher metabolic rate and vice versa. In vivo, the variability in the activity and the efficiency of the mitochondrial complexes and substrate availability generates a continuous change in mitochondrial oxygen affinity that in turn likely affects the mitochondrial efficiency. Moreover, the ATP-induced inhibition of COX activity after the treatment was unchanged. This strongly suggests that COX IV-2 is involved in the control of energy expenditure in vivo and offers a possible explanation for the lower RMR observed in the subjects with a higher expression of this subunit isoform.

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ADP-stimulated respiration at physiological oxygen tensions (5 kPa) seen in the control cells was completely abolished in the COX IV-1/H11002/H11001 cells. ATP inhibition of control cells containing COX IV-1 was highly dependent on oxygen tension; it was absent at 20 kPa and present already at 5 kPa. This may reflect the excess capacity of COX over other components of the ETS in normoxia. At rest, skeletal muscle post capillary oxygen tension roughly ranges from 1 to 6 kPa (34). Our results thus suggest that in vivo COX activity is regulated by oxygen tension already at rest. The results also suggest that ATP likely plays a role in the utilization of COX IV-2 and COX IV-1 in response to changes in oxygen levels. The abundance of ATP at rest ensures an efficient respiration through increased utilization of COX IV-2, whereas during exercise with decreasing ATP levels, an activation of COX IV-1 takes place. The advantage of a switch toward a slightly more inefficient COX during exercise (proton slip) would be a higher maximal power output (36) and a lower p50mito (Fig. 4) that may improve the oxygen extraction and affect the arteriovenous oxygen difference by increasing the diffusion gradient between capillary and mitochondria. Previous findings from cross-sectional studies indicate a positive association between RMR and VO2max but have failed to find any causal link between these two variables. On an organ level, the major limitation in the oxygen transport chain is imposed by the maximal cardiac output (22) since the total oxidative capacity of the mitochondrial pool by far surpasses the amount of oxygen that can be delivered from the central circulation. However, at maximal cardiac output small changes in p50mito and therefore peripheral oxygen extraction can have substantial effects on VO2max (19, 35, 40). The positive relationship between VO2max and RMR could be assumed to be an indirect effect of training status and thereby mitochondrial mass or function, since mitochondrial properties have been linked to resting metabolic rate (38). However, in the present study we find no evidence of an association between, mitochondrial mass or function and RMR (see Table 1). Instead, this study indicates that the COX IV-2/COX IV-1 ratio, p50mito, VO2max and RMR all are interrelated and that lower RMR comes with a lower VO2max as a trade-off.

Another fundamental question is the relationship between metabolic rate and production of ROS. The traditional view is that a small fraction of electrons escapes the ETS prematurely to produce ROS and therefore a high metabolic rate would mean a high ROS production (10). More recently, this concept has been seriously challenged and a contrasting theory holds that a low metabolic rate comes with a higher mitochondrial membrane potential and therefore a lower turnover of oxygen which yields more ROS (21). COX subunit isoform switch may confer protection against hypoxia-dependent inhibition of
respiration and improve efficiency of ATP production but it has been proposed to be at the expense of increased ROS production as shown in isolated mitochondria derived from astrocytes (37). Accordingly, Oliva and colleagues (25) found a higher ROS production in glioma cells that express COX IV-2 compared with cells that express COX IV-1. In addition, glioma cells expressing COX IV-1 also had higher activities of catalase, superoxide dismutase and an elevated ratio of GSH/GSSG indicating that cells expressing COX IV-1 may have a better protection against ROS (25).

Further, Singh and colleagues (37) observed an increased ROS production in astrocytes as a result of NPA induced COX IV-2 expression which was attenuated in cells where COX IV-2 was knocked down with siRNA. By contrast, Fukuda and colleagues (9) showed that COX IV-2 contributes to an optimized electron transfer during hypoxia and thereby contributes to a lower ROS production. This is in agreement with the improved viability observed in COX IV-1/2− cells during the hypoxic challenge and the 60% lower H2O2 production in the COX IV-1/2− myotubes. Our results support the hypothesis that mitochondrial ROS production is positively correlated with respiration rate (10). In our cellular ROS experiment setup we could only detect H2O2 that escaped scavenging by cellular detoxification systems. The reduced H2O2 emission and improved H2O2 tolerance in our COX IV-1/2− myotubes may simply reflect a higher buffer capacity of these cells due to the lower basal ROS production. Nonetheless, excessive oxidative stress is a contributing factor in several diseases, and the COX IV-2-dependent reduced levels of ROS may have implications in pathogenesis.

In conclusion, we have found that COX IV-2 protein is constitutively expressed in skeletal muscle of healthy individuals. The abundance of this isoform correlated strongly to whole body resting metabolic rate measured in the same subjects. Moreover, concomitant overexpression and knock down of COX IV-2 and COX IV-1 in human myotubes leads to lower basal- and leak respiration, further supporting a central role for COX IV-2 in regulation of metabolic rate. In addition, COX IV-2 overexpressing cells generated less H2O2 and were more resistant to oxidative and hypoxic stress. Together these data suggest an important role of COX IV-2 in control of energy expenditure and ROS homeostasis in humans. This may have implications on vital metabolic functions including weight control, glucose handling, and exercise capacity.

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**DISCLOSURES**

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

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