Biochimica et Biophysica Acta 1837 (2014) 226–231

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Increased intrinsic mitochondrial function in humans with mitochondrial haplogroup H

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ARTICLE INFO

Article history:
Received 17 June 2013
Received in revised form 21 October 2013
Accepted 25 October 2013
Available online 1 November 2013

Keywords:
Caucasian mitochondrial haplogroup
Mitochondrial OXPHOS capacity
Intrinsic mitochondrial function

ABSTRACT

It has been suggested that human mitochondrial variants influence maximal oxygen uptake (VO_{2max}). Whether mitochondrial respiratory capacity per mitochondrion (intrinsic activity) in human skeletal muscle is affected by differences in mitochondrial variants is not known. We recruited 54 males and determined their mitochondrial haplogroup, mitochondrial oxidative phosphorylation capacity (OXPHOS), mitochondrial content (citrate synthase (CS)) and VO_{2max}. Intrinsic mitochondrial function is calculated as mitochondrial OXPHOS capacity divided by mitochondrial content (CS). Haplogroup H showed a 30% higher intrinsic mitochondrial function compared with the other haplo group U. There was no relationship between haplogroups and VO_{2max}. In skeletal muscle from men with mitochondrial haplogroup H, an increased intrinsic mitochondrial function is present.

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1. Introduction

Mitochondrial DNA (mtDNA) is the only genome that is maternally inherited [15] and the polymorphisms that have occurred over evolutionary time have permitted a distribution of these normal variants into mtDNA haplogroups. This has, in turn, allowed for studies of human origin and demographic evolution [38]. The mtDNA haplogroups are basically organized in a hierarchic fashion, where the subgroups branch off from ancestor groups [37]. The various mitochondrial haplotypes have been associated with different disease states and with longevity [32].

mtDNA encodes only for a small number of proteins, but these are essential subunits for the mitochondrial energy-generating enzymes of oxidative phosphorylation (OXPHOS). Thus different mitochondrial haplogroups may potentially differ bioenergetically as producers of ATP required for cell homeostasis and function and consumption of oxygen. In humans this has been investigated in a few studies using the cytoplasmic hybrid technique [2,3,17], but no studies exist using the high resolution respirometry technique of mitochondrial oxygen consumption in permeabilized human skeletal muscle. The myocytes are characterized by very dynamic and potentially very high ATP turnover rates, which therefore demands tightly regulated oxidative phosphorylation system.

Using the cytoplasmic hybrid technique one study investigated mitochondrial OXPHOS capacity and coupling efficiency in haplogroups H and T (Caucasian) and found no differences in either intrinsic mitochondrial function (corrected for mitochondrial protein content) or coupling efficiency in the mitochondria [3]. In contrast another group also using cybrids (mitochondrial haplogroups Uk and H) that were constructed from human platelets found differences in mitochondrial intrinsic function [17]. A similar approach was used in arctic (A, C and D) and tropical (L1, L2 and L3) haplogroups, again using isolated mitochondria from cybrids, and no difference was present in intrinsic mitochondrial function [2].

It has previously been reported that maximal oxygen uptake (VO_{2max}) differs between the different haplogroups [23,24], but consensus has not been reached [29]. VO_{2max} correlates weakly with mitochondrial content [11,39] and it has recently been reported that mitochondrial OXPHOS capacity correlates with mitochondrial content [21]. No correlation has been reported between VO_{2max} and intrinsic mitochondrial function.

The present study was undertaken to examine if mitochondrial OXPHOS capacity and intrinsic mitochondrial function in human skeletal muscle is affected by differences in mitochondrial haplogroup variants. The potential differences in mitochondrial OXPHOS capacity between haplogroups have never been investigated before in permeabilized human skeletal muscle fibers. Furthermore we examined if a correlation is present between mitochondrial OXPHOS capacity and maximal oxygen uptake in the different haplogroups.

2. Materials and methods

2.1. Subjects

A total of 54 Caucasian individuals participated in the study, subject characteristics are given in Table 1. All participants were fully informed of the nature and the possible risks associated with the study before informed consent was given. The study was

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http://dx.doi.org/10.1016/j.bbabio.2013.10.009

Table 1
Characteristic of the subjects in the different haplogroups.

	U	H	Composite
	$n = 13$	$n = 21$	$n = 20$
NGT/T2DM	8/5	10/11	10/10
Age (years)	43 ± 5	46 ± 3	45 ± 3
Weight (kg)	93 ± 5	101 ± 3	99 ± 4
BMI (kg/m^2)	28 ± 1	31 ± 1	30 ± 1
VO_{2max} (ml/min/kg)	32 ± 3	27 ± 2	32 ± 3

Data are means ± SE. Abbreviations: BMI: Body mass index; NGT: Normal glucose tolerance; T2DM: Patients with type 2 diabetes; VO_{2max}: Maximal oxygen uptake. Composite include the T, J, I, L, W and HV haplogroups.

approved by the ethical committee of Frederiksberg and Copenhagen municipality, and adhered to the Principles of the Declaration of Helsinki.

Due to the low number of subjects in haplogroups (J, W, I, L, T, HV) we grouped these into one composite group. Besides from haplogroup L the other haplogroups are not distinct from each other (see Fig. 1).

Subjects met at the laboratory fasted (10–12 hours) in the morning. After a 15 minutes rest a blood sample was taken and a muscle biopsy was obtained from the m. vastus lateralis using the Bergstrom technique [10]. The biopsy material was divided into two or three portions. One was immediately frozen in liquid nitrogen and stored at −80 °C, another part was placed in a relaxing buffer (see below) and immediately hereafter procedures for measurement of mitochondrial OXPHOS capacity, using high resolution respirometry began (see below). In a subgroup of participants a third part was embedded in tissue-tek and used for histochemical analysis (stored at −80 °C). After the muscle biopsies were obtained subjects rested for 20 min after which maximal oxygen uptake was measured as previously described [7,20,27].

2.2. Total DNA extraction from skeletal muscle biopsies

The frozen muscle samples (−80 °C) were grinded in a porcelain mortar with liquid nitrogen inside the cold chamber (4 °C). Muscle powder was then transferred to a sterile eppendorf and resuspended in 500 of RSB tampon (10 mM Tris–HCl pH 7.5, 10 mM NaCl, 25 mM EDTA). Cells were lysed with a 1% final concentration of SDS and 40 μg/ml Proteinase K was then added. Afterwards, this reaction mixture was incubated at 37 °C overnight.

Upon incubation, nucleic acids separation was achieved using a mixture of phenol–chloroform–alcohol 25:25:1 (Phenol IAC). Firstly, ammonic acetate was added to a final concentration of 1.125 M to form the ammonic salts of the nucleic acids to facilitate their posterior precipitation. 1.5 volumes of Phenol IAC were added and after a vigorous stir with a vortex for a minute, the reaction mixture was centrifuged at 20 °C and 700 ×*g* during 5 min. The upper aqueous phase containing nucleic acids was recollected with the help of a sterilized glass Pasteur pipette and was transferred to a sterile falcon tube of 50 ml. Again, 1.5 volumes of Phenol IAC were added and the process of centrifugation was repeated.

This last aqueous phase was recollected and transferred to a new sterile falcon tube, and 1.5 volumes of chloroform–isoamilic alcohol 24:1 were added in order to remove traces of phenol that could interfere in latter processes such as the PCR. The mixture was vigorously stirred and centrifuged again at 20 °C and 700 ×*g* during 5 min to enable a good separation of the two phases.

Finally, a double volume of cool ethanol 99% (−20 °C) was added to the recollected aqueous phase in a new falcon tube. With gentle mixing the precipitation of the DNA occurs forming a chalky ball. This clew was transferred with a glass Pasteur pipette and a bit of ethanol to a sterile eppendorf tube and was then centrifuged at 4 °C and 8000 ×*g* for 30 min. The ethanol was poured off and the DNA pellet was dried at vacuum in a SpeedVac at 30 °C for 30 min to eliminate all the ethanol.

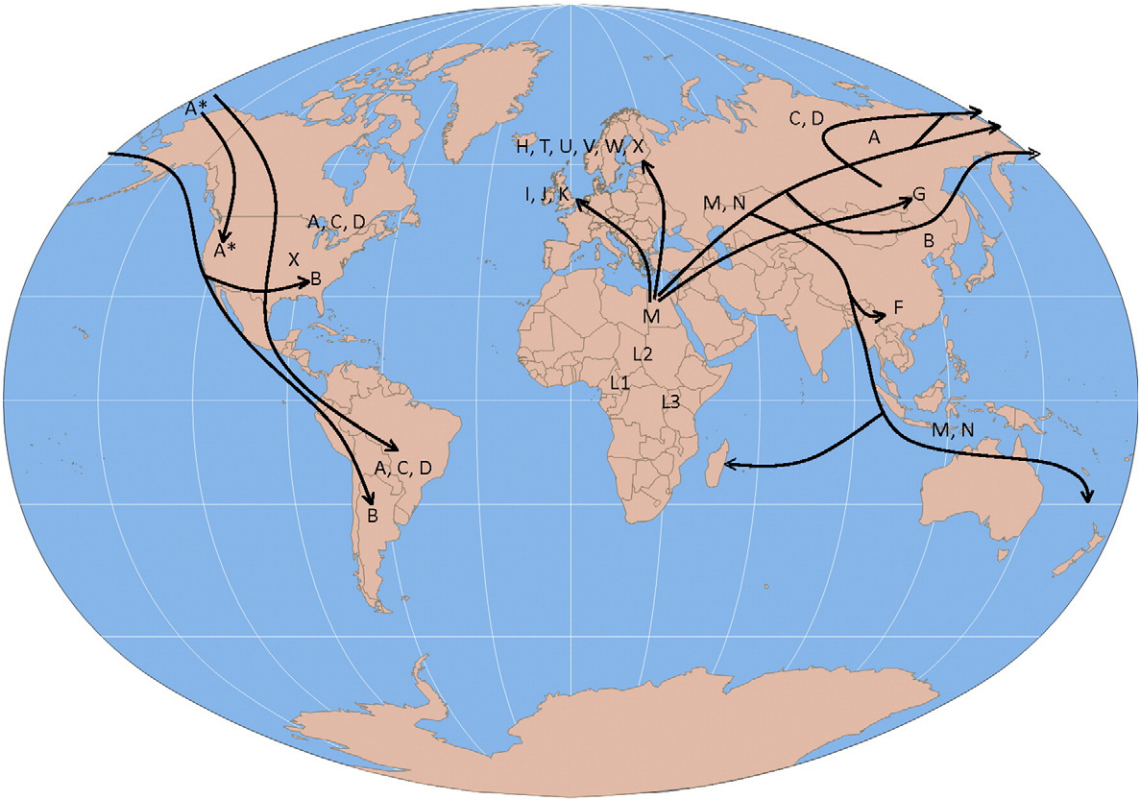

Fig. 1. The map indicates the migration of the three oldest haplogroups (L1, L2, L3) from Africa. The map is redrawn based on information found in the paper by Shriver and Kittles [33].

At last, the dried pellet was resuspended in 500 μl of sterile water (FLUKA) at 37 °C with gentle agitation in a thermoblock for at least 24 h.

2.3. Nucleic acids quantification

Total DNA concentration was quantified of using a NanoDrop® ND-1000 Spectrophotometer, that measures doubled stranded DNA (dsDNA) concentration from 1.5 to 3700 ng/μl in a total volume of 2 μl.

2.4. Mitochondrial haplogrouping strategy

Mitochondrial Caucasian haplogroups were determined by qRT-PCR, or by sequencing hypervariable region I (HV-I), and confirming by restriction fragment length polymorphism (RFLP) if necessary. The mtDNA haplogroups-defining single nucleotide polymorphisms (SNPs), according to previously reported [28,35,36] (Supplementary Table S1), were determined using TaqMan reagents. For each SNP, reagents include two primers around the SNP and two probes: a fluorophore VIC-labeled probe specific for one allele and other fluorophore FAM-labeled probe specific for the other allele. DNA was amplified in a final volume of 25 μl, using 12.5 μl of TaqMan Gene Expression Master Mix (Applied Biosystems, Austin, TX, USA), 0.9 μM of each primer, 0.2 μM of each probe and 10 ng of total DNA. The amplification was performed using universal conditions. NonCaucasian haplogroups were determined as described elsewhere [8] and confirming by HV-I sequencing.

The HV-I sequence was achieved from position 15977 to 16450. With this aim a PCR was carried out using primers: L15977 5′-CCACCATT AGCACCCAAAGC-3′ and H16450 3′-CGAGGAGAGTAGCACTCTTG-3′, and the amplification programme: 95 °C–5 min, [95 °C–45 s, 62 °C–45 s, 72 °C–2 min] ×35 cycles, 72 °C–5 min. Once the PCR was realized and verified by electrophoresis the presence of the unique desired DNA fragment, this amplification was purified using SpinClean™ PCR Purification Kit (MBiotech) following the recommended instructions. After purification all DNA samples were quantified with the NanoDrop.

The automatic sequencing of the samples was carried out at the sequencing service SECUGEN S.L. of the Biologic Investigation Centre of Madrid. With this purpose samples were prepared following its specifications: 15 μl of sample at a concentration of 10 ng/μl with 1.5 μl of the specific primer (L15977 o H16455) at a concentration of 5 μM. With the obtained sequences it was possible to read approximately 450 bp using the software FinchTV 1.4.0, © Geospiza Inc. 2004-2006.

The polymorphisms found were compared with the previously HV-I reported sequences in supplementary data from ref. [28] at the web http://www.stats.gla.ac.uk/~vincent/founder2000/index.html to determine haplogroups. If necessary, haplogroups were also confirmed by RFLPs (Table S1) [28,34–36].

2.5. Measurement of mitochondrial OXPHOS capacity

The skeletal muscle tissue was subjected to mechanical dissection with sharp forceps in relaxing buffer on ice. The relaxing buffer contained (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 20 imidazole, 20 taurine, 6.56 $MgCl_2$, 5.77 ATP, 15 phosphocreatine, 0.5 dithiothreitol, and 50 K-MES, pH 7.1. Skeletal muscle fibers were permeabilized by gentle agitation for 30 min at 4 °C in the relaxing solution supplemented with 50 μg/ml saponin. Fibers were then washed in ice-cold respiration medium (content is described later) 2 × 10 minutes during gentle agitation. The method has been described in details elsewhere [18].

Mitochondrial OXPHOS capacity was measured at 37 °C in a high resolution respirometer (Oxygraph 2k; Oroboros Instruments, Innsbruck, Austria), following the principles described by Pesta & Gnaiger [26]. The respiration medium consisted of 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 1 g/l BSA essentially fatty acid free, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM K-HEPES, pH 7.1. The software DatLab (Oroboros Instruments, Innsbruck, Austria) was used for data analysis. The use of permeabilized fibers provides the possibility to study mitochondria "in situ" in the cell, and very small muscle samples (2–4 mg) are needed to study mitochondrial respiration. In order to avoid any potential oxygen diffusion limitation all experiments were conducted after hyper-oxygenation (450 nmol O_2/ml).

Substrate and inhibitor were added, one protocol was employed, made in duplicate. The aim of the protocol was to look at maximal coupled state 3 OXPHOS capacity: The following substrates were added. State 2 respiration was assessed with malate (M, 2 mM), followed by Octanoyl Carnitine (MO, 1.5 mM), state 3 respiration was reached with ADP (MO_D, 5 mM). Subsequently, glutamate (GMO_D, 10 mM) was added followed by succinate ($GMOS_D$, 10 mM), this state is referred to as maximal coupled state 3 respiration (state 3), cytochrome *c* (10 μM) was added to control for outer mitochondrial membrane integrity. Finally oligomycin [2 μg/ml] was added to inhibit the ATP synthase (state 4o). Mitochondrial coupling efficiency can be estimated as the respiratory control ratio (RCR) between state 3 (maximal ATP production) and respiration when ATP production is blocked (state 4o). Increased coupling efficiency of the mitochondria is seen with increasing RCR [16].

Mitochondrial intrinsic capacity was calculated as mitochondrial OXPHOS capacity divided by CS activity.

2.6. Muscle characteristics

Citrate synthase activity was measured as previously described [5]. Fiber type distribution was analyzed histochemically as described elsewhere [4,14].

2.7. Statistics

In order to do appropriate statistics on the samples we grouped the following haplogroups (J, W, I, L, T, HV) into one composite group. In the result section on mitochondrial OXPHOS capacity and intrinsic mitochondrial function measurements, we have shown all haplogroups including the one containing the six different haplogroups.

Data are presented as means ± SE. *P* values less than 0.05 were considered significant. Statistical analysis of differences in mitochondrial OXPHOS capacity rates between the studied groups was carried out with a two-way ANOVA for repeated measures. The restrictive assumptions, normality and equal variance, were checked before the statistical analysis was conducted. Significant main effect or interactions between the variables were further analyzed with the Holm–Sidak post hoc analysis. Differences between the three groups were evaluated with a one-way ANOVA. If the normality test failed, the data were $\log_{10}$ transformed and reanalyzed. All statistical analyses were performed using the software program SigmaStat 3.1 (Systat Software, San Jose, CA, USA).

3. Results

Subject characteristics are given in Table 1. No differences were present in age, weight, body mass index (BMI) or maximal oxygen uptake (VO_{2max}) between the subjects stratified according to haplogroups. Even though VO_{2max} was not significantly different between the groups, the haplogroup H had a 16% lower VO_{2max} compared with the two other haplogroups.

The mitochondrial OXPHOS capacity expressed per mg of tissue as well as LEAK (state 4o)) was not different between groups (U: 30 ± 2, 58 ± 4 and 24 ± 3; H: 28 ± 2, 60 ± 4 and 20 ± 2; Composite: 25 ± 2, 58 ± 3 and 23 ± 2 pmol sec^{-1} mg^{-1} in complex I, complex I + II linked OXPHOS capacity and state 4o, respectively). When looking at haplogroup L from the composite group, it seems as if this haplogroup has a higher mitochondrial OXPHOS capacity (L: 80 pmol sec^{-1} mg^{-1} complex I + II linked OXPHOS capacity) compared with the other haplogroups from the composite group. However, when the respiratory flux rates are normalized to mitochondrial content (by citrate synthase

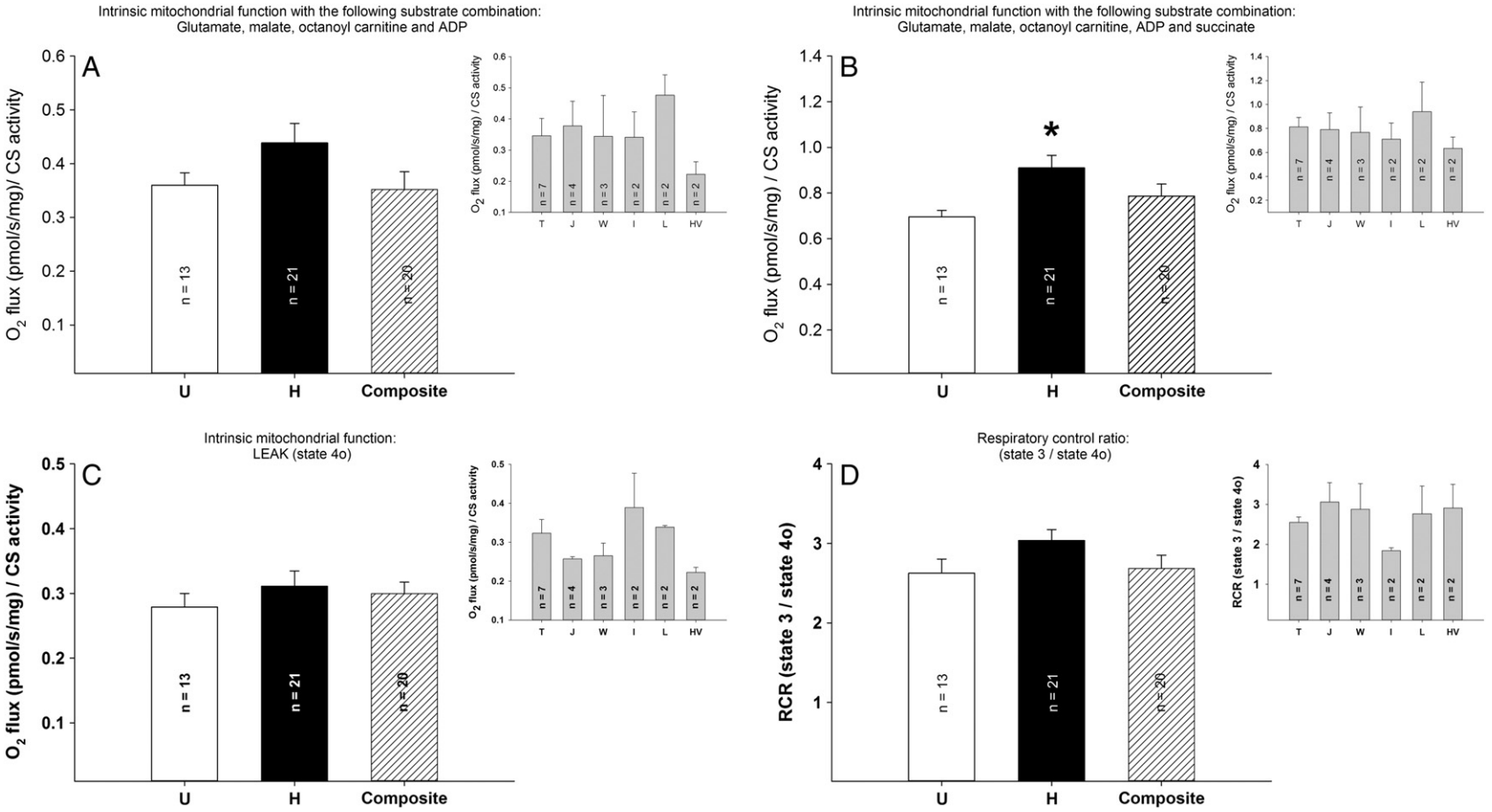

Fig. 2. Intrinsic mitochondrial function in permeabilized skeletal muscle fibers (vastus lateralis) obtained from 54 male subjects, stratified according to mitochondrial haplogroup U, H and a composite haplogroup. The small inserted graph is displaying the different haplogroups represented in the composite haplogroup. No statistics was performed on the subgroups in the composite haplogroup due to the low representation of each haplogroup. A) State 3 respiration with the following substrates: Glutamate, Malate, and octanoyl carnitine (GMO_D). B) Same as in (A) but with the addition of succinate ($GMOS_D$). C) Same as in (B) but with the addition of oligomycin (4o). D) Respiratory control ratio (RCR) calculated as $GMOS_D$/4o. Data are shown as mean ± SE. * denote significantly ($P < 0.05$) higher respiration in H compared with U and the composite haplogroup.

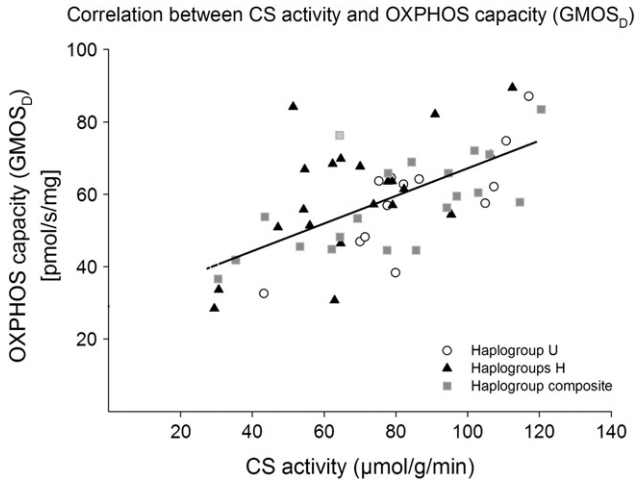

Fig. 3. A correlation between CS activity and mitochondrial OXPHOS capacity ($GMOS_D$) in all 54 subjects ($P < 0.000$; $R = 0.618$). The same significant correlation was seen when the three different haplogroups (H: $P = 0.001$; $R = 0.794$; U: $P = 0.003$; $R = 0.609$; Composite: $P = 0.001$; $R = 0.672$) were analyzed separately.

(CS) activity), haplogroup H had an increased intrinsic mitochondrial function when complex I and II linked substrates were used (maximally coupled OXPHOS capacity) compared with the two other haplogroups (Fig. 2B). No differences were present in intrinsic mitochondrial function with complex I linked substrates (Fig. 2A) as well as LEAK respiration (Fig. 2C). Respiratory Control Ratio (RCR) was not significantly different between the haplogroups (Fig. 2D), although there was a trend ($P = 0.102$) towards a higher RCR in haplogroup H, this trend was driven entirely by the higher intrinsic mitochondrial function with complex I + II linked substrates (haplogroup H). The CS activity was not different in the three haplogroups (85 ± 6, 69 ± 5 and 79 ± 6 µmol/g/min in haplogroup U, H and the composite group, respectively). When correlating CS activity and mitochondrial OXPHOS capacity from all 54 subjects a significant positive correlation was present ($P < 0.000$; $R = 0.618$) (Fig. 3). The same significant correlation was seen when the three different haplogroups (H: $P = 0.001$; $R = 0.794$; U: $P = 0.003$; $R = 0.609$; Composite: $P = 0.001$; $R = 0.672$) were analyzed separately. Correlating mitochondrial OXPHOS capacity with maximal oxygen uptake (l/min or l/min/kg) in the whole group (54 subjects) revealed a significant correlation ($P = 0.001$; R = 0.469 and $P = 0.001$; $R = 0.475$, respectively).

No differences were found in fiber type distribution between the haplogroups (type I: 42 ± 3; 47 ± 4; 46 ± 5 %; type IIA: 43 ± 4; 39 ± 3; 40 ± 4 %; type IIX: 15 ± 2; 13 ± 3; 14 ± 4 %, in U, H and composite haplogroups, respectively). Two subjects in haplogroup L (part of the composite group) had 81% type I fibers and only 2% type IIX fibers.

4. Discussion

The major and novel finding is the increased intrinsic mitochondrial function in haplogroup H compared with the haplogroup U and the composite haplogroup (Fig. 1). The influence of the mitochondrial haplogroup on the mitochondrial OXPHOS capacity has not previously been studied in human skeletal muscle fibers.

Haplogroup H has in other studies been attributed a beneficial trait in terms of survival after sepsis [9] and with respect to a high sperm motility [30], but in these an increased intrinsic mitochondrial function in H haplogroup was not observed [30]. Whether these beneficial observations in haplogroup H can be explained by differences in intrinsic mitochondrial function is hard to say, but need to be explored.

It is striking that this difference in mitochondrial OXPHOS capacity was measurable, given the fact that many mitochondrial functions are predominantly controlled by proteins encoded from the genes in the nuclear DNA, and only 13 proteins are encoded in the mitochondrial DNA. However these are essential subunits for the mitochondrial energy-generating enzymes of oxidative phosphorylation (OXPHOS). The finding of an increased intrinsic mitochondrial function in haplogroup H compared with the other groups could partly be explained by a non-significant difference in CS activity. A study in cybrids constructed from human platelets ($n = 5$ and 5 in haplogroup Uk and H, respectively) on a osteosarcoma 143B rho0 nuclear background [17], found no differences between the two haplogroups when data were expressed per cell, which is also in line with an earlier study [3]. But when the data were corrected for a surrogate measure of mitochondrial electron transport chain units, a significantly higher basal respiratory rate (on endogenous substrates), leak (4o) and uncoupled respiratory rate was found in the Uk compared with the H haplogroup, i.e. seemingly opposite to the present data. Several differences in design and methods exist between the present study and the aforementioned study [17]. While the same type of respirometer (Oroboros Instruments, Innsbruck, Austria) was used in both studies, in the present study we used permeabilized muscle fibers which has the advantage that the mitochondria are still located within an integrated cellular system, where the cytoskeleton is largely intact [18]. This approach was not used in studies of cybrids [17], and perhaps the OXPHOS system was not sufficiently tested in the cybrids. Next, we have used a validated [21] method for mitochondrial mass normalization of mitochondrial OXPHOS capacity. Finally, the conclusion was based upon samples from altogether ten human subjects [17] while in the present study we have used fifty-four human subjects.

In this study each of the groups characterized by their haplotype had a similar relative distribution of subjects with type 2 diabetes or normal glucose tolerance. This does not influence the data, because patients with type 2 diabetes do not differ from non-diabetic patients with respect to intrinsic mitochondrial function [12,20,22,27]. This is also supported by the findings in the present study, where no differences exist in intrinsic mitochondrial function between patients with type 2 diabetes and control subjects.

There was no significant difference in the fiber type distribution between groups, so potential fiber type specific differences in mitochondrial function cannot explain the difference in intrinsic mitochondrial function among the haplogroups. In this study we also aimed to study a link between maximal mitochondrial OXPHOS capacity and VO_{2max} to follow up on a previously published study [24] in which a higher VO_{2max} in haplogroup H compared to haplogroup J was reported. However, in the present study VO_{2max} in the subjects with haplogroup H was similar to haplogroup U and the composite group. If anything, the VO_{2max} tended to be lowest in the haplogroup H (Tab. 1). Overall there does not seem to be a consistent link between mitochondrial haplogroup and VO_{2max}. In this respect it is important to note that maximal oxygen uptake is influenced by many factors. Although several of these were controlled in this study, for example physical activity patterns and total lean mass, the influence of a specific haplogroup on VO_{2max} would not a priori be expected to be prominent. A correlation between mitochondrial OXPHOS capacity and maximal oxygen uptake was found in the present study.

In addition to a higher VO_{2max} in haplogroup H compared to haplogroup J mentioned above, Martínez-Redondo and colleagues also observed a higher mitochondrial oxidative damage in haplogroup H, which they speculated could be related to the reported functional differences in the amino acid variations in complex I between these two haplotypes [24,31]. This is in agreement with observations that complex I and III are the main sources for ROS production [13,19]. In the present study we did not measure oxidative damage and ROS production. The proportion of oxygen converted to ROS in the mitochondria range from 1% to 15% [1,6,25] and it is therefore tempting to suggest and speculate that the higher mitochondrial oxygen consumption (intrinsic mitochondrial function) in haplogroup H may at least in part be linked to a higher ROS production in this group. Our data do not support this speculation about a higher ROS production in haplogroup H. A trend for a

higher RCR was seen in haplogroup H in the present study, but this was entirely driven by a higher intrinsic mitochondrial function with complex I and II linked substrates (maximal state 3 respiration). Further studies are needed where ROS production is measured to investigate if the ROS production could explain these findings.

In summary, in this study we have found that intrinsic mitochondrial function in skeletal muscle is increased in humans with mitochondrial haplogroup H compared with haplogroup U and a composite haplogroup consisting of haplogroups T, J, I, L, W and HV. This study is the first to demonstrate this phenomenon in skeletal muscle mitochondria measured ex vivo. The higher intrinsic mitochondrial function in haplogroup H is not related to whole body maximal oxygen uptake rates.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2013.10.009.

Acknowledgments

We thank the healthy subjects and patients who volunteered to participate in this study. Regitze Kraunsøe and Jeppe Bach are thanked for expert technical assistance. The financial supports from The Nordea Foundation, The Novo Nordisk Foundation, Kathrine and Vigo Skovgaards Foundation, Aase and Ejnar Danielsens Foundation, Simon Fougner Hartmanns Foundation, and The Danish Council for Independent Research — Medical Sciences (271-07-0271) are gratefully acknowledged.

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