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REVIEW

Early adjustments in mitochondrial structure and function in skeletal muscle to high altitude: design and rationale of the first study from the Kilimanjaro Biobank

G. J. M. Stienen [1,2]

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Abstract

The physiological acclimatisation and adaptation processes in skeletal muscle at high altitude are of high medical and social relevance not only to understand limitations in physical performance at high altitude but also to understand the consequences of hypoxemia and tissue hypoxia in critically ill patients. Of particular importance in these processes are the alterations in content and function of mitochondria and myoglobin. The majority of studies on oxygen delivery to the tissues and utilisation by the cellular metabolism at high altitude were performed after prolonged stay at high altitude and in altitude-adapted highlanders. However, these studies do not provide insight in the sequence of events during the physiological acclimatisation and adaptation processes. Therefore, it is important to identify the *early* alterations in structure and function of the major determinants of the oxygen transport via myoglobin and oxygen utilisation by the mitochondria in skeletal muscle at high altitude. To achieve this goal, it is of interest to collect, analyse and compare quadriceps muscle biopsies and venous blood samples of climbers, guides and porters before and after climbing Mount Kilimanjaro and in participants of the Kilimanjaro Marathon before and after the run. The samples will be carefully documented and stored in the Kilimanjaro Biobank and will be made available to other research groups.

Keywords Muscle adaptation · Mitochondria · Myoglobin · Physical fitness · Age

Introduction

Each year, thousands of visitors from all over the world climb Mount Kilimanjaro (Fig. 1), the highest mountain of Africa (5895 m), or participate in the Kilimanjaro marathon (average altitude 1000 m above sea level). As such, the Mount Kilimanjaro not only represents a focal point in Tanzania of internationally orientated socio-economical activities but also offers unique research opportunities (Greene et al. 1981; De Mol et al. 2011; Van Adrichem et al. 2015; Lawrence and Reid 2016; Dekker et al. 2019). The location of Kilimanjaro Christian Medical Centre is ideally suited to conduct research on the medical aspects of ascending to high altitude, where oxygen pressure is reduced to approximately 50% of that at sea level. In contrast with high altitude research performed for instance in settlements or during prolonged stay in the Himalayas and in the Andes, Mount Kilimanjaro provides excellent opportunities to study the *early* events and triggers of the acclimatisation and adaptation processes in skeletal muscle at high altitude. We therefore will start to collect, analyse and compare quadriceps muscle biopsies and venous blood samples of climbers, guides and porters before and after climbing Mount Kilimanjaro and in participants of the Kilimanjaro Marathon before and after the run. The samples will be carefully documented and stored in the Kilimanjaro Biobank and will be made available to other research groups.

Background

The acclimatisation and adaptation processes in skeletal and cardiac muscle at high altitude are of high medical and social relevance not only to understand limitations in physical

✉ G. J. M. Stienen
gjm.stienen@icloud.com

[1] Department of Physiology, Kilimanjaro Christian Medical University College, PO Box 2240, Moshi, Tanzania

[2] Department of Physiology, Amsterdam UMC, Amsterdam Cardiovascular Sciences, Vrije Universiteit, Amsterdam, the Netherlands

Fig. 1 View on the snow-capped Mount Kilimanjaro in northern Tanzania from Kilimanjaro Christian Medical University College (inset). The peak on the left: Uhuru peak, altitude 5895 m above sea level; peak on the right: Mawenzi peak, altitude 5149 m above sea level. The new research building (finished in 2018) of the College is shown on the left side of the inset

performance at high altitude but also to understand the consequences of hypoxemia and tissue hypoxia in critically ill patients (Murray et al. 2018). Of particular importance in these acclimatisation and adaptation processes are the alterations in content and function of the mitochondria and of myoglobin. The mitochondria are the intracellular organelles where oxygen is taken up in order to provide chemical energy (ATP) required for proper function of the muscle cells and perform external work. Myoglobin is required for adequate transport of oxygen from the capillary vessels to the intermyofibrillar and perinuclear mitochondria in muscle cells.

Studies on skeletal muscle during the last 30 years have provided evidence that at high altitude mitochondrial volume density is reduced, in particular of subsarcolemmal mitochondria, and that changes occur in the mitochondrial oxidative enzyme activity and protein expression in climbers returning from extreme altitude (> 5500 m) (Hoppeler et al. 1990; Levett et al. 2012; Murray and Horscroft 2016). The changes in myoglobin expression at high altitude in humans are less clear: expression levels were maintained in climbers returning from the summit of the Everest (Levett et al. 2015) but were found to be decreased after 7–9 days at 4559 m, along with a down regulation of other iron-related proteins, possibly to support the erythropoietic response (Robach et al. 2007). In addition, muscle proteomic studies have revealed a variety of changes (Viganò et al. 2008; Cerretelli et al. 2009; Flueck 2009; Chicco et al. 2018) and a recent study on white blood cells provided insights in the early temporal regulation of transcription factors, inflammatory state and ROS homeostasis in the human hypoxic response (Malacrida et al. 2019).

It should be noted that the majority of studies indicating alterations in oxygen delivery to the tissues and utilisation by the cellular metabolism at high altitude were performed after prolonged stay at high altitude and in altitude-adapted highlanders, subject to natural selection over thousands of years. These studies have provided important insights in the regulatory role of the hypoxia-inducible factors (HIF-1α and HIF-2α), the mitochondrial biogenesis factor (PGC-1α) and the transcriptional regulator of fatty acid metabolism, peroxisome proliferator-activated receptor alpha (PPARα) (Simonson et al. 2010; Murray et al. 2018) and—amongst others—the EPAS1 haplotype (Ge et al. 2012). However, surprisingly little is known on the initial cause-and-effect relationship of the acclimatisation and adaptation processes in skeletal and cardiac muscle at high altitude.

Our previous studies in skeletal and cardiac muscle have provided insight in the alterations in mitochondrial structure and function, in particular with respect to the spatial organisation of the intermyofibrillar mitochondria relative to the calcium release units, mitochondrial plasticity (mitochondrial fusion and fission) and in calcium handling by the mitochondria via the mitochondrial calcium uniporter (MCU) and the mitochondrial sodium calcium exchanger (mNCE) (Canato et al. 2010; Scorzeto et al. 2013; Fowler et al. 2015; Wüst et al. 2016, 2017; Baartscheer et al. 2017; van den Berg et al. 2017; Wüst and Stienen 2018; Marcucci et al. 2018; Miranda-Silva et al. 2020). The aim of the current project will be to extend these previous studies and address important aspects of mitochondrial plasticity and dynamics in health and disease.

Objectives

The general objective is to characterise the early alterations in structure and function of the major determinants of the oxygen

transport via myoglobin and oxygen utilisation by the mitochondria in skeletal muscle at high altitude. It is anticipated that the results of this study will provide insight into the limitations of physical performance at high altitude and in the consequences of hypoxemia and tissue hypoxia in critically ill patients.

The specific objectives are to answer the following research questions:

1. Do early alterations in expression and function occur of proteins involved in mitochondrial fusion (Mfn-1 and Mfn-2), mitochondrial fission (dynamin-related proteins, DRP1 and OPA1), mitochondrial biogenesis (PGC-1α), the MCU and its regulators and the mNCE?
2. Do early alterations in expression and function occur in tissue myoglobin, blood haemoglobin and tissue and blood iron status?
3. What are the changes in expression and activity of citrate synthase and the components in the mitochondrial Electron Transfer System, including complexes I–V and the respiratory supercomplexes?
4. Are these precursor effects in agreement with the long-term changes observed in mitochondrial structure and function after acclimatisation and adaptation at high altitude?
5. How do these early alterations depend on physical fitness or training level and age of the individuals?

Rationale of the study

To address these questions, we will analyse and compare quadriceps muscle biopsies and venous blood samples of climbers, guides and porters *before* and *after* climbing Mount Kilimanjaro (peak 5895 m) in Tanzania. In these subjects, the effects of age and physical fitness/training level will be assessed. In addition, to assess the impact of altitude on physical performance during exercise, we will collect skeletal muscle biopsies, blood samples and measures of physical fitness, training level and markers of cardiac and skeletal muscle damage of participants *before* and *after* the Kilimanjaro Marathon (~ 1000 m). These later samples will be used in conjunction with data collected during marathons near or below sea level in the Netherlands.

It is our ambition to build at the Kilimanjaro Christian Medical Centre an internationally oriented base of expertise and knowledge on acclimatisation and adaptation at high altitude and on the effects of hypoxemia and tissue hypoxia in critically ill patients. The Kilimanjaro Biobank will be a specialised Biobank collecting samples according to generally accepted standards (Kauffmann and Cambon-Thomsen 2008; Hewitt and Watson 2013; Lal et al. 2015; Harati et al. 2019).

Methods

Subjects

In 90 healthy volunteers (60 climbers, 15 guides and 15 porters), exercise tests will be performed on a bicycle ergometer and blood samples and muscle biopsies will be obtained before and after climbing Kilimanjaro Mountain. In addition, 60 participants will be enrolled in the Kilimanjaro Marathon part of the study, in which the same measurements will be performed before and after the Kilimanjaro Marathon. We will actively approach tour organisers, travel agencies and our local contacts to bring our study under the attention of potential participants.

Ethical considerations and group sizes

Healthy subjects will be included in the study after written informed consent according to the "Guidelines on Ethics for Health research" in Tanzania (ISBN 9087675018). Several groups (fitness, age) will be studied at two time-points, before and after the climb/marathon. Therefore, the study contains cross-sectional as well as longitudinal elements. A power calculation indicates that the required number of participants per group (n) is equal to:

$$n = \frac{2\left(Z_{\alpha} + Z_{1-\beta}\right)^2 \sigma^2}{\Delta^2} = 12.4,$$

for $P < 0.05$ (single-sided, $Z_{\alpha} = 1.65$), a power of 80%, ($Z_{1\text{-}\beta} = 0.816$), a standard deviation (σ) of 0.1 and an effect size (Δ) of 0.1. Considering a drop-out of 20% based on previous experience, $n = 15$.

Hence, 15 subjects per group are required to ascertain whether meaningful differences are present in climbers before and after the Kilimanjaro climb and participants of the Kilimanjaro marathon (repeated measures). To assess the effect of age and fitness, 4 subgroups will be formed of 15 participants each: young (Y) < 40 years; old (O) ≥ 40 years; exercise/training intensity 4 months before the climb: low (L) < 4 h/week; high (H) ≥ 4 h/week or before the run < 40 km/week (L) and ≥ 40 km/week (H). Hence, the groups are defined as YL, YH, OL and OH. Subjects will be enrolled in each of the groups in a random fashion, until the groups are filled. The data from guides and porters will be used to allow comparison between climbers, guides and porters.

Inclusion and exclusion criteria

Inclusion criteria are the following: physically able, healthy subjects with an age range of 16–80 years and a body mass index (BMI) of 20–30 kg/m^2 who have not undergone major medical treatment or have been systematically using

medication within the last 5 years prior to the study. Exclusion criteria are the following: subjects with physical disability or a history of chronic disease (e.g. HIV; metabolic syndrome or underweight; cancer; cardiovascular, respiratory or renal disease).

Physical performance and training level

Maximum power output is as follows: 1-s peak power will be obtained during a 30-s Wingate test; blood lactate concentration will be obtained after the exercise from the fingertip; endurance performance will be obtained from the average power output during a 15-km time trial; physical performance will be normalised to lean body mass$^{2/3}$; fat mass estimated from 4 skin folds; maximal isometric knee-extension torque will be determined using a custom-made dynamometer (van der Zwaard et al. 2018). Training level will be assessed by means of a questionnaire on the physical/training activity in the 4 months prior to the climb and marathon.

Maximum oxygen consumption ($\dot{V}O_{2max}$) and lactate thresholds

$\dot{V}O_{2max}$ and lactate thresholds will be determined during the 15-km time trial and/or during a maximal incremental exercise test on a bicycle ergometer using open circuit spirometry (van der Zwaard et al. 2018).

Blood sampling

Resting blood samples will be collected in EDTA-coated tubes by venepuncture and analysed for red blood cells, haemoglobin concentration, haematocrit, iron status and mean corpuscular volume (Ruiter et al. 2015; van der Zwaard et al. 2018). Blood samples taken during and after exercise will be used to determine lactate content.

Skeletal muscle biopsies

This study had been approved by the College Research Ethics and Review Committee (CRERC) of the Kilimanjaro Christian University College (no. 2349). After written informed consent, biopsies will be obtained under local anaesthesia (Lidocaine) from quadriceps muscle using a 16-gauge Cook Medical or Bard Magnum Biopsy system (microbiopsies ~ 50 mg wet weight) or using a Bergstrom needle (~ 200 mg wet weight) (Hayot et al. 2005; Manders et al. 2015). Biopsy samples will be aligned to muscle fibre arrangement, frozen in liquid nitrogen, cut in 10-μm-thick sections using a cryostat at − 20 °C and collected on polylysine-coated slides and stored at − 80 °C prior to the analyses described below.

Protein and mRNA analyses

Myosin isoform composition, content and activity of mitochondrial complexes I–V and supercomplexes thereof as well as the expression of Mfn-1 and Mfn-2, DRP1 and OPA1 will be determined by SDS-PAGE, Blue-Native Gel Electrophoresis and Western immunoblotting. Measurements of the maximal enzymatic activities of complexes I, II, III and IV and citrate synthase will be performed using Konelab 20XT (Thermo Fisher, Breda, the Netherlands) as described previously (Stienen et al. 1996; Wüst et al. 2016). Total RNA will be extracted using a RiboPure kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR will be performed by means of a StepOne Real-Time PCR system (Applied Biosystems) to determine mRNA expression levels using commercially available primers.

Quantitative histochemistry

Fibre-type composition of the biopsies will be determined by immunofluorescence using appropriate antibodies. Mitochondrial oxidative capacity and distribution will be determined by succinate dehydrogenase (SDH) activity using quantitative histochemistry (Bekedam et al. 2003). Capillarisation will be determined by UEA staining (van der Zwaard et al. 2018). The myoglobin concentration will be determined as described previously (van Beek-Harmsen et al. 2004).

Electron microscopy

The subcellular localisation, distribution, size and volume fraction of the mitochondria will be determined by electron microscopy as described previously (Scorzeto et al. 2013).

Data collection and analysis

The processed blood samples and muscle biopsies will be transported to the collaborating laboratories for analysis. Data collection and repeated measures analysis (ANOVA) of the muscle biopsies will be performed in close collaboration with the participants abroad. A 3-way ANOVA with the following factors will be used: climb/run, age, fitness/training level. Difference with a P value of < 0.05 will be considered significant.

Limitations of the study

A limitation of this study is that it is difficult to distinguish between the effects of altitude and activity during the climb/run. However, the climbers, porters and guides are generally

well prepared and the level of activity during the climb will be similar to the prior (training) activity. Moreover, inclusion of subjects with different degrees of fitness will allow us to gain insight in the impact of the level of activity/training on the magnitude of the changes in mitochondrial structure and function. Another limitation is that it is a priori difficult to assess the potential clinical relevance of the study for critically ill patients. However, we expect that the findings will provide important mechanistic insight in the consequences of hypoxemia and tissue hypoxia in critically ill patients and thus may lead to novel treatment options.

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Compliance with ethical standards

Conflict of interest The author declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent will be obtained from all individual participants to be included in the study.

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