METABOLIC BASIS TO SHERPA ALTITUDE ADAPTATION

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Abstract

The Himalayan Sherpas, a human population of Tibetan descent, are highly adapted to life in the hypobaric hypoxia of high altitude. Mechanisms involving enhanced tissue oxygen *delivery* in comparison with Lowlander populations, have been postulated to play a role in such adaptation. Whether differences in tissue oxygen *utilization* (i.e. metabolic adaptation) underpin this adaptation is not however known. We sought to address this issue, applying parallel molecular, biochemical, physiological and genetic approaches to the study of Sherpas and native Lowlanders, studied before and during exposure to hypobaric hypoxia on a gradual ascent to Mount Everest Base Camp (5,300 m). When compared with Lowlanders, Sherpas demonstrated a lower capacity for fatty acid oxidation in skeletal muscle biopsies, along with enhanced efficiency of oxygen utilization, improved muscle energetics and protection against oxidative stress. This in part appeared to be related to a putatively advantageous allele for the *PPARA* gene, which was enriched in the Sherpas compared with the Lowlanders. Our findings suggest that metabolic adaptations underpin human evolution to life at high altitude, and could impact upon our understanding of human diseases in which hypoxia is a feature.

186 words (250 max)

Significance Statement

A relative fall in tissue oxygen levels (hypoxia) is a common feature of many human diseases including heart failure, lung diseases, anemia and many cancers, and can compromise normal cellular function. Hypoxia also occurs in healthy humans at high altitude due to low barometric pressures. Human populations resident at high altitude in the Himalayas have evolved mechanisms that allow them to survive and perform, including adaptations that preserve oxygen delivery to the tissues. Here we studied one such population, the Sherpas, and found metabolic adaptations, underpinned by genetic differences, which allow their tissues to use oxygen more efficiently, thereby conserving muscle energy levels at high altitude, and possibly contributing to the superior performance of elite climbing Sherpas at extreme altitudes.

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Introduction

At high altitude, low barometric pressure is accompanied by a fall in the partial pressure of inspired O_2, resulting in *hypobaric hypoxia*. The cellular response to hypoxia is orchestrated by the Hypoxia Inducible Factor (HIF) transcription factors, with HIF-1α and HIF-2α respectively mediating responses to short-term and more sustained hypoxia (1). In normoxia, prolyl-hydroxylases target HIFα subunits for destruction (2). Under low O_2 partial pressures, however, HIF-1α/HIF-2α are stabilized and dimerize with the nuclear HIF-1β subunit. This dimer interacts with hypoxia-response elements in promoter regions to increase expression of specific genes, e.g. *EPO* (encoding erythropoietin) and *VEGFA* (vascular endothelial growth factor A) (3).

The Tibetan Plateau has an average altitude of some 4,500 m. Humans were first present on the Plateau ~30,000 years ago, with the earliest permanent settlements appearing 6-9,000 years ago (4) – a period sufficient to drive the natural selection of genetic variants (and associated features) favouring survival and performance in sustained hypoxia (5, 6). Evidence supports the selection of genetic variants encoding components of the hypoxia-inducible factor (HIF) pathway, such as *EPAS1* (encoding HIF-2α) (7) and *EGLN1* (prolyl-hydroxylase-2, PHD2) (8) in Tibetan populations. One population, the Sherpas, migrated from Tibet to eastern Nepal ~500 years ago and exhibit remarkable physical performance at extreme altitude (9).

Whilst the human adaptive response to hypoxia is incompletely understood, mitigation against the fall in convective O_2 delivery plays an important role. In Lowlanders, increased ventilation and cardiac output, and the production of more O_2-carrying red blood cells help to sustain O_2 delivery

and content (10, 11). Likewise, exhaled concentrations of nitric oxide (NO), a key regulator of blood flow, are higher in Tibetans than Lowlanders (12), as are circulating NO metabolites and limb blood flow (13). The rise in red cell mass in response to hypobaric hypoxia is not as great in Tibetans as in Lowlanders, however (14, 15), suggesting that adaptation involves more than just increased O_2 delivery. In fact, acclimatization also involves alterations in O_2 *use*. In Lowlander muscle, mitochondrial density declines with sustained exposure to extreme altitude (16-18), whilst exposure to more moderate high altitude is associated with a reprogramming of muscle metabolism (19) even without altered mitochondrial density (20), including downregulation of electron transfer complexes (19) and tricarboxylic acid (TCA) cycle enzymes (21), loss of fatty acid oxidation (FAO) capacity (19, 20) and improved oxidative phosphorylation coupling efficiency (20). Sherpas have lower muscle mitochondrial densities than unacclimatized Lowlanders (22), but little is known of their metabolic adaptation to hypoxia, or any genetic selection which might underpin it. A role has been suggested for peroxisome proliferator-activated receptor alpha (PPARα), a transcriptional regulator of FAO in liver, heart and muscle. HIF downregulates PPARα in some tissues (23), whilst there is evidence for selection of variants in its encoding gene (*PPARA*) in some Tibetan subgroups (8, 24). We hypothesized that metabolic adaptation, and PPARα in particular, play a central role in the Sherpa adaptation to hypobaric hypoxia.

Results and Discussion

Selection of *PPARA* Variants in Sherpas

Lowlander and Sherpa subjects were participants of the research expedition, Xtreme Everest 2 (25). The Lowlanders comprised 10 investigators selected to operate the Everest Base Camp (EBC) laboratory. Sherpas (n = 15) were a sex-matched (73% male, *cf.* 70% in Lowlanders) and age-matched (26.8 ± 1.2 yr, *cf.* 28.0 ± 1.6 yr in Lowlanders) group living in Kathmandu and the Solukhumbu and Rolwaling valleys. No subject ascended higher than 4,200 m in the 3 months preceding the trek, nor above 2,500 m in the preceding 3 weeks. In addition, Sherpas presented evidence of sole Sherpa ancestry for 2 generations (i.e. 4 Sherpa grandparents). The frequency of putatively advantageous *PPARA* alleles (8) was higher in Sherpas than Lowlanders (Fig. 1A; Table S1), with genotype frequencies of the cohorts being significantly different at 2 single nucleotide polymorphisms (SNPs), rs6520015 and rs7292407 (P = 0.0091), though not rs9627403. This reflected patterns reported in some other Tibetan groups (26).

Muscle Hypoxia and Circulating NO Metabolites

Baseline testing, including blood sampling, muscle biopsy sampling, high-resolution respirometry of permeabilized muscle fibers and oral glucose tolerance tests (OGTT) took place in London (35 m) for Lowlanders and Kathmandu (1,300 m) for Sherpas (25). All subjects then followed an identical ascent (Fig. 1B) from Kathmandu to EBC (5,300 m) whereupon further testing took place at an early timepoint (A1; 15-20 d post-departure for Lowlanders, 11-12 d for Sherpas), and a late timepoint (A2; 54-59 d post-departure) for Lowlanders only. At the time of sampling, both groups had passed through the acute phase of hypoxic exposure (<24 h) (1) and had been sufficiently exposed to chronic hypoxia for acclimatization to have occurred. Indeed, arterial hemoglobin-O_2 saturations

were similarly low in both groups (Fig. 1C), whilst muscle expression of the HIF-target *VEGFA* increased in all subjects (Fig. 1D), indicating a molecular response to hypoxia. Following measurements at A1, the Lowlanders remained at EBC for 2 months to carry out research, presenting an opportunity to collect data pertaining to longer-term metabolic acclimatization. Interestingly, *VEGFA* expression was no longer elevated by this timepoint, suggesting further acclimatization had occurred.

To our surprise, there were no differences in circulating N-nitrosamine (RNNO), S-nitrosothiol (RSNO), nitrate (NO_3^-) or nitrite (NO_2^-) concentrations between Lowlanders and Sherpas at baseline (Fig. 1E-H). In Lowlanders, a transient increase in plasma RNNO levels occurred upon arrival at EBC ($P < 0.05$) but disappeared by the later timepoint (Fig. 1E). In Sherpas, plasma nitrate levels fell at altitude ($P < 0.05$; Fig. 1G) and nitrite levels increased ($P < 0.05$; Fig. 1H), whilst in Lowlanders nitrite levels fell by the later timepoint ($P < 0.05$). The absence of large differences in NO metabolites between the groups at baseline or at altitude, suggested an adaptive phenotype in Sherpas that is distinct from other Tibetan highlanders (13).

Lower Fatty Acid Oxidation Capacity in Sherpas

Skeletal muscle biopsies revealed marked differences in gene expression and FAO capacity between Sherpas and Lowlanders. Expression of *PPARA* mRNA was 48% lower in Sherpas than Lowlanders ($P < 0.05$; Fig. 2A), thus the putatively advantageous *PPARA* allele is associated with diminished expression. Correspondingly, expression of the PPARα target *CPT1B* was 32% lower in Sherpas at baseline compared with Lowlanders ($P < 0.05$; Fig. 2B). The *PPARA* gene contains 139 SNPs. rs6520015 is one of the tagging SNPs reported by Simonson *et al* (8), however it appears to be a

non-coding variant. It is thus uncertain whether the SNP itself affects transcriptional regulation, or whether it tags a functional variant elsewhere, modifying expression or mRNA stability. Ascent to EBC did not alter *PPARA* expression in either group, yet despite this *CPT1B* expression decreased by 44% in Lowlanders ($P < 0.05$) but did not decrease further in Sherpas. This suggests that the Lowlander response to hypoxia involves decreased PPARα transcriptional activity without changes in *PPARA* expression, similar to hypoxic rat skeletal muscle (27).

Gene expression changes do not necessarily reflect protein levels or activity, therefore we measured activity of the β-oxidation enzyme 3-hydroxyacyl-CoA dehydrogenase (HADH), finding it to be 27% lower in Sherpas than Lowlanders at baseline ($P < 0.05$), and not changing in either group following ascent (Fig. 2C). Moreover, fatty acid oxidative phosphorylation capacity (FAO_P) was measured as the oxygen flux in saponin-permeabilized muscle fibers with octanoylcarnitine, malate and ADP, using high-resolution respirometry (28). FAO_P was 24% lower in Sherpas than Lowlanders at baseline ($P < 0.01$), and did not change in either group following ascent (Fig. 2D, Fig. S1). *Ex vivo* measurements may be particular to assay conditions used, therefore we also measured muscle metabolite levels to indicate changes in metabolism *in vivo*. Total carnitine concentrations decreased in Lowlanders with time spent at EBC ($P < 0.05$), though were not significantly different to those in Sherpas at baseline (Fig. 2E). The ratio of long chain acylcarnitines to total carnitines, however, increased in Lowlanders with time at altitude ($P < 0.05$; Fig. 2F), suggesting incomplete FAO results in accumulation of potentially-harmful lipid intermediates (29). In Sherpa muscle, however, the long chain acylcarnitine to total carnitine ratio was lower than in Lowlanders at baseline ($P < 0.05$), perhaps resulting from lower expression of CPT-1. In further contrast with Lowlanders, the long chain acylcarnitine to total carnitine ratio remained low in Sherpa muscle at altitude.

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179 **TCA Cycle Regulation at High Altitude**

180 We therefore sought to understand whether there were differences between the populations in
181 other aspects of mitochondrial metabolism. The TCA cycle enzyme citrate synthase (CS) is a
182 candidate marker of mitochondrial content in human muscle (30). At baseline, Sherpas had a 26%
183 lower muscle CS activity than Lowlanders ($P < 0.05$; Fig. 3A), in agreement with findings of 17-33%
184 lower mitochondrial volume density in Sherpa *vastus lateralis* compared with Lowlanders (22). In
185 accordance with lower CS activity, concentrations of 6- and 5-carbon intermediates downstream of
186 CS (citrate, aconitate, isocitrate, α-ketoglutarate) were lower in Sherpas than Lowlanders ($P <$
187 0.001). However, concentrations of 4-carbon intermediates (succinate, fumarate, malate,
188 oxaloacetate) were not different (Fig 3B-I). This suggests an alternative strategy to supply the TCA
189 cycle with succinate. Intriguingly, recent analysis of a large SNP dataset from low and high altitude-
190 adapted populations in the Americas and Asia (31) aimed to identify pathways of convergent
191 evolution, and highlighted fatty acid ω-oxidation as the most significant cluster of overlapping gene
192 sets between high altitude groups (32). ω-oxidation, is normally a minor pathway in vertebrates,
193 becoming more important when β-oxidation is defective (33), and through successive cycles oxidizes
194 fatty acids to adipate and succinate in the endoplasmic reticulum, after which succinate enters the
195 mitochondria with anaplerotic regulation of the TCA cycle (34).

196

197 Upon ascent to altitude, 6- and 5-carbon TCA cycle intermediates increased in Sherpa muscle ($P <$
198 0.05; Fig. 3B-E), suggesting improved coupling of intermediary metabolism, TCA cycle and oxidative
199 phosphorylation. In Lowlanders, however, citrate, aconitate and isocitrate decreased at altitude (P
200 < 0.05; Fig. 3B-D), despite no significant change in CS activity, perhaps reflecting impairments

upstream. Interestingly, α-ketoglutarate concentrations were maintained in Lowlanders at altitude (Fig. 3E), despite decreased succinate downstream, which could be explained by the fall in both α-ketoglutarate dehydrogenase and isocitrate dehydrogenase, reported previously in Lowlanders following an identical ascent to EBC (21). α-ketoglutarate plays regulatory roles in hypoxia, including a suppression of HIF stabilization (35), but also supporting glutathione synthesis (36). Taken together, these results indicate different TCA cycle regulation in Sherpas and Lowlanders. The replete TCA cycle of Sherpas at altitude contrasts sharply with the depletion of TCA cycle intermediates in Lowlanders, and suggests a coupling of the TCA cycle in Sherpa muscle to their distinct intermediary substrate metabolism.

Greater Mitochondrial Coupling Efficiency in Sherpas

To further understand whether mitochondrial function differs between Sherpas and Lowlanders, we used high-resolution respirometry, to probe electron transfer system (ETS) capacity and coupling efficiency in permeabilized muscle fibers. At baseline, there was no significant difference between the two groups in OXPHOS or ETS capacities with either malate and glutamate (N-pathway through Complex I) or succinate as substrates (S-pathway through Complex II; Fig. 4A,B; Fig. S2), but Sherpas had a lower OXPHOS capacity with malate, glutamate and succinate combined to reconstitute TCA cycle function (NS-pathway; $P < 0.01$; Fig. 4C). There were no early changes in either group upon ascent. By the later timepoint however, succinate-linked respiration had fallen in Lowlanders ($P < 0.05$), consistent with previous findings of decreased succinate dehydrogenase (Complex II) levels in subjects with sustained exposure >5,300 m (21).

In addition, we measured muscle fiber respiration in the absence of ADP (LEAK), i.e. O_2 consumption without ADP phosphorylation. Expressing LEAK relative to OXPHOS capacity, it is possible to calculate OXPHOS coupling efficiency (37, 38). At baseline, Sherpa muscle mitochondria had lower LEAK respiration and greater coupling efficiency than Lowlander mitochondria ($P < 0.001$; Fig. 4D,E), indicating more efficient use of O_2. Upon ascent to EBC and with sustained time at altitude, LEAK decreased in Lowlanders ($P < 0.01$), though it remained higher than in Sherpas (Fig. 4D), and coupling efficiency improved ($P < 0.05$; Fig. 4E). In Sherpas at altitude, LEAK did not change although coupling efficiency decreased ($P < 0.01$). One possible explanation for these differences in coupling efficiency might be the altered expression of uncoupling protein 3 (UCP3). *UCP3* is a transcriptional target of PPARα and lower UCP3 levels at altitude might improve the efficiency of O_2 utilization. In previous studies, however, muscle UCP3 expression increased with acute hypoxia (17, 39), which may offer some protective benefit considering its possible role as an antioxidant (39). Notably though, UCP3 levels decreased with more sustained exposure to extreme altitude (17). Here, *UCP3* was upregulated in Sherpas at altitude in association with decreased coupling efficiency ($P < 0.05$; Fig. 4F). However, *UCP3* expression also increased in Lowlanders in the short-term ($P < 0.01$) in whom there was decreased LEAK respiration. Moreover, *UCP3* expression returned to baseline in Lowlanders with longer-term exposure with no further change in LEAK respiration. Overall, our results indicate that Sherpa muscle mitochondria are characterized by a lower OXPHOS capacity and greater, albeit declining, efficiency, whilst in Lowlanders OXPHOS efficiency improved with acclimatization.

Glycolysis and Glucose Metabolism

Next we investigated the capacity to derive cellular energy via glycolysis, which is increased in hypoxic cells (40), as this may allow ATP levels to be maintained when O_2 is limited. Hexokinase activity was the same in both groups at baseline, and did not change at altitude (Fig. 5A), however lactate dehydrogenase (LDH) activity was 48% higher in Sherpa muscle than in Lowlanders ($P < 0.05$), indicating greater capacity for anaerobic lactate production (Fig. 5B). Fasting blood glucose was the same in Sherpas and Lowlanders at baseline, and decreased upon ascent in Lowlanders ($P < 0.01$; Fig. 5C), who also showed faster clearance of glucose during an OGTT ($P < 0.001$; Fig. 5D) in agreement with previous reports (41). In Sherpas, however, there was no indication of altered glucose homeostasis. Meanwhile, over time at altitude glycolytic intermediates increased in Lowlander muscle (Fig. 5E) with increased glucose-6-phosphate/fructose-6-phosphate and 2-phosphoglycerate/3-phosphoglycerate (Table S2). In contrast, total glycolytic intermediates did not change in Sherpa muscle, although 2-phosphoglycerate/3-phosphoglycerate decreased. These findings, might to some extent be explained by altered HIF activities. Many genes encoding glycolytic enzymes are upregulated by HIF-1 (42), whilst hypoglycemia is seen in Chuvash polycythemia, an autosomal recessive disorder in which HIF degradation is impaired (43). Taken together, our findings suggest an increased reliance on glucose by Lowlanders under resting conditions at altitude compared with Sherpas, but a greater capacity for lactate production in Sherpas which may prove effective upon exertion.

Energetics and Oxidative Stress

Finally, to understand the implications of Sherpa metabolic adaptation we investigated muscle energetics and redox homeostasis. Lowlanders at altitude showed progressive loss of muscle phosphocreatine (PCr; $P < 0.001$; Fig. 6A), indicating a loss of energetic reserve, which may relate to

downregulation of muscle creatine kinase, as reported previously (21). By contrast, in Sherpa muscle, PCr increased at altitude ($P < 0.01$). Similarly, Sherpa muscle ATP levels, which were lower than in Lowlanders at baseline ($P < 0.05$), increased at altitude ($P < 0.001$; Fig. 6B), illustrating that Sherpa metabolism is better suited to maintaining muscle energetics at altitude than Lowlander metabolism in either the short-term or following acclimatization. Moreover, with short-term exposure, markers of oxidative stress (reduced/oxidized glutathione and methionine sulfoxide) increased in Lowlander muscle, but not Sherpa muscle (Fig. 6C,D), indicating superior redox homeostasis in the Sherpas. Antioxidant protection may represent another outcome of convergent evolution, having been reported in Andean subjects in association with protection of fetal growth (44), whilst glutathione levels are raised in Chuvash polycythemia suggesting a possible role for HIF activation (45).

Conclusions

It has long been suspected that Sherpa people are better adapted to life at high altitude than Lowlanders (46). Recent findings have suggested a genetic basis to adaptation in populations around the world (6), and here we show that Sherpas have a metabolic adaptation associated with improved muscle energetics and protection against oxidative stress. Genetic selection on the *PPARA* gene is associated with decreased expression, and thus lower fatty acid β-oxidation and improved mitochondrial coupling compared with Lowlanders, with a possible compensatory increase in fatty acid ω-oxidation. Sherpas also have a greater capacity for lactate production. With acclimatization to altitude, Lowlanders accumulate potentially-harmful lipid intermediates in muscle as a result of incomplete β-oxidation, alongside depletion of TCA cycle intermediates, accumulation of glycolytic intermediates, a loss of PCr despite improved mitochondrial coupling, and a transient increase in

292 oxidative stress markers. In Sherpas, however, there are remarkably few changes in intermediary
293 metabolism at altitude, but increased TCA cycle intermediates and PCr and ATP levels, with no sign
294 of oxidative stress.
295
296 Genetic selection, by definition, requires an increased likelihood of advantageous gene variants
297 being passed on to offspring. This might occur if the disadvantageous variant is associated with
298 poorer survival to reproductive age and beyond, including greater fetal/neonatal mortality.
299 Evidence supports precisely such effects with fetal growth at altitude being poorer in Lowlander
300 populations than many native highlanders (47), including Tibetans (48) and Sherpas (49). Likewise,
301 gene variants may affect survival through childhood or fecundity/fertility in the hypoxic
302 environment. We cannot speculate on the mechanism by which *PPARA* variants prove
303 advantageous, however PPAR isoforms are expressed in the placenta (50) and influence female
304 reproductive function (51). It would be of interest to seek association of the *PPARA* variants with
305 birth weight and measures of placentation in high altitude natives and Lowlanders exposed to
306 hypoxia.
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308 Our findings suggest a metabolic basis to Sherpa adaptation, which may permit the population to
309 survive and perform at high altitude. Such adaptations may also underpin the superior performance
310 of elite climbing Sherpas at extreme high altitude.
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312 **Materials and Methods**
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314 Subjects were selected from the participants of Xtreme Everest 2 (25). All Lowlanders were born
315 and lived below 1,000 m, not descended from a high altitude-dwelling population and of European
316 (Caucasian) origin. Subjects gave written consent, and underwent medical screening. All protocols
317 were approved by UCL Research Ethics Committee and Nepal Health Research Council. Vastus
318 lateralis biopsies were taken from the mid-thigh, muscle fibers prepared for respirometry (28) and
319 respiration measured using substrate-uncoupler-inhibitor titrations (Tables S3, S4). Enzyme
320 activities were assayed as described (27). RNA was extracted and Taqman® assays used to analyse
321 gene expression (Table S5). For metabolite analysis, a methanol/chloroform extraction (52) was
322 followed by liquid chromatography mass spectrometry (LC-MS). OGTTs were carried out on fasted
323 subjects on the day after biopsies. Blood plasma NO metabolites were quantified as described
324 (53). Genomic DNA was isolated from whole blood and PPARA SNPs genotyped using TaqMan® for
325 allelic discrimination (Applied Biosystems, UK; Table S1). To compare cohorts at baseline, an
326 unpaired two-tailed Student's t-test was used (significance at $P \leq 0.05$). Genotype frequencies were
327 compared using a Chi-squared test. To assess the effects of altitude, a one-way ANOVA with
328 repeated measures was used. Post-hoc pairwise comparisons were carried out with a Tukey
329 correction.

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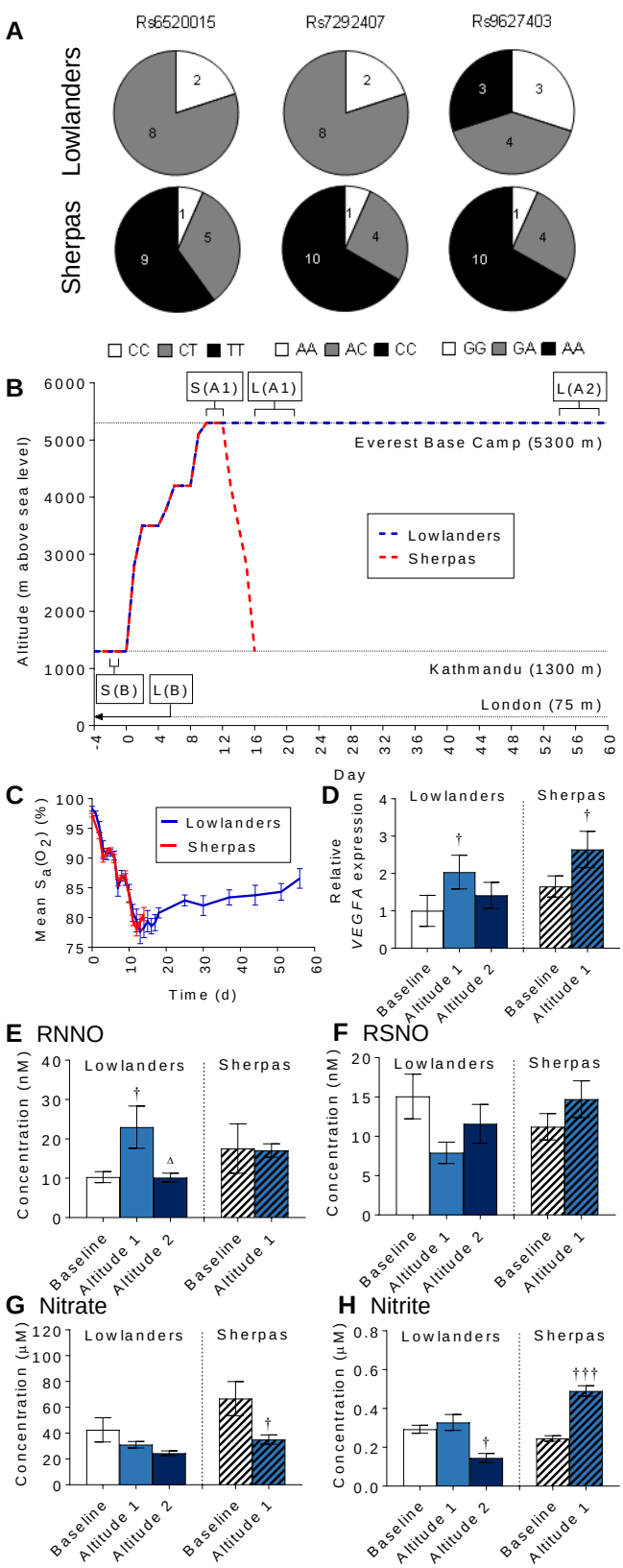
463 **Figure Legends**

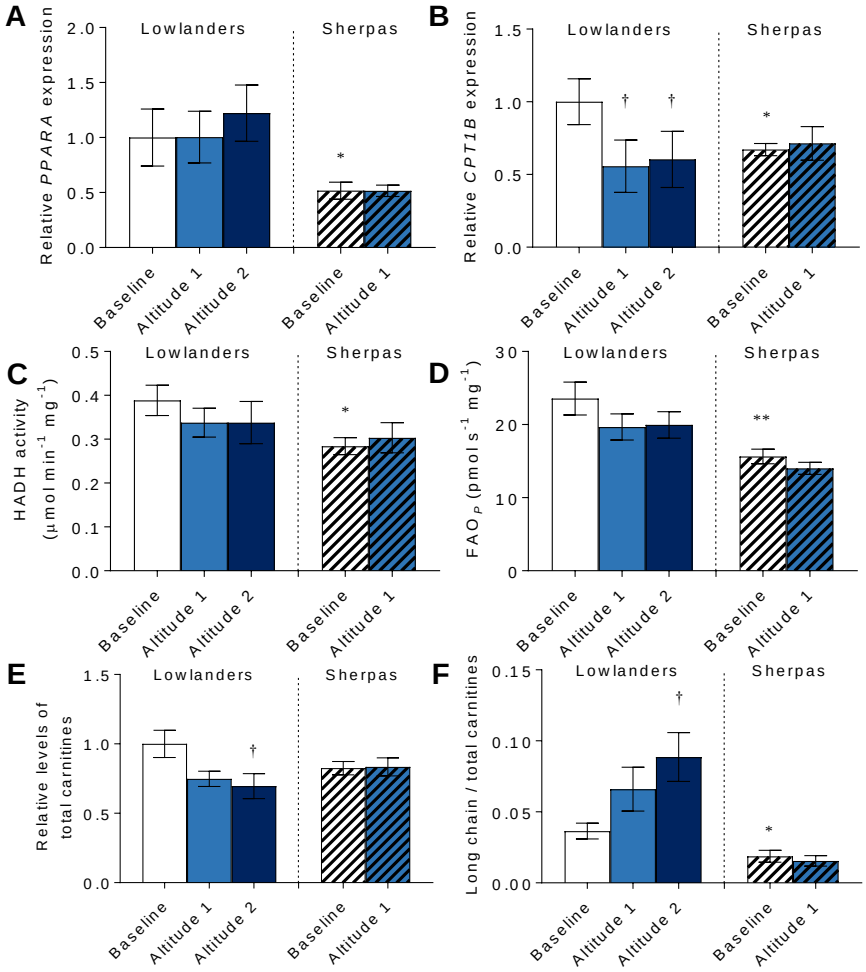
464 **Figure 1** Subject genetics, ascent profile, arterial blood O_2 saturation, muscle hypoxia and
465 circulating NO metabolites. A) Genotypes of Lowlanders and Sherpas at 3 *PPARA*
466 SNPs - subjects homozygous for the putatively advantageous allele in black,
467 heterozygous subjects in gray and subjects homozygous for the non-advantageous
468 allele in white (digits in segments refer to number of subjects with genotype); B)
469 Ascent profile including timing of biopsies; C) Arterial hemoglobin-O_2 saturations;
470 D) Muscle *VEGFA* expression, and E-H) plasma nitrogen oxides in Lowlanders (L) and
471 Sherpas (S) at baseline (B) and early (A1) and late (A2) altitude. Mean ± SEM (*n* = 4-
472 15). †*P* ≤ 0.05; †††*P* ≤ 0.001 B vs A1 within cohort. $^{\Delta}$*P* ≤ 0.05 A1 vs A2 within cohort.

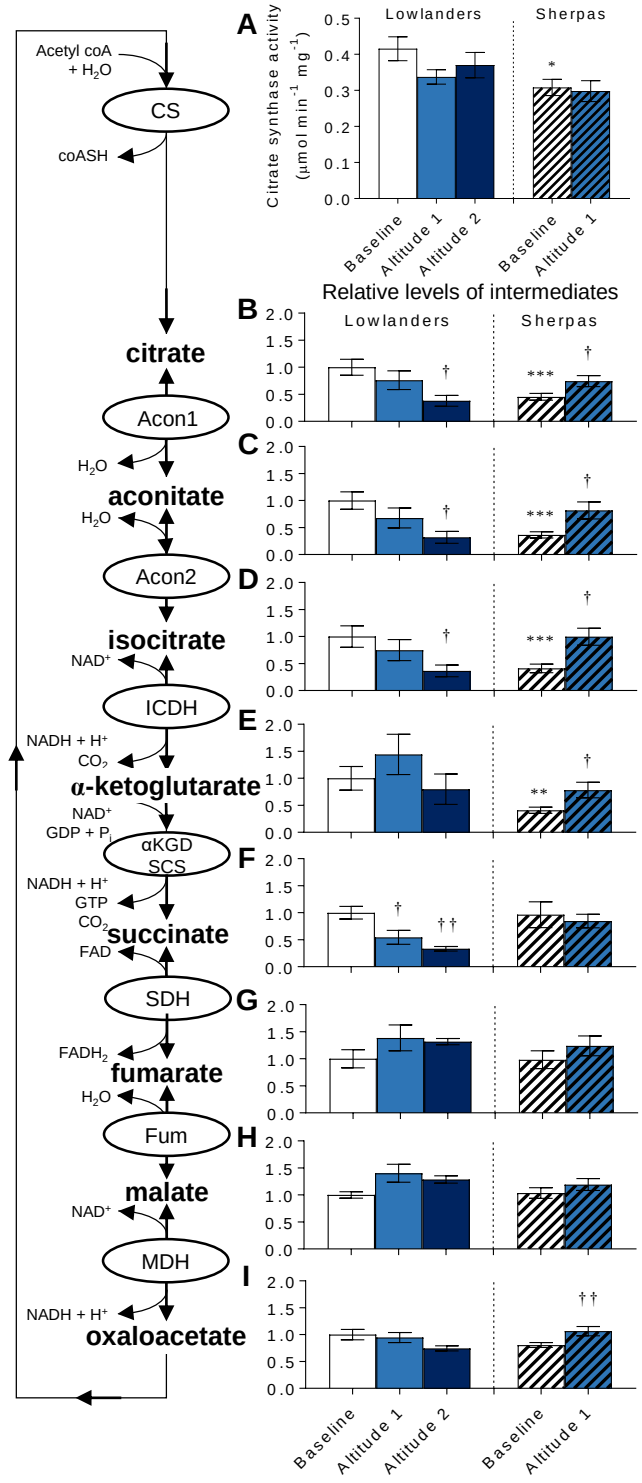
473 **Figure 2** Fatty acid oxidation and regulation in muscle. A) *PPARA* expression; B) *CPT1B*
474 expression; C) HADH activity; D) Oxidative phosphorylation with
475 octanoylcarnitine&malate (FAO_P); E) Total carnitine; F) Long chain/total carnitine
476 ratio in Lowlanders and Sherpas. Gene expression and carnitine levels are expressed
477 relative to Lowlanders at baseline. Mean ± SEM (n = 6-13). **P* ≤ 0.05; ***P* ≤ 0.01
478 Lowlanders vs Sherpas at baseline. †*P* ≤ 0.05 baseline vs altitude within cohort.
479
480 **Figure 3** TCA intermediates and activity in muscle. A) Citrate synthase activity and B-I) TCA
481 cycle intermediates in Lowlanders and Sherpas. Metabolite levels are expressed
482 relative to Lowlanders at baseline. Mean ± SEM (*n* = 7-14). **P* ≤ 0.05; ***P* ≤ 0.01;
483 ****P* ≤ 0.001 Lowlanders vs Sherpas at baseline. †*P* ≤ 0.05; ††*P* ≤ 0.01; baseline vs
484 altitude within cohort.
485
486 **Figure 4** Mitochondrial oxygen consumption, efficiency and uncoupling protein expression.
487 A) N-OXPHOS (GM_P), B) S-ETS capacity (S_E) and C) NS-OXPHOS capacity (GMS_P) in
488 permeabilized muscle fibers from Lowlanders and Sherpas. D)
489 Octanoylcarnitine&malate-supported LEAK (FAO_L) and E) OXPHOS coupling
490 efficiency. F) Muscle *UCP3* expression relative to Lowlanders at baseline. Mean ±
491 SEM (*n* = 7-11). ***P* ≤ 0.01; ****P* ≤ 0.001 Lowlander vs Sherpas at baseline. †*P* ≤
492 0.05; ††*P* ≤ 0.01 baseline vs altitude within cohort. $^{\Delta}$*P* ≤ 0.05; $^{\Delta\Delta}$*P* ≤ 0.01 altitude 1 vs
493 2 within cohort.
494
495 **Figure 5** Muscle glycolysis and blood glucose homeostasis. A) Hexokinase and B) Lactate
496 dehydrogenase activity. C) Fasting blood glucose and D) glucose clearance during
497 OGTT. E) Total muscle glycolytic intermediates relative to Lowlanders at baseline.
498 Mean ± SEM (*n* = 5-14). **P* ≤ 0.05 Lowlanders vs Sherpas at baseline. †*P* ≤ 0.05; ††*P*
499 ≤ 0.01; †††*P* ≤ 0.001 baseline vs altitude within cohort.

500 **Figure 6** Muscle energetics and oxidative stress. A) Phosphocreatine, B) ATP, C)
501 Oxidized/reduced glutathione (GSSG/GSH) and D) Sulfoxide/total methionine
502 (MetSO/Met), all expressed relative to Lowlanders at baseline. Mean ± SEM (*n* = 8-

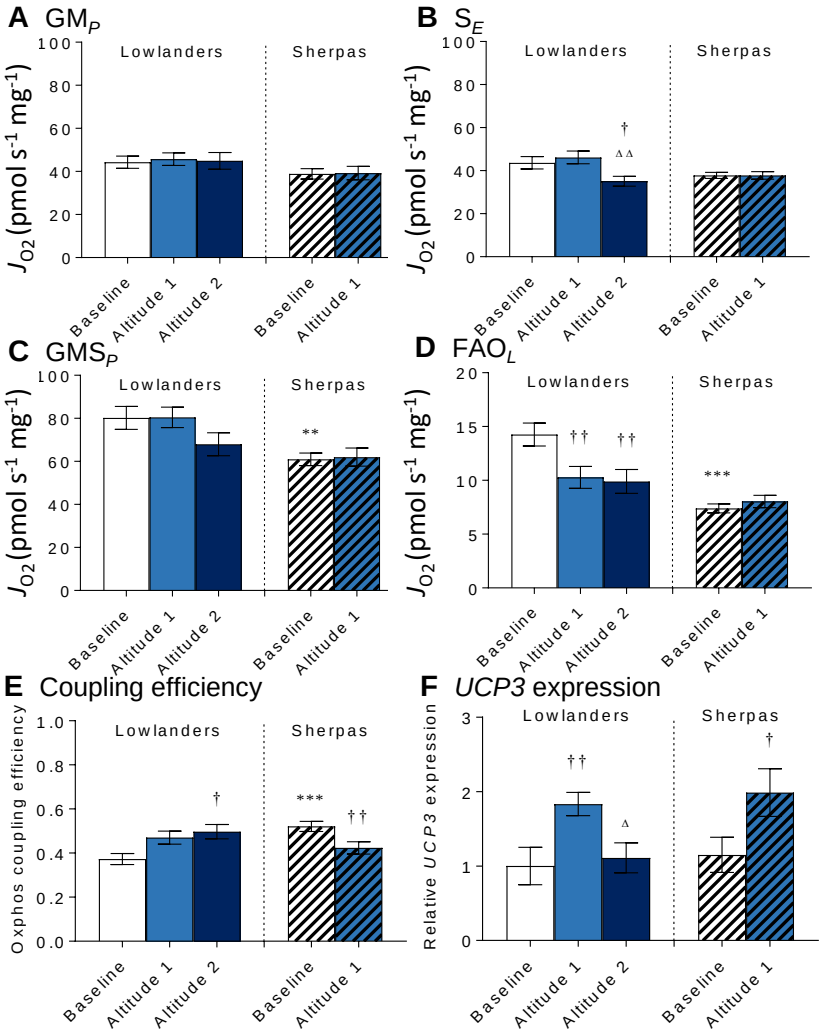
503 14). ††$P \leq 0.01$; †††$P \leq 0.001$ baseline vs altitude within cohort. $^{\Delta}P \leq 0.05$ altitude 1
504 vs 2 within cohort.

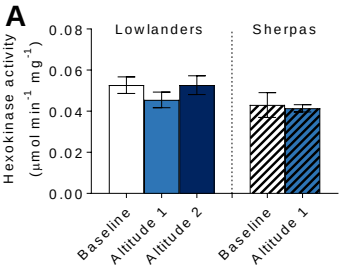

A
Rs6520015
Rs7292407
Rs9627403
Lowlanders
Sherpas
CC CT TT
AA AC CC
GG GA AA
B
S(A1)
L(A1)
L(A2)
Everest Base Camp (5300 m)
Lowlanders
Sherpas
Kathmandu (1300 m)
S(B)
L(B)
London (75 m)
Altitude (m above sea level)
Day
C
Lowlanders
Sherpas
Mean Sa(O2) (%)
Time (d)
D
Lowlanders
Sherpas
Relative VEGFA expression
Baseline
Altitude 1
Altitude 2
E RNNO
F RSNO
G Nitrate
H Nitrite
Concentration (nM)
Concentration (µM)

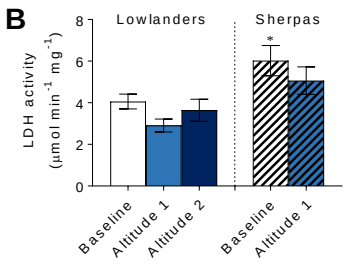

A
Relative PPARA expression
2.0
1.5
1.0
0.5
0.0
Lowlanders
Sherpas
*
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1
B
Relative CPT1B expression
1.5
1.0
0.5
0.0
Lowlanders
Sherpas
†
†
*
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1
C
HADH activity (μmol min-1 mg-1)
0.5
0.4
0.3
0.2
0.1
0.0
Lowlanders
Sherpas
*
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1
D
FAOp (pmol s-1 mg-1)
30
20
10
0
Lowlanders
Sherpas
**
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1
E
Relative levels of total carnitines
1.5
1.0
0.5
0.0
Lowlanders
Sherpas
†
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1
F
Long chain / total carnitines
0.15
0.10
0.05
0.00
Lowlanders
Sherpas
†
*
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1

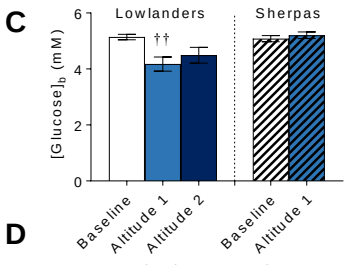

Acetyl coA
+ H2O
CS
coASH
citrate
Acon1
H2O
aconitate
H2O
Acon2
isocitrate
NAD+
ICDH
NADH + H+
CO2
α-ketoglutarate
NAD+
GDP + Pi
αKGD
SCS
NADH + H+
GTP
CO2
succinate
FAD
SDH
FADH2
fumarate
H2O
Fum
malate
NAD+
MDH
NADH + H+
oxaloacetate

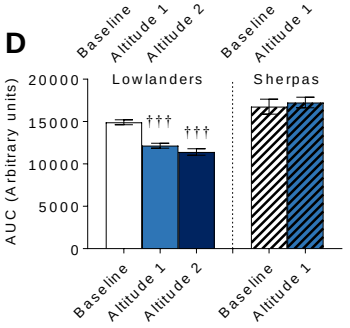
A
Lowlanders
Sherpas
Citrate synthase activity
(μmol min-1 mg-1)
0.5
0.4
0.3
0.2
0.1
0.0
*
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1
Relative levels of intermediates
B
C
D
E
F
G
H
I
Lowlanders
Sherpas
2.0
1.5
1.0
0.5
0.0
***
**
†
††
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1

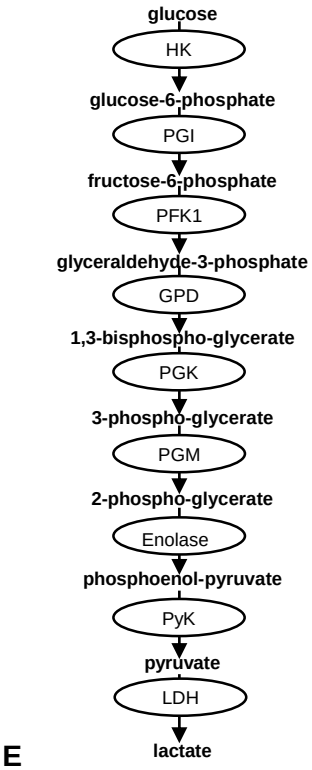

A GM$_P$
B S$_E$
C GMS$_P$
D FAO$_L$
E Coupling efficiency
F *UCP3* expression
Lowlanders
Sherpas
J_{O2} (pmol s^{-1} mg^{-1})
Oxphos coupling efficiency
Relative *UCP3* expression
Baseline
Altitude 1
Altitude 2

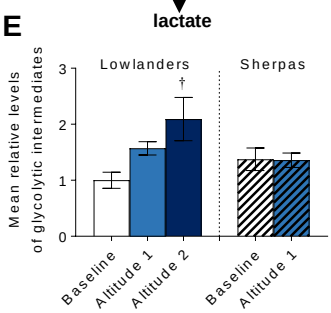

A
Hexokinase activity (μmol min-1 mg-1)
0.08
0.06
0.04
0.02
0.00
Lowlanders
Sherpas
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1

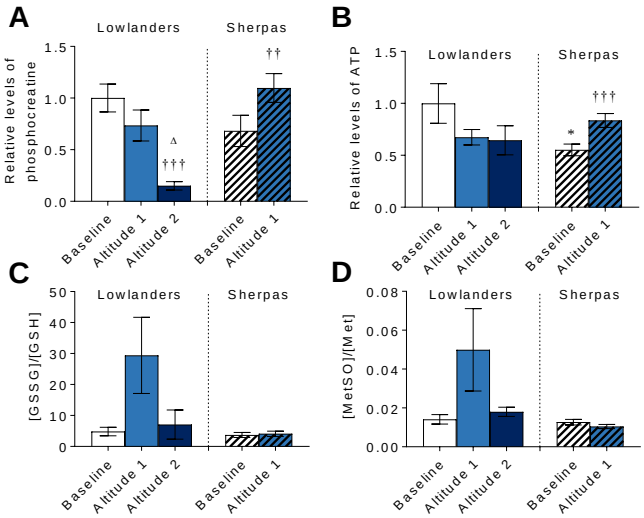

B
LDH activity (μmol min-1 mg-1)
8
6
4
2
0
Lowlanders
Sherpas
*
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1


C
[Glucose]b (mM)
6
4
2
0
Lowlanders
Sherpas
††
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1


D
AUC (Arbitrary units)
20000
15000
10000
5000
0
Lowlanders
Sherpas
†††
†††
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1


glucose
HK
glucose-6-phosphate
PGI
fructose-6-phosphate
PFK1
glyceraldehyde-3-phosphate
GPD
1,3-bisphospho-glycerate
PGK
3-phospho-glycerate
PGM
2-phospho-glycerate
Enolase
phosphoenol-pyruvate
PyK
pyruvate
LDH
lactate


E
Mean relative levels of glycolytic intermediates
3
2
1
0
Lowlanders
Sherpas
†
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1


A
Lowlanders
Sherpas
Relative levels of phosphocreatine
1.5
1.0
0.5
0.0
Δ
†††
††
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1

B
Lowlanders
Sherpas
Relative levels of ATP
1.5
1.0
0.5
0.0
*
†††
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1

C
Lowlanders
Sherpas
[GSSG]/[GSH]
50
40
30
20
10
0
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1

D
Lowlanders
Sherpas
[MetSO]/[Met]
0.08
0.06
0.04
0.02
0.00
Baseline
Altitude 1
Altitude 2
Baseline
Altitude 1

SUPPLEMENTARY INFORMATION FOR:

METABOLIC BASIS TO SHERPA ALTITUDE ADAPTATION

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Materials and Methods

Study design

The design and conduct of Xtreme Everest 2 has been described previously (25). Healthy Lowlanders (*n* = 10, 7 male) and healthy age- and gender-matched Sherpas (*n* = 15, 11 male) were selected from recruited participants. All Lowlander subjects were born and lived below 1,000 m, were not descended from a high altitude-dwelling population (e.g. Tibetan, Andean, Ethiopian) and were of European (Caucasian) origin. These subjects were Xtreme Everest 2 investigators selected to be resident at the Everest Base Camp laboratory throughout the expedition for the purpose of conducting research. The researchers were selected on the basis of their availability and ability to contribute to the scientific programme of the expedition, but not due to any proven ability to perform at high altitude, indeed a number of these subjects were altitude naïve at the time of departure. Sherpa subjects were drawn from communities in the Solokhumbu and Rolwaling valleys and were required to provide evidence that all parents and grandparents were Nepali Sherpas. Two of the Sherpa subjects were first cousins, but no other subjects (Sherpa or Lowlander) were related.

Subjects gave written consent for participation, and were subjected to medical screening prior to the expedition, which involved completion of a health questionnaire and a check-up with the Chief Medical Officer. Potential participants with serious cardiac or respiratory disease were excluded. A local, medically-qualified translator was present at all times to ensure effective communication between scientific investigators and Sherpa subjects. All protocols were approved by the UCL Research Ethics Committee and the Nepal Health Research Council (NHRC). All subjects from both cohorts were free from altitude exposure for at least three months prior to the expedition and were physically active, but neither particularly sedentary nor highly trained. Subjects were flown from Kathmandu, Nepal (1,300 m) to Lukla in the Solokhumbu region (2,800 m), before ascending on foot

to Everest Base Camp (5,300 m) by a matched 10-day ascent profile. Diet was not strictly controlled, however all subjects were presented with similar, communal fare at tea houses, lodges and camps throughout the expedition, and this did not include large quantities of foods known to be rich in nitrogen oxides, such as green leafy vegetables or cured meats.

Muscle sample collection and preparation

Biopsies of the *vastus lateralis* muscle were taken from the mid-thigh using Tilley-Henckel forceps under local anaesthesia (2% lignocaine, 1:80,000 adrenaline) of the skin and superficial muscle fascia. A 5 mm incision was made and 150 mg wet weight tissue collected, with repeat biopsies taken adjacent to previous biopsies. Sherpa biopsies were taken in Kathmandu and again 11-12 d after departure (1-2 d at 5,300 m). Lowlander biopsies were taken in London, UK (35 m) before the expedition, 15-20 d after departure (5-10 d at 5,300 m), and again 54-59 d after departure (44-49 d at 5,300 m). Atmospheric parameters from the three laboratories have been reported elsewhere (25). The London and Kathmandu biopsies were taken to assess baseline (B) metabolic profile. Thereafter, biopsies were taken at Everest Base Camp within 21 d of the start of the ascent to assess the effects of shorter term high altitude exposure (A1) on metabolism, while biopsies taken after 55 d indicated the effects of more sustained high altitude exposure (A2).

Ideally, biopsies would have been carried out on the subjects at the same times following the onset of exposure, however Sherpas and Lowlanders were studied at different days after arrival at Everest Base Camp for logistical reasons. The Lowlander subjects, being Xtreme Everest 2 investigators, needed to establish camp, construct the laboratory, and unpack, calibrate and validate equipment for high-resolution respirometry upon arrival. Since these measurements cannot be made on frozen samples, biopsy sampling only occurred once respirometry could be carried out. The Sherpa

subjects, however, arrived and departed within guided trekking groups after the laboratory had been established. These treks followed pre-ordained ascent/descent schedules to and from Kathmandu, with the subjects spending 3 nights at Everest Base Camp. There was therefore a narrow window of opportunity during which Sherpas could be studied. Following measurements at the A1 time-point, the Lowlander subjects were scheduled to remain at Everest Base Camp for a further two months for the purpose of carrying out research, presenting us with the additional opportunity of collecting further valuable data pertaining to longer-term metabolic acclimatization to hypobaric hypoxia in a group resident at 5,300 m. It was not, however, possible for us to collect comparable longer-term data for the Sherpa subjects on this expedition, though we acknowledge that this would have been of interest.

No food or caffeine was allowed within the 12 h preceding each biopsy. The muscle sample was immediately placed in ice-cold biopsy preservation medium (BIOPS): CaK_2EGTA (2.77 mM), K_2EGTA (7.23 mM), $MgCl_2.6H_2O$ (6.56 mM), taurine (20 mM), phosphocreatine (15 mM), imidazole (20 mM), dithiothreitol (0.5 mM), MES (50 mM) and Na_2ATP (5.77 mM), pH 7.10, which was filtered and stored at -40 °C or lower until use to prevent bacterial growth. Following this, the muscle sample was cleared of any fat or connective tissue and divided into sections as follows: 15 mg, snap-frozen in liquid nitrogen for metabolomics; 20 mg, snap-frozen in liquid nitrogen for gene expression and enzyme activity assays; 50 mg, retained in ice-cold biopsy preservation medium for high-resolution respirometry. Frozen samples were flown back to the UK on liquid nitrogen and stored at -80 °C until use.

Measurement of NO metabolites

Venesection was performed for the measurement of circulating biomarkers. Plasma was separated from blood cells by centrifugation of whole blood at 800 *g* for 15 min and immediately frozen in 1 ml aliquots in liquid nitrogen. Samples were stored under liquid nitrogen for the duration of the expedition, transported back to the UK on dry ice and kept at −80 °C until analysis.

NO metabolite concentrations were quantified immediately after thawing of frozen plasma aliquots in the presence of an excess of N-ethylmaleimide (NEM, in PBS; 10 mM final concentration). For the analysis of circulating total nitroso species, aliquots of NEM-treated EDTA plasma were directly injected into a triiodide-containing reaction chamber, and the NO produced from the reduction of protein nitroso species was quantified by gas phase chemiluminescence (CLD 77sp, EcoMedics), as described (52). The concentration of nitroso species in these samples was estimated from the difference in quantification of NO signal after sample pre-treatment with mercuric chloride with sulfanilamide vs. sulfanilamide alone. For nitrite/nitrate analysis, NEM-treated samples were deproteinised with ice-cold methanol (1:1 v/v), separated by centrifugation and subjected to analysis by high pressure liquid chromatography using a dedicated nitrite/nitrate analyser (ENO20, Eicom). Sample processing was performed in a staggered fashion to ensure reproducible processing times, and reported values are corrected for background contaminant levels.

Genetics

Total genomic DNA was isolated from whole blood samples using LGC Genomics' DNA extraction service (www.lgcgroup.com/services/extraction/dna-extraction/). In brief, samples were extracted using detergent-driven cell lysis, followed by guanidinium isothiocyanate-mediated DNA binding to silica. Contaminants were removed by washing and DNA subsequently eluted into a low salt buffer (10 mM Tris, 1mM EDTA). Three single nucleotide polymorphisms (SNPs) on the *PPARA* gene

(rs9627403, rs7292407, rs6520015) were genotyped using the TaqMan® platform for allelic discrimination (Applied Biosystems, Paisley, UK). Polymerase chain reaction (PCR) amplification was performed on 384-well plates using TaqMan® Predesigned SNP Genotyping Assays and using conditions recommended by the manufacturer (Applied Biosystems, Paisley, UK). Reactions were analysed by individuals blinded to subject/racial status and phenotypic data using the Applied Biosystems TaqMan® 7900HT system and the sequence detection system software v2.4. All samples were genotyped twice, with 100% concordance. The TaqMan® SNP genotyping Assay ID numbers for each *PPARA* SNP are shown in Table S2.

Oral glucose tolerance test

Oral glucose tolerance tests (OGTT) were carried out to assess whole-body insulin sensitivity. After an overnight fast, subjects were challenged with an oral dose of 75 g glucose dissolved in water. Blood was collected at 0, 15, 30, 60, 90, and 120 min after ingestion and the blood-glucose concentration was measured using a standard AccuChek Glucometer (Roche Applied Science). The area under the curve (AUC) was then calculated using the trapezoidal rule. OGTT was performed on Lowlanders in London prior to altitude exposure, 16-21 days after departure (6-11 days at 5,300 m) and 55-60 days after departure (45-50 days at 5,300 m). OGTT was performed on Sherpa subjects in Kathmandu prior to altitude exposure, and 12-13 days after departure (2-3 days at 5,300 m). For all subjects, OGTT was performed the day following biopsy collection to avoid confounding experiments on muscle metabolism.

High-resolution respirometry

Skeletal muscle fiber bundles were prepared from the respirometry-designated sample according to previously described methods (28). After permeabilization, fiber bundles were blotted on filter

paper and weighed using a microbalance (Mettler-Toledo). Respiration of fiber bundles was then measured in a mitochondrial respiration medium (MiR05) containing EGTA (0.5 mM), $MgCl_2.6H_2O$ (3 mM), K-lactobionate (60 mM), taurine (20 mM), KH_2PO_4 (10 mM), HEPES (20 mM), sucrose (110 mM), defatted BSA (1 g L^{-1}), pH 7.4, using two substrate-uncoupler-inhibitor titration (SUIT) protocols and shown in Table S3 and S4. Respirometry was performed such that there was crossover of personnel between the three laboratories.

Malate (M; 5 mM) and octanoylcarnitine (Oct; 0.2 mM) were added initially to stimulate LEAK respiration (FAO_L; Fig. 4D; Table S3; Fig. S1). ADP (saturating concentration ≥ 10 mM) activated phosphorylation of ADP to ATP, resulting in OXPHOS limited by the capacity of β-oxidation (FAO_P, F-OXPHOS; Fig. 2D). Addition of glutamate (G; 10 mM) followed by succinate (S; 10 mM) saturated convergent electron entry to the Q-junction in the FN-pathway ($OctGM_P$; Fig. 4A) and the FNS-pathway ($OctGMS_P$), respectively. Cytochrome *c* (10 µM) addition was used as a quality control to confirm outer mitochondrial membrane integrity; all assays with an increase in O_2 consumption of >15% following cytochrome *c* addition were excluded from further analysis. FCCP was used (step-wise titration of 0.25 µM) to uncouple oxidative phosphorylation and investigate ETS capacity ($OctGMS_E$; Fig. 4C). Finally, rotenone was added (0.5 µM) to inhibit Complex I (and thus FAO (38)) and isolate succinate-linked ETS capacity (S_E; Fig. 4B). The OXPHOS coupling efficiency (Fig. 4E) was calculated as follows to give an indication of mitochondrial coupling (37):

$$j_{\approx P} = \frac{P - L}{P}$$

$j_{\approx P}$ = OXPHOS coupling efficiency; P = OXPHOS capacity following ADP addition; L = LEAK respiration prior to ADP addition.

A second SUIT protocol was used to interrogate ETS function in the absence of fatty acid substrates (Table S4; Fig. S2). Malate (5 mM) was added initially, followed by glutamate (10 mM) to measure LEAK respiration. ADP (saturating concentration ≥ 10 mM) activated phosphorylation of ADP to ATP, resulting in N-pathway OXPHOS capacity. Addition of succinate (10 mM) stimulated convergent electron entry to the Q-junction through Complexes I and II (NS-pathway). Cytochrome *c* (10 µM) addition was used as a quality control to confirm outer mitochondrial membrane integrity; all assays with an increase in O_2 consumption of >15% following cytochrome *c* addition were excluded from further analysis. FCCP was used (step-wise titration of 0.25 µM) to uncouple oxidative phosphorylation and investigate ETS capacity. Finally, rotenone was added (0.5 µM) to inhibit Complex I and isolate succinate-linked ETS capacity.

Enzyme activity assays

Enzyme activity assays were performed as described previously (19). Briefly, approximately 10 mg of *vastus lateralis* from each individual was homogenized with an Eppendorf pestle in an Eppendorf tube containing 300 µl of homogenisation buffer containing HEPES (20 mM), EDTA (1 mM), Triton X-100 (0.1% v/v). The samples were then centrifuged (380 *g*, 30 s, 4 °C) and the supernatant was collected. This was centrifuged again (380 *g*, 30 s, 4 °C) and the supernatant collected to obtain a homogeneous suspension. Protein concentration of chamber and tissue homogenates was measured using the Quick Start Bradford protein assay (Bio-Rad). All assays were performed in a spectrophotometer (Evolution 220, Thermo Scientific) at 37 °C in a reaction volume of 1 ml. Citrate synthase activity was quantified with homogenate diluted to 10 µg protein ml^{-1} in an assay buffer containing Tris (20 mM), 5,5'-dithiobis-2-nitrobenzoic acid (0.1 mM) and acetyl-CoA (0.3 mM) at pH 8.0. The reaction was initiated by the addition of oxaloacetate (0.5 mM) and absorbance change at 412 nm was measured. 3-hydroxy acyl dehydrogenase (HADH) activity was assayed with

homogenate diluted to 20 µg protein ml^{-1} in an assay buffer containing imidazole (50 mM), NADH (0.15 mM) and Triton X-100 (0.1% v/v), pH 7.4. The reaction was initiated by the addition of 0.1 mM acetoacetyl-CoA (0.1 mM) and absorbance change at 340 nm was measured. Hexokinase activity was quantified with homogenate diluted to 60 µg protein ml^{-1} in an assay buffer containing imidazole (20 mM), ATP (1 mM), $7H_2O.MgCl_2$ (5 mM), dithiothreitol (5 mM), NAD^+ (2 mM), and glucose-6-phosphate-dehydrogenase (3.125 U), pH 7.4. Glucose (5 mM) was added to trigger the reaction and absorbance change at 340 nm was measured. Activity of LDH was quantified with homogenate diluted to 2 µg protein ml^{-1} with an assay buffer containing HEPES (50 mM) and NADH (0.3 mM), pH 7.0 and the reaction was triggered by the addition of pyruvate (0.5 mM). The reaction was monitored by measuring absorbance at a wavelength of 340 nm.

Reverse transcription quantitative polymerase chain reaction

RNA was extracted from frozen skeletal muscle samples using a Qiagen RNeasy Fibrous Tissue Mini kit as per the manufacturer's instructions, except that the incubation step with DNase I was excluded as this was found to lower RNA yields. The Taqman® assays used are detailed in Table S5.

Mass Spectrometry

A methanol/chloroform extraction protocol was used, as described previously (51). First, 600 µl chloroform:methanol (2:1 mixture) was added to cryovials containing ~20 mg frozen skeletal muscle and a metallic bead. Samples were lysed in a tissue lyser (Qiagen, 3 x 2 min, 22 s^{-1}) and sonicated for 15 min. Metallic beads were then removed, before 200 µl chloroform and 200 µl distilled water were added. Samples were thoroughly vortexed prior to centrifugation (~20,000 *g*, 15 min), which resulted in clear separation of an aqueous phase (upper), protein pellet (middle) and organic phase (lower). The aqueous and organic fractions were carefully extracted using a positive displacement

pipette and transferred to separate Eppendorf tubes. A further 600 µl chloroform:methanol (2:1 mixture) was added to the protein pellet and lysis, mixing and centrifugation steps repeated to maximise metabolite recovery. Both the aqueous and organic fractions were dried under nitrogen and stored at -80 °C until further analysis.

Due to their high polarity, compounds which contain phosphate were measured using hydrophilic interaction liquid chromatography (HILIC) where an aqueous layer is formed on the surface of the stationary phase and this layer allows retention of the analytes. Samples were reconstituted in 200 µL acetonitrile: water (7:3 mixture) vortexed and analysed. The instrumentation comprised an Acquity Ultra Performance Liquid Chromatography unit (Waters Ltd, Elstree, UK) interfaced with an AB Sciex 5500 triple quadrupole (AB Sciex, Macclesfield, UK). Mobile phases were run at 0.6 ml min^{-1} where mobile phase A consisted of 10 mM ammonium acetate adjusted to pH 9.5 with ammonia, and mobile phase B was acetonitrile. Mobile phase B was held for 1 min at 70%, decreased to 40% over 2.5 min, returned to 70% by 3.6 min, and maintained for 2.4 min. The total run time was 6 min. Data were acquired in both positive and negative ionisation modes using capillary spray voltages of 3.5 kV and 2.5 kV, respectively. The ion transfer tube was set to operate at 356°C and the vaporiser temperature was set to 420 °C. Sheath, auxiliary and sweep gases were set to 52, 16 and 2 arbitrary units, respectively.

Other aqueous metabolites were measured using the same instrumentation, but the chromatographic separation was performed using an ACE C18-PFP 3 µm column (2.1 x 150 mm) (Advanced Chromatography Technologies Ltd.). The mobile phase gradient was run at 0.5 ml min^{-1} using water (mobile phase A) and acetonitrile (mobile phase B). The gradient started 0% B, and increased to 60% B from 1.6 to 4.5 min, followed by re-equilibration for 2 min. The total run-time was 6.5 min. Data were acquired in both positive and negative ionisation modes using capillary spray

223 voltages of 3.5 kV and 2.5 kV, respectively. The ion transfer tube was set to operate at 350 °C, whilst
224 the vaporizer temperature was set to 400 °C. Sheath, auxiliary and sweep gases were set to 50, 15
225 and 2 arbitrary units, respectively.

226 Half of the organic fraction and half of the aqueous fraction were combined with 200 µl of
227 acetonitrile containing an internal standard mix of eight deuterated carnitines (1.63 µM [d9]
228 free carnitine, 0.3 µM [d3] acetyl carnitine, 0.06 µM [d3] propionyl carnitine, 0.06 µM [d3] butyryl
229 carnitine, 0.06 µM [d9] isovarelyl carnitine, 0.06 µM [d3] octanoyl carnitine, 0.06 µM [d9] myristoyl
230 carnitine, and 0.12 µM [d3] palmitoyl carnitine) (Cambridge Isotope Laboratories, Inc.) and dried
231 under nitrogen. Samples were derivatized with 100 µl of 3 M HCl in butanol for 15 min at 65 °C. The
232 resulting mixture was dried under nitrogen and finally reconstituted in 4:1 acetonitrile: 0.1% formic
233 acid in water and vortexed and placed into autosampler vials. The strong mobile phase used for
234 analysis was acetonitrile with 0.1% formic acid (B) and the weak mobile phase was 0.1% formic acid
235 in water (A). The analytical UPLC gradient used a Synergi Polar RP phenyl ether column (50 × 2.1
236 mm, 2.5 µm, Phenomenex) starting with 30% B in 0.1% formic acid followed by a linear gradient to
237 100% B for 3 min and held at 100% B for the next 5 min with a further 2 min re-equilibration. The
238 total run time was 10 min and the flow rate was 0.5 ml min^{-1} with an injection volume of 2 µl.
239 Analytes were measured using an MRM method with the daughter ion being set to 85.0 Da for each
240 compound.

241 Protein pellets were dissolved in 1 ml of 1 M NaOH solution and heated for 10 min at 80 °C. Samples
242 were then centrifuged (13,000 rpm, 10 min). Sample protein concentration was quantified using a
243 bicinchoninic acid (BCA) assay kit (Sigma BCA1-1KT) and absorbance at a wavelength of 562 nm was
244 then quantified using a spectrophotometer (Evolution 220, Thermo Scientific).

Data were processed using the vendor software and normalized to total protein content and to the intensity of the internal standards.

It was not feasible, in a field study such as this, to avoid autoxidation during sample processing, which is known to affect redox, thus the redox ratios reported do not correspond to true physiological levels, which would have been obtainable with direct addition of thiol alkylating agents. Nevertheless, we demonstrate that ratios change substantially, reflecting redox differences in relation to oxidative load. Addition of a thiol-alkylating agent would have compromised the analysis of other metabolites.

Statistics

To compare Sherpa and Lowlander cohorts at baseline, an unpaired two-tailed Student's t-test was performed (considering significance at $P \leq 0.05$). Genotype frequencies were compared between Sherpas and Lowlanders using a Chi-squared test. To assess the effects of an ascent to high altitude on both cohorts, a one-way ANOVA with repeated measures was performed. If a significant difference was reported, post-hoc pairwise comparisons were carried out with a Tukey correction.

Data Sharing

All data is available from the University of Cambridge data repository: https://doi.org/10.17863/CAM.9072

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286

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289 Medicine, the Centre for Human Integrative Physiology at the University of Southampton and the
290 Duke University Medical Centre.

291

292 Membership, roles, and responsibilities of the Xtreme Everest 2 Research Group can be found
293 at www.xtreme-everest.co.uk/team.

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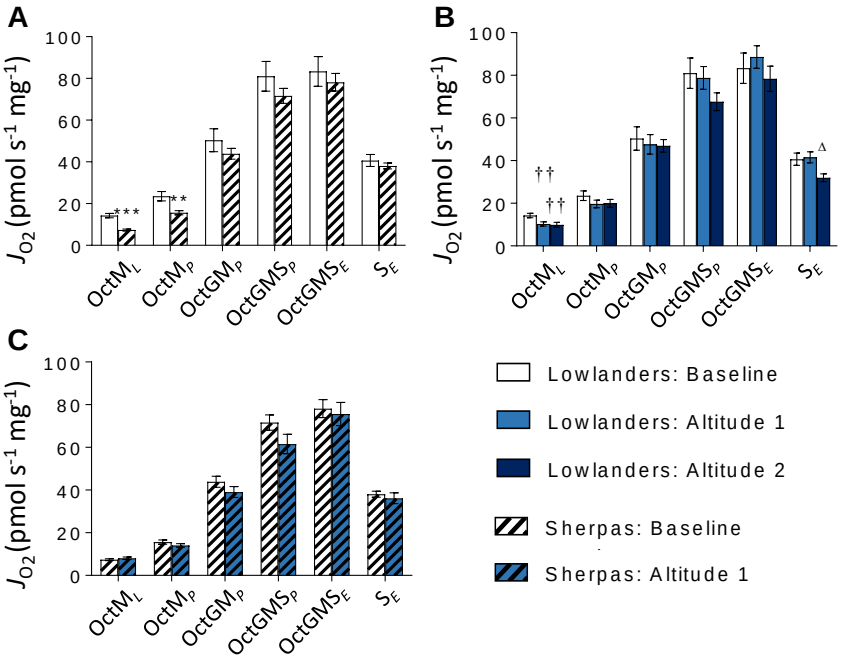
306

SI Figure Legends

Figure S1 **Mitochondrial respiratory function by substrate-uncoupler-inhibitor titration protocol #1, in the presence of octanoylcarnitine.** Fatty acid oxidation-LEAK ($OctM_L$), Fatty acid oxidation-OXPHOS ($OctM_P$), N-OXPHOS ($OctGM_P$), NS-OXPHOS ($OctGMS_P$), NS-ETS capacity ($OctGMS_E$) and S-ETS capacity (S_E)**. A)** Lowlanders vs Sherpas at baseline (B); **B)** Lowlanders at baseline (B), early (A1) and late (A2) altitude timepoints; **C)** Sherpas at baseline (B) and early (A1) altitude timepoints. Mean ± SEM (n = 10-11). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ Lowlanders vs Sherpas at baseline. $^{\dagger\dagger}P \leq 0.01$; B vs A1 within cohort. $^{\Delta}P \leq 0.05$ A1 vs A2 within cohort.

Figure S2 **Mitochondrial respiratory function by substrate-uncoupler-inhibitor titration protocol #2, in the absence of octanoylcarnitine.** N-LEAK (GM_L), N-OXPHOS (GM_P), NS-OXPHOS (GMS_P), NS-ETS capacity (GMS_E) and S-ETS capacity (S_E) **A)** Lowlanders vs Sherpas at baseline (B); **B)** Lowlanders at baseline (B), early (A1) and late (A2) altitude timepoints; **C)** Sherpas at baseline (B) and early (A1) altitude timepoints. Mean ± SEM (n = 10-11). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ Lowlanders vs Sherpas at baseline. $^{\dagger}P \leq 0.05$; baseline vs altitude within cohort. . $^{\Delta\Delta}P \leq 0.01$ A1 vs A2 within cohort.


A
B
C
J_{O_2} (pmol s^{-1} mg^{-1})
100
80
60
40
20
0
***
**
††
††
Δ
OctM$_L$
OctM$_P$
OctGM$_P$
OctGMS$_P$
OctGMS$_E$
S$_E$
Lowlanders: Baseline
Lowlanders: Altitude 1
Lowlanders: Altitude 2
Sherpas: Baseline
Sherpas: Altitude 1

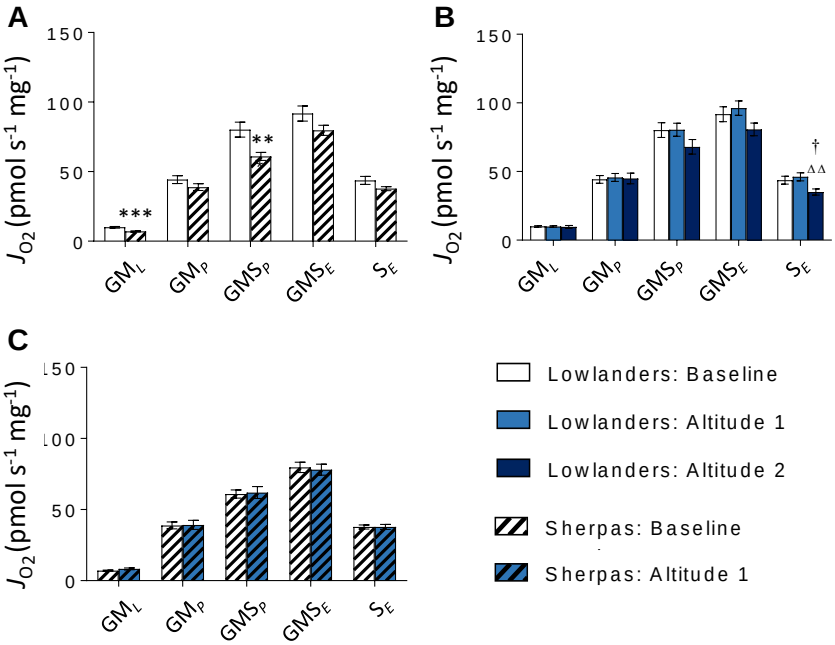

A
B
C
J_{O_2} (pmol s^{-1} mg^{-1})
150
100
50
0
***
**
†
ΔΔ
GM_L
GM_P
GMS_P
GMS_E
S_E
Lowlanders: Baseline
Lowlanders: Altitude 1
Lowlanders: Altitude 2
Sherpas: Baseline
Sherpas: Altitude 1

Table S1. PPARA SNP positions and putatively advantageous alleles as identified by Simonson et al. (8), and TaqMan® SNP genotyping Assay ID information

***PPARA* SNP**	**HG 18 Position***	**Selected Allele**	**Alternate Allele**	**TaqMan® SNP genotyping Assay ID**
rs9627403	Chr22: 44827140	A	G	C__30661738_10
rs7292407	Chr22: 44832376	C	A	C_189279291_10
rs6520015	Chr22: 44842095	T	C	C__26019862_10

* Based on UCSC Genome Browser Human Reference Build 18 (S4)

Table S2. Relative levels of glycolytic intermediates

Intermediate	L(B)	L(A1)	L(A2)	S(B)	S(A1)
Glucose-6-phosphate and fructose-6-phosphate	1.00 ± 0.17	1.44 ± 0.25	3.29 ± 0.95†	1.33 ± 0.43	1.76 ± 0.35
Dihydroxyacetone phosphate	1.00 ± 0.18	1.20 ± 0.25	0.86 ± 0.13	1.23 ± 0.26	1.40 ± 0.12
2-phosphoglycerate and 3-phosphoglycerate	1.00 ± 0.16	2.07 ± 0.28†	2.13 ± 0.22†	1.56 ± 0.22	0.92 ± 0.08††

Key: L, Lowlanders; S, Sherpas; B, baseline; A1, early altitude; A2, late altitude. † $P < 0.05$, †† $P < 0.01$ B vs A1 within cohort.

Note: Two pairs of metabolites, glucose- and fructose-6-phosphate, and 2- and 3-phosphoglycerate could not be distinguished from each other, so combined levels are shown. Levels of all intermediates are shown relative to Lowlanders at baseline as mean ± standard error of the mean, n = 7-14 per group.

Table S3. Substrate-uncoupler-inhibitor titration protocol #1.

No.	Substrate/Uncoupler/Inhibitor	State	Figure
1	Malate (5 mM)		4D/S1
	Octanoylcarnitine (0.2 mM)	$OctM_L$	
2	ADP (10 mM*)	$OctM_P$	2D/S1
3	Glutamate (10 mM)	$OctGM_P$	S1
4	Succinate (10 mM)	$OctGMS_P$	S1
5	Cytochrome *c* (10 µM)	$OctGMSc_P$	
6	FCCP (0.25-1.5 µM†)	$OctGMS_E$	S1
7	Rotenone (0.5 µM)	S_E	S1

Reagents were added in the order 1-7 (left column) to give the final concentrations shown in parentheses. *10 mM was the minimum amount of ADP added. Higher concentrations were required to reach saturation in some cases. †FCCP was titrated in 0.25 µM steps until an inhibitory effect was observed.

Table S4. Substrate-uncoupler-inhibitor titration protocol #2.

No.	Substrate/Uncoupler/Inhibitor	State	Figure
1	Malate (5 mM)		
	Glutamate (10 mM)	GM_L	S2
2	ADP (10 mM*)	GM_P	4A/S2
3	Cytochrome *c* (10 µM)	GMc_P	
4	Succinate (10 mM)	GMS_P	4C/S2
5	FCCP (0.25-1.5 µM†)	GMS_E	S2
6	Rotenone (0.5 µM)	S_E	4B/S2

Reagents were added in the order 1-6 (left column) to give the final concentrations shown in parentheses. *10 mM was the minimum amount of ADP added. Higher concentrations were required to reach saturation in some cases. †FCCP was titrated in 0.25 µM steps until an inhibitory effect was observed.

Table S5. Details of Taqman assays selected to assess gene expression.

Gene	Amplicon size	Assay number
ACTB*	63	Hs01060665_g1
HPRT1*	82	Hs02800695_m1
PPIA*	97	Hs04194521_s1
RNA18S*	90	Hs03928985_g1
VEGFA	59	Hs00900055_m1
PPARA	62	Hs00947536_m1
UCP3	74	Hs01106052_m1
CPT1B	133	Hs03046298_s1

* Housekeeping genes used as controls for normalisation of target genes.