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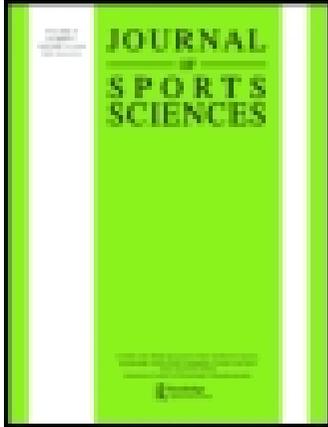
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Effects of an 8-weeks erythropoietin treatment on mitochondrial and whole body fat oxidation capacity during exercise in healthy males

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Abstract

The present investigation was performed to elucidate if the non-erythropoietic ergogenic effect of a recombinant erythropoietin treatment results in an impact on skeletal muscle mitochondrial and whole body fatty acid oxidation capacity during exercise, myoglobin concentration and angiogenesis. Recombinant erythropoietin was administered by subcutaneous injections (5000 IU) in six healthy male volunteers (aged 21 ± 2 years; fat mass $18.5 \pm 2.3\%$) over 8 weeks. The participants performed two graded cycle ergometer exercise tests before and after the intervention where $\text{VO}_{2\text{max}}$ and maximal fat oxidation were measured. Biopsies of the *vastus lateralis* muscle were obtained before and after the intervention. Recombinant erythropoietin treatment increased mitochondrial O_2 flux during ADP stimulated state 3 respiration in the presence of complex I and II substrates (malate, glutamate, pyruvate, succinate) with additional electron input from β -oxidation (octanoylcarnitine) (from 60 ± 13 to 87 ± 24 $\text{pmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$ $P < 0.01$). β -hydroxyacyl-CoA-dehydrogenase activity was higher after treatment ($P < 0.05$), whereas citrate synthase activity also tended to increase ($P = 0.06$). Total myoglobin increased by 16.5% ($P < 0.05$). Capillaries per muscle area tended to increase ($P = 0.07$), whereas capillaries per fibre as well as the total expression of vascular endothelial growth factor remained unchanged. Whole body maximal fat oxidation was not increased after treatment. Eight weeks of recombinant erythropoietin treatment increases mitochondrial fatty acid oxidation capacity and myoglobin concentration without any effect on whole body maximal fat oxidation.

Keywords: erythropoietin, skeletal muscle, fat oxidation

Introduction

Erythropoietin is a hypoxia-induced hormone released by the renal cortex that promotes proliferation and differentiation of erythroid progenitor cells. Erythropoietin increases the number of erythroid cells in the peripheral blood and thus increases the oxygen carrying capacity of blood (Fisher, 2010). Therefore treatment with recombinant erythropoietin will lead to an increase in aerobic capacity, due to its ability to increase maximum O_2 uptake in direct relation to haematocrit (Lundby et al., 2007).

Intriguingly, increased blood oxygen carrying or diffusion capacity fails to explain completely the endurance performance gains derived from recombinant erythropoietin treatments in humans (Thomsen et al., 2007). This suggests a possible role for

erythropoietin by peripheral adaptations in target tissues such as skeletal muscle (Christensen, Lundby, et al., 2012; Joshi, Abraham, Shiwen, Baker, & Tsui, 2014; Rundqvist et al., 2009). Recently, an elegant study performed in healthy males addressed the whole body metabolic effects of a 10 weeks erythropoiesis-stimulating agent treatment. Christensen and colleagues (2013) demonstrated a calorogenic effect of the treatment alone or in combination with endurance training, reflected by an increased resting energy expenditure (RER) after treatment. This increase was not explained by effects in either fat-free mass or thyroid function, but a possible role for increased thermogenesis was suggested by an upregulation of the mitochondrial uncoupler UCP2. Based on these observations, further research about the functionality of

erythropoietin in skeletal muscle due to its potential metabolic and physical performance benefits is encouraged. More specifically, it remains unknown whether whole body fat oxidation during exercise until exhaustion and/or mitochondrial fat oxidation are in fact higher with prolonged erythropoietin stimulation. The possible underlying mechanisms explaining a higher fat oxidation in muscles with EPO involve increased mitochondrial biogenesis, and/or increased oxygen (O₂) transport and supply to the mitochondria, through higher capillarisation and/or concentration of intracellular O₂ carriers, i.e., myoglobin. Myoglobin is expressed in red muscles in response to mitochondrial demand for oxygen in the working muscles (Wittenberg & Wittenberg, 2003), but the potential effects of an enhanced myoglobin protein concentration and therefore oxygen delivery to the mitochondria by erythropoietin stimulation needs further research.

Our hypothesis is that a part of the non-erythropoietic ergogenic effect of a recombinant erythropoietin treatment results in an effect on skeletal muscle mitochondrial fatty acid oxidation capacity. We also hypothesized that the whole body maximal fat oxidation during exercise is increased with a recombinant erythropoietin treatment, as well as the intracellular oxygen carrier myoglobin. Therefore, we determined the effect of a prolonged erythropoietin treatment (8 weeks) on maximal mitochondrial fatty acid oxidation, whole body maximal fat oxidation capacity, myoglobin

expression, as well as angiogenesis in healthy lean males.

Materials and methods

Participants

Six healthy male participants not previously exposed to recombinant erythropoietin agreed to participate in this investigation (Table I). Results describing basic haematological changes are shown in Table I, whereas maximal mitochondrial respiratory capacity and haematocrit changes after EPO administration to these participants have already been published (Plenge et al., 2012). Participants were asked to maintain their daily physical activity level as usual throughout the entire study. All participants were fully informed of the nature and possible risks associated with the study; each volunteer provided informed consent and the ethical committee of Copenhagen municipality (file number H-2-2010-104) approved the investigation adhering to the ethics standards for sport and exercise research.

General procedures

Study participants reported to the laboratory after an overnight fast (10–12 h) at 09.00 h on 4 occasions. The two initial visits were performed before the treatment and the two last visits 1 week after the treatment to ensure the measurement of prolonged

Table I. Subject characteristics, performance, muscle enzymatic activities and angiogenesis.

	Pre		Post	
	Mean ± s	CI (95%)	Mean ± s	CI (95%)
Age (year)	21 ± 2	18.9–23.1		
BMI (kg · cm ⁻²)	22.7 ± 0.5	22.2–23.2	22.5 ± 0.6	21.9–23.1
Body fat (%)	18.5 ± 2.3	16.1–20.9	17.4 ± 1.7	15.6–19.1
Lean body mass (kg)	56.1 ± 2.8	53.2–59	56.4 ± 2.7	53.6–59.2
Hgb (mmol · L ⁻¹)	9.7 ± 0.4	9.3–10.1	10.9 ± 0.8*	10.1–11.7
MCH (fmol)	1.9 ± 0.1	1.8–2.0	1.9 ± 0.1	1.8–2.0
MCHC (mmol · L ⁻¹)	20.9 ± 0.3	20.6–21.2	20.6 ± 0.2	20.4–20.8
VO _{2max} (mL kg · min ⁻¹)	53.7 ± 2.8	50.8–56.6	58.1 ± 3.3*	54.6–61.6
MFO (g · min ⁻¹)	0.40 ± 0.02	0.38–0.42	0.42 ± 0.08	0.34–0.50
Fat _{max} (Watt)	217 ± 16	200–234	235 ± 22*	212–258
Time MFO (min)	19.3 ± 1.5	17.7–20.9	21.0 ± 1.9*	19.0–23.0
Mb (mg · g ⁻¹)	127 ± 15	111–143	139 ± 15*	123–155
CS activity (μmol · g ⁻¹ · min ⁻¹)	129 ± 23	105–153	139 ± 27 [†]	111–167
Mean fibre CSA (10 ³ μm ²)	5.1 ± 0.5	4.6–5.6	5.0 ± 0.8	4.2–5.8
Cap per area (cap mm ²)	488 ± 29	458–518	555 ± 78 [†]	473–637
Cap per fibre (cap · fiber ⁻¹)	2.5 ± 0.4	2.1–2.9	2.5 ± 0.6	1.9–3.1

Note: BMI; body mass index; Hgb; total haemoglobin concentration; MCH; mean corpuscular haemoglobin; MCHC; mean corpuscular haemoglobin concentration; VO_{2max}; maximal oxygen uptake. MFO; maximal fat oxidation. Fat_{max}; exercise intensity where maximal fat oxidation occurs. Time MFO (min); maximal fat oxidation test duration; Mb; Myoglobin; CS; citrate synthase. CSA; cross-sectional area; Cap; Capillaries. Data are means ± s. CI; confidence interval (95%); * P < 0.05 Pre vs. Post recombinant erythropoietin treatment.

instead of acute effects of erythropoietin. Both pre and post treatment visits were separated by at least 3 days. In visit one, participants underwent physical performance measurements whereas visit two was devoted for blood extraction and muscle biopsies. Participants were instructed to abstain from alcohol and strenuous exercise the day before each visit. Body composition was measured with a dual-energy X-ray absorptiometry scan (Lunar Prodigy Advance; Lunar, Madison, WI, USA). Maximal fat oxidation was determined by a graded exercise test (test 1) on a bicycle ergometer (ER800; Jaeger, Würzburg, Germany), commencing at 60 W (submaximal fat oxidation) for 5 min, followed by 35 W increases every 3 min until the respiratory exchange rate (RER) was above 1.0 for a full interval of 3 min. After that, participants continued exercising until exhaustion at graded increase intensity steps of $35 \text{ W} \cdot \text{min}^{-1}$, where maximal oxygen uptake ($\text{VO}_{2\text{max}}$) was determined. Participants also performed another graded cycling exercise test (test 2) until exhaustion to confirm the $\text{VO}_{2\text{max}}$ values from test 1. The detailed description of test 2 has been reported in Plenge et al. 2012. No differences were observed between $\text{VO}_{2\text{max}}$ values measured by either test 1 or 2. Online equipment (Oxycon Pro; Jaeger, Würzburg, Germany) were used to measure O_2 consumption and CO_2 production and were calibrated every day before use with a known gas composition. At their second visit, during fasting conditions, a blood sample was taken followed by a muscle biopsy obtained from *m. vastus lateralis* using the Bergstrom needle modified for suction. The muscle specimens were cleaned to remove any visible blood, fat or connective tissues. A portion of the obtained muscle tissue was frozen immediately in liquid nitrogen and stored at -80°C for later biochemical analysis. Another portion of muscle sample was mounted in embedding medium and frozen in iso-pentane, cooled to its freezing point for subsequent capillary staining. The time needed to obtain and freeze the muscle biopsies was below 30 secs. Finally, a small portion of the biopsied muscle was placed in an ice-cold relaxing medium [biopsy preservation solution (BIOPS); 10 mM Ca-EGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM $\text{K}^+ / 4$ -morpholinoethanesulfonic acid, 0.5 mM dithiothreitol, 6.5 mM MgCl_2 , 5.8 mM ATP, 15 mM phosphocreatine, pH 7.1] to measure mitochondrial respirometry.

Recombinant human erythropoietin treatment

The treatment aimed to increase the haematocrit to approximately 50% and to maintain that value throughout the study period, and the procedures followed have been described previously in full detail

(Plenge et al., 2012). Briefly, participants were given one daily subcutaneous rhEPO (NeoRecormon, Roche, Schweiz) injection for 1 week (5000 IU) and followed by titrating the recombinant erythropoietin dosage depending on their haematocrit level (giving either 0, 2500, or 5000 IU) once per week for 7 weeks. Haematocrit and haemoglobin were therefore controlled every week by a blood gas analyser (Radiometer, ABL 800, Denmark). The participants were also instructed to take daily iron supplements of 100 mg throughout the study period, starting 2 weeks prior to the treatment, in order to ensure the lack of iron deficiency that would hamper erythropoiesis (Schaefer & Bahner, 2000).

High resolution respirometry

Individual fibre bundles were placed in an ice-cooled petri dish containing BIOPS and then separated using forceps with sharp tips under a microscope and all visibly (under microscope) damaged fibres were discarded. To ensure complete permeabilisation, the fibres were incubated by gentle agitation at 4°C for 30 min in 3 mL BIOPS solution containing $50 \mu\text{g} \cdot \text{mL}^{-1}$ saponin. After chemical permeabilisation, the tissue was washed twice for 10 min in ice-cold mitochondrial respiration medium (MIR05; EGTA (0.5 mM), $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ (3 mM), K-lactobionate (60 mM), Taurine (20 mM), KH_2PO_4 (10 mM), HEPES (20 mM), sucrose (110 mM), BSA ($1 \text{ g} \cdot \text{L}^{-1}$), at pH 7.1). The muscle bundles were then blotted and measured for wet weight on a precision balance. Two to three milligrams of muscle fibres were transferred immediately into the respirometer chambers (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) containing 2.0 mL BIOPS at a chamber temperature of 37°C .

Oxygen concentration ($\mu\text{M} = \text{nmol} \cdot \text{mL}^{-1}$) and oxygen flux ($\text{pmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$; negative time derivative of oxygen concentration, divided by muscle mass per volume) were recorded using DatLab software (Oroboros Instruments). All measurements were done at hyperoxic conditions ($200\text{--}450 \text{ nmol } \text{O}_2 \cdot \text{mL}^{-1}$) in order to avoid oxygen limitation in the respirometers. Four instruments were operated in parallel. Standardised instrumental and chemical calibrations were undertaken on the Oxygraph-2k high-resolution respirometer and applied to correct the back-diffusion of oxygen into the chamber, leak from the exterior, oxygen consumption by the chemical medium and O_2 sensor. All muscle respirometry measurements were done in duplicate. A substrate-inhibitor titration protocol was applied as follows. Baseline respiration (state 2; absence of adenylates) with electron supply of NADH to complex I was assessed by addition of

malate (2 mM). Then, octanoylcarnitine (0.2 mM steps) was titrated to support electron entry from fatty acid β -oxidation through electron-transferring from flavoprotein and complex I to coenzyme Q. Active respiration was stimulated by ADP (2.5 mM; state 3). Further, we added complex I substrates glutamate and pyruvate (both 10 mM). Succinate (10 mM) was now added for combined complex I+II linked respiration, and therefore showing maximal coupled physiological respiratory capacity of the electron transport system. Intactness of the outer mitochondrial membrane was tested by quantifying respiration after titration of cytochrome C ($10 \mu\text{mol} \cdot \text{L}^{-1}$). Finally, residual O_2 respiration was measured after addition of antimycin A, which blunts complex III, and therefore the entire electron transport chain.

Capillary density, β -hydroxy-acyl-CoA-dehydrogenase and Citrate Synthase determinations

Muscle capillary density was analysed and quantified as described by Qu, Andersen, and Zhou (1997). Subsequently, fibre size and capillary density were determined in a minimum of 200 muscle fibres per subject using a computerised quantification system (Tema Scanbeam, Hadsund, Denmark). All visible damaged cells (disrupted areas) were discarded during the analysis.

The maximal activity of the enzymes β -hydroxy-acyl-CoA-dehydrogenase and citrate synthase was determined fluorometrically, using the methodology described previously (Andersen, Schjerling, Andersen, & Dela, 2003).

Myoglobin concentration

The concentration of myoglobin was measured spectrophotometrically using a modified version of the method by Reynafarje (1963) that takes advantage of the characteristic myoglobin absorbance spectrum to detect specifically myoglobin but not any other haeme proteins. A 19.25 mL buffer g^{-1} of tissue (0.04 M potassium phosphate, pH 6.6) was added to muscle biopsies weighing ~ 30 mg and samples were homogenised for 5×1 min with 1 min intervals in between on ice using a TissueLyser LT homogeniser (Quiagen). Samples were centrifuged for 50 min at 15,000 g at 4°C , and the supernatant was equilibrated with pure CO gas for 3 min. A pinch of dithionite was then added and the sample was equilibrated with CO for 1 more min. Finally, the absorbance was recorded at 538 and 568 nm using a HP 8543UV-visible diode array spectrophotometer and the concentration of myoglobin (Mb) was calculated using the formula:

$$C_{\text{mg/g wet weight}}^{\text{Mb}} = (\text{OD}_{538} - \text{OD}_{568}) \times 117.3 \quad (1)$$

where OD = optical density. Longer CO equilibration times made no changes to the Mb concentration measurements and samples were therefore considered as being 100% CO saturated.

Total protein extraction, electrophoresis, and western blot analysis

Muscle protein extracts were prepared as described previously (Guerra et al., 2007). Total protein content was quantified using the bicinchoninic acid assay (Smith et al., 1985). Briefly, proteins were solubilised in sample buffer containing 0.0625 M Tris -HCl, pH 6.8, 2.3% (wt/vol) sodium dodecyl sulfate, 10% (vol/vol) glycerol, 5% (vol/vol) beta-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue. Equal amounts (50 μg) of each sample were electrophoresed on SDS-PAGE 10% Criterion gels (Bio-Rad, Copenhagen, Denmark) and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes in a tank buffer system [transfer buffer contained 25 mM Tris, 192 mM glycine and 20% methanol (wt/vol)]. To determine the total amount of vascular endothelial growth factor (VEGF), an antibody directed against the total form of this angiogenesis marker (Cat. no. j806; Santa Cruz Biotechnology, Inc., Heidelberg, Germany, validated in (Ringholm et al., 2011)) was diluted in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBS-T) (BSA-blocking buffer). To control the differences in loading and transfer efficiency across membranes, they were incubated with a monoclonal mouse anti-alpha-tubulin antibody diluted in TBS-T with 5% blotting grade blocker, non-fat, dry milk (blotto-blocking buffer). No significant changes were observed in alpha-tubulin protein levels during the experiments (data not shown). Antibody-specific labelling was revealed by incubation with an HRP-conjugated goat anti-rabbit antibody (1:20,000) diluted in 5% blotto blocking buffer and visualised with the ECL Western blotting detection system using a CCD (LAS-3000 Luminescent Image Analyser; Fujifilm, Tokyo, Japan) and quantified by the "Multi Gauge" analysis software (ver. 3.0; Fujifilm). In addition, two human muscle samples obtained from a healthy young man were used as internal controls on all gels.

Calculations

The whole body fat oxidation was calculated from the respiratory quotients during the last 60 s of each exercise step in the graded exercise test using

standard indirect calorimetry equations (Fraysn, 1983). As previously described, the maximal whole body fat oxidation and the exercise intensity that elicits this are then estimated based on the mathematical modelling of each subject's data (Nordby, Saltin, & Helge, 2006).

Statistics

Variables were checked for normal distribution by using the Kolmogorov-Smirnov test. Paired *t*-tests were run to elucidate significance between single variables pre- and post-erythropoietin treatment. The expected power based on the paired *t*-test performed for the mitochondrial respirometry data (β -oxidation) was 88.3% given the observed changes and the calculated *s*.

A one way-ANOVA test was used to detect changes in the time-course of respiration measurements before and after intervention. Values are reported as the mean \pm *s* of the mean. $P \leq 0.05$ was considered significant. Statistical analysis was performed using SPSS v.15.0 for Windows (SPSS Inc., Chicago, IL). Effect size statistics were calculated using Cohen's *d* (G*Power Version 3.1.2), being the cut-offs small (from 0.2 to 0.5), medium (from 0.5 to 0.8) or large (over 0.8).

Results

Blood and performance responses to recombinant erythropoietin treatment

Subject characteristics are provided in Table I. Participants' BMI, whole body lean mass and fat mass remained unchanged through the study. Recombinant erythropoietin treatment significantly increased haematocrit and the maximal oxygen uptake rate (VO_{2max}). These results have already been published in a previous article from our laboratory (Plenge et al., 2012). Total haemoglobin concentration was significantly increased after the intervention ($P < 0.05$, Cohen's *d* = 2.08; Table I), whereas both mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were unaltered after the completion of the study (Table I).

The maximal whole body fat oxidation remained unchanged after 8 weeks of the recombinant erythropoietin treatment (Table I). However, both the time and the exercise intensity needed to reach maximal fat oxidation were significantly increased after treatment by 7.7% and 8.1%, respectively ($P < 0.05$, Cohen's *d* = 1.03 and 0.96, respectively, Table I).

Mitochondrial respiration

After 8 weeks of the intervention, a 1.9-fold increase was observed in state 3 O_2 flux after the addition of malate, octanoylcarnitine and ADP to the permeabilised muscle fibres (electron input from complex I and fatty acid β -oxidation, $P < 0.05$, Cohen's *d* = 1.79; Figure 1). The titration of additional substrates (glutamate, pyruvate and succinate for complexes I and II, respectively) resulted in an increase in respiratory rates such that maximal coupled state 3 flux rate with convergent electron supply was further elevated (60 ± 13 vs. 87 ± 24 $\text{pmol} \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$ $P < 0.05$; Cohen's *d* = 1.53; Figure 1A). The addition of cytochrome C did not result in significant increases in O_2 flux, ensuring the intactness of the outer mitochondrial membrane (data not

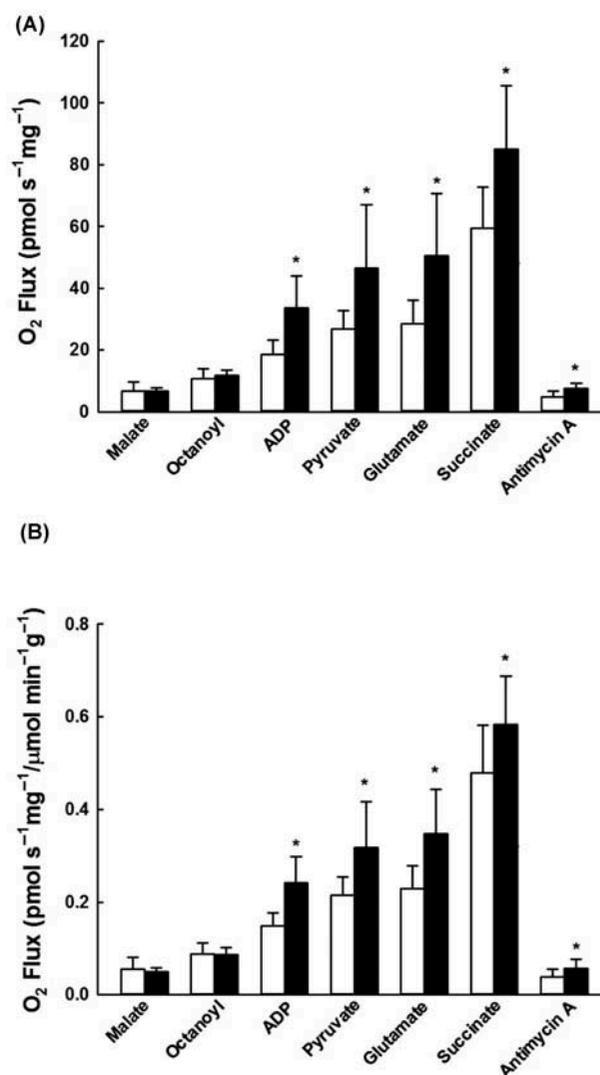


Figure 1. Mitochondrial respiration. O_2 flux in permeabilised skeletal muscle fibres before (white bars) and after (black bars) recombinant erythropoietin treatment. Data shown as O_2 flux per mg of tissue (A), and further normalised to the citrate synthase activity (B). Data shown as means \pm *s*. * $P < 0.05$ Pre vs. Post.

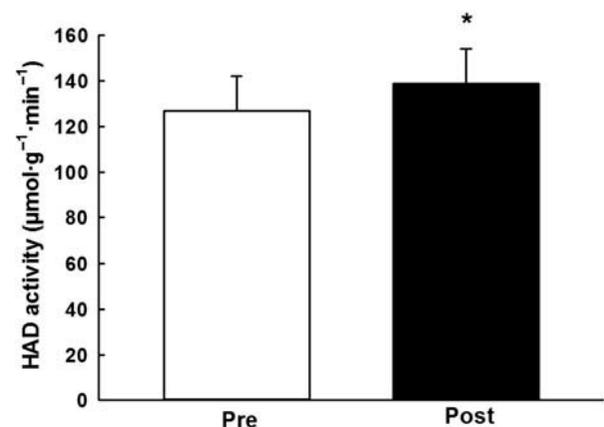


Figure 2. Muscle β -hydroxy-acyl-CoA-dehydrogenase (HAD) activity. Data shown as means \pm s. * $P < 0.05$ Pre vs. Post.

shown). Subsequent inhibition of complex III by antimycin A blunted the O_2 flux in all samples, but the remaining residual oxygen consumption was elevated after treatment ($P < 0.05$; Cohen's $d = 1.69$; Figure 1A). Mitochondrial enzyme citrate synthase activity tended to increase with the intervention, but did not reach a statistical significance ($P = 0.06$, Cohen's $d = 0.44$; Table I). When mitochondrial O_2 flux was normalised by citrate synthase activity (a marker of mitochondrial content), the significant increases observed on O_2 flux per mg of wet weight were maintained (All $P < 0.05$, Figure 1B). Moreover, mitochondrial β -oxidation measured by enzymatic β -hydroxy-acyl-CoA-dehydrogenase activity was 9% higher after the 8 weeks recombinant erythropoietin program ($P < 0.05$, Cohen's $d = 0.87$; Figure 2).

Adding succinate to the chamber resulted in an increase in respiratory rates which was higher during post-treatment than in pretreatment. Thus, substrate control ratio for succinate was 0.5 ± 0.1 and 0.6 ± 0.1 ($P < 0.05$, Cohen's $d = 1.1$), respectively.

Muscle oxygen buffering capacity and angiogenesis

The total number of capillaries per mm of analysed muscle area tended to increase with the intervention (from 488 ± 29 to 555 ± 78 cap \cdot mm $^{-2}$, $P = 0.07$, Cohen's $d = 1.25$ Table I); however, the number of capillaries per fibre (Table I), as well as the total protein expression of the vascular endothelial growth factor (Figure 3) remained unchanged.

To obtain further insights into the interactions between iron and oxygen regulation in skeletal muscle tissue, we analysed myoglobin protein concentration. A significant increment of 16.5% in the amount of total myoglobin was detected after treatment ($P < 0.05$; Cohen's $d = 0.88$; Table I).

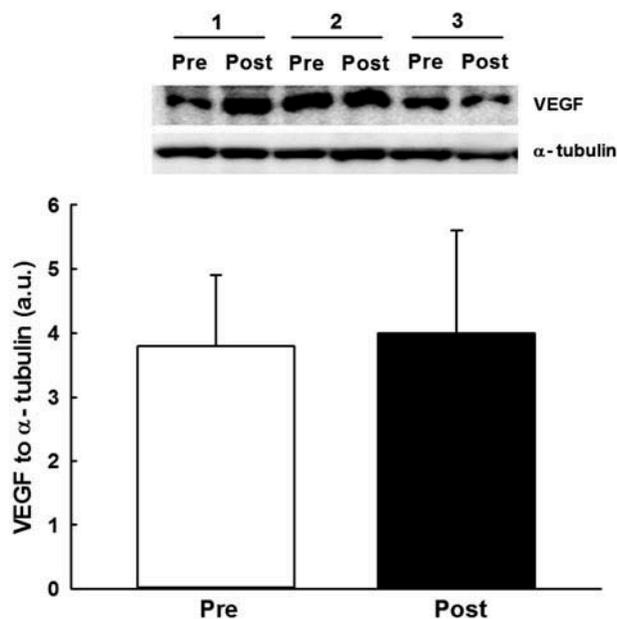


Figure 3. Muscle vascular endothelial growth factor (VEGF) expression. Top panel: representative Western blot assay from three of the participants to determine the total amount of VEGF expression to α -tubulin before (white bars) and after (black bars) rhEPO treatment. Bottom panel: densitometric analysis of VEGF immunoblots (arbitrary units of band densities). Values are relative to α -tubulin. Data shown as means \pm s.

Discussion

In agreement with our hypothesis, we have shown that both mitochondrial fatty acid oxidation and myoglobin expression are significantly increased by 8 weeks of the recombinant erythropoietin treatment. The results were supported by higher muscle β -hydroxy-acyl-CoA-dehydrogenase enzymatic activity, but these changes did not lead to a higher whole body maximal fat oxidation during exercise until exhaustion.

Recombinant erythropoietin increases mitochondrial fat oxidation but not systemic maximal fat oxidation

The primary novel finding in this study is that ADP stimulated state 3 mitochondrial O_2 flux capacity with electron flux from complex I+II and fatty acid β -oxidation is substantially increased after 8 weeks of the recombinant erythropoietin treatment. Moreover, when mitochondrial O_2 flux is normalised by citrate synthase activity (i.e., mitochondrial volume marker) the levels of oxidative phosphorylation in presence of fatty acids remain elevated. The independent measurement of hydroxy-acyl-CoA-dehydrogenase enzymatic activity as an oxidoreductase that specifically targets the third step of β -oxidation (the oxidation of L-3-hydroxyacyl CoA by NAD^+) was also higher after treatment. These results provide experimental evidence for an increased mitochondrial fatty acid

oxidation with erythropoietin. Animal and human studies evaluating the metabolic effects of erythropoietin mainly show increases in RER and protection against high-fat-diet-induced obesity by an increased muscle fat oxidation (Christensen, Lundby, et al., 2012; Christensen et al., 2013; Hojman et al., 2009; Meng, Zhu, Bi, Yang, & Wang, 2013). In young healthy males, Christensen, Vendelbo, et al. (2012) demonstrated that RER was higher after an acute high dose ($400 \text{ IU} \cdot \text{kg}^{-1}$) infusion of recombinant erythropoietin, together with a tendency for an increased basal whole body fat oxidation. However, specific energy sensing muscle key proteins regulating influx of fatty acids to the mitochondria, such as AMP-activated protein kinase and Acetyl CoA carboxylase were not altered by the treatment. The same group has confirmed the raise in RER after a prolonged erythropoiesis-stimulating agent treatment in healthy sedentary participants, with no underlying molecular explanation other than an increased thermogenesis through higher mRNA expression of the mitochondrial uncoupler UCP2 (Christensen et al., 2013). In partial agreement with the thermogenesis theory suggested by the authors, we have previously reported higher uncoupled mitochondrial respiration in the same participants included in the present investigation (Plenge et al., 2012). On the contrary, some other studies have not found any effect of prolonged erythropoietin administration on muscle hexokinase or membrane transport system levels (Juel, Thomsen, Rentsch, & Lundby, 2007), erythropoietin receptor-mediated signalling cascades (Christensen, Lundby, et al., 2012), muscle phenotype and angiogenesis (Lundby, Hellsten, Jensen, Munch, & Pilegaard, 2008), suggesting that the effects of erythropoietin on skeletal muscle are likely indirect, i.e., through increased oxygen delivery.

The results obtained from this investigation cannot rule out a possible indirect effect of erythropoietin on the skeletal muscle by means other than erythropoietin receptor, and further research is needed to elucidate the intracellular mechanisms responsible for the higher mitochondrial fat oxidation capacity observed.

Other authors have shown that muscle mitochondrial oxidative phosphorylation capacity is in excess to systemic O_2 delivery during cycling (Boushel & Saltin, 2013). This finding further support our results, because a higher O_2 delivery capacity of the bloodstream, together with the increased myoglobin concentration and the higher mitochondrial respiration induced by the erythropoietin treatment, did not increase maximal whole body fatty acid oxidation, despite a higher $\text{VO}_{2\text{max}}$. Our results imply that in healthy young males maximal whole body fat oxidation during exercise is not affected by an increase in either the oxygen delivery capacity of the blood or

the capacity to oxidise fat from the mitochondria. However, in diseases where dysfunctional mitochondria are present, an increased oxygen delivery may exert an effect on systemic fat oxidation.

Recombinant erythropoietin increases myoglobin concentration, and has no effect on angiogenesis

In an attempt to further elucidate alternative intracellular mechanisms leading to erythropoietin effects on mitochondria, we evaluated the intracellular concentration of myoglobin, the haemoprotein responsible for transporting O_2 within the skeletal muscle cell which is found in all mammals, at approximately $400\text{--}500 \mu\text{mol} \cdot \text{kg}^{-1}$ wet weight (Wittenberg & Wittenberg, 2003). The intracellular concentration of myoglobin is closely associated with the work performed by the tissue, and correlates with cytochrome C oxidase expression as well as capillary density (Lawrie, 1953; Reis & Wooten, 1970). Moreover, data on cardiomyocytes support an intracellular gradient of oxygenated myoglobin, with maximal oxygenation at the cell membrane, and a progressive lowering towards the respiring mitochondria (Takahashi, Endoh, & Doi, 2000). Unfortunately, we cannot establish a cause-effect relationship between our myoglobin and mitochondrial respiration measurements, because the high resolution respirometry technique used relies on oxygen saturation conditions in the chamber, to avoid oxygen limitations during measurements (Gnaiger, 2001). In consequence, this fact should be taken into consideration as a limitation of this study, and our myoglobin and mitochondrial respiration results should be analysed separately.

We observed a 16% increase in our participants' myoglobin concentration after 8 weeks of recombinant erythropoietin administration at the basal state. An earlier study found a decreased myoglobin mRNA expression 10 h after acute injections of 15,000 IU recombinant erythropoietin (Lundby et al., 2008). The discrepancy with our results may be explained by the application of different techniques to assess myoglobin levels (protein concentration vs. mRNA expression), the different time points selected to measure the gene expression of myoglobin, and the fact that maybe a single acute infusion of recombinant erythropoietin does not provide sufficient stimulus to produce a positive myoglobin mRNA expression. Studies performed in rats support our results, by co-localisation of myoglobin and mitochondrial complex IV, which suggest a direct myoglobin-mediated O_2 delivery to the mitochondria that in turn may play a potentially significant role for respiration (Yamada et al., 2013). In consequence, more studies to further elucidate the potential role of myoglobin on the skeletal muscle

adaptations to a recombinant erythropoietin treatment are needed.

Erythropoietin appears to promote angiogenesis by enhancing the level of vascular endothelial growth factor in endothelial cells (Alvarez Arroyo et al., 1998). We did not observe a significant effect of 8 weeks recombinant erythropoietin treatment on the skeletal muscle vascular endothelial growth factor expression as an early stage of angiogenesis. In agreement, we did not observe a significant increment in the number of capillaries per analysed muscle fibres, despite a tendency to increase the number of capillaries per analysed muscle area. Another study performed in humans reached similar results (Lundby et al., 2008), suggesting that erythropoietin alone cannot initiate angiogenesis in human skeletal muscle under basal conditions.

Limitations of the study

Two considerations should be addressed as limitations of this investigation. First, the lack of a control group not receiving any recombinant erythropoietin treatment, and second, the low number of participants recruited, although the statistical power was enough to show significant results for the dependent variables. In consequence, more studies are needed where a control group and more participants are included to confirm our results.

In summary, this study provides evidence for a higher mitochondrial fatty acid oxidation, as well as higher myoglobin concentration after a prolonged recombinant erythropoietin treatment. Maximal whole body fat oxidation during exercise is not affected by an increase in either the oxygen delivery capacity of the blood or the capacity to oxidise fat from the mitochondria in healthy males. However, recombinant erythropoietin treatment could be useful for clinical populations of patients where dysfunctional mitochondria are involved.

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