Erythropoietin enhances whole body lipid oxidation during prolonged exercise in humans

Corinne Caillaud · Philippe Connes · Helmi Ben Saad · Jacques Mercier

Abstract Animal studies have suggested that erythropoietin, besides its well-known hematopoietic effects, can modulate metabolism and prevent fat accumulation. We investigated the effects of repeated injections of recombinant human erythropoietin (EPO) on the balance of substrate oxidation during aerobic exercise in humans. Twelve healthy aerobically trained males received subcutaneously either moderate dose of EPO (50 U/kg, EPO) or saline injections (NaCl 0.9 %, control) three times a week for 4 weeks. Body weight, % fat, maximal aerobic capacity, and substrate utilization during exercise were assessed before and after treatment, while hemoglobin and hematocrit were monitored regularly during the treatment. Carbohydrate and fat oxidation were evaluated via indirect calorimetry, during a submaximal exercise performed at 75 % of the participants' maximal aerobic capacity ($\dot{V}O_{2max}$) for 60 min. Results showed that 4 weeks of EPO treatment significantly enhanced fat oxidation (+56 % in EPO versus −9 % in control) during exercise, independent of its effects on hematological parameters or $\dot{V}O_{2max}$. This study shows that EPO can modulate substrate utilization during exercise, leading to enhanced fat utilization and lower use of carbohydrates. This opens new research directions exploring whether systemic EPO levels, in physiological conditions, participate to the modulation of fat oxidation.

Keywords Indirect calorimetry · Metabolism · Submaximal exercise · Lipid oxidation · Human

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Introduction

Erythropoietin is a glycosylated protein hormone, well known as the primary factor controlling red blood cells formation. Since the introduction, in 1989, of human recombinant erythropoietin (EPO) for the treatment of anemia in end-stage kidney disease (ESKD), studies showed that EPO also has extra-hematopoietic effects, primarily as a tissue protective agent with anti-oxidant and anti-apoptotic properties [3, 28, 33]. Recently, EPO has emerged as a potential modulator of metabolism. Animal studies showed that exogenous EPO [12, 15] prevented increases in body weight and fat mass in mice fed with a high-fat diet. Models using genetically modified mice (Tg-mice) expressing the erythropoietin receptor (EPOR) only in hematopoietic cells, showed that disruption of EPO signaling in metabolic tissues quickly led to the development of obesity and insulin resistance [29]. Conversely, in other models, mice overexpressing erythropoietin were protected against obesity [17]. In vitro, skeletal muscle homogenates overexpressing EPO displayed increased oxidation of exogenous palmitate [15]. These animal studies suggest that the balance between fat utilization and storage may be modulated by EPO. Whether EPO promotes fat oxidation or modifies body composition in humans is still unknown. Early investigations conducted in patients with ESKD did not report modifications of body mass index [2, 20, 31]. In young healthy subjects, acute dosage with EPO increases energy expenditure at rest within 2 h following injection [6] with non-significant changes in fat oxidation. However, the metabolic influence of EPO in humans may exert its full effect during muscle activity, when fuel utilization increases in response to energy demand. In that case, an increased utilization of fat by active muscles will make a significant difference in the balance between fat storage and utilization.

We hypothesized that EPO treatment could promote fat oxidation during exercise. To verify this hypothesis, we investigated the balance of substrate utilization during aerobic exercise in healthy subjects both before and after 4 weeks of moderate dose EPO treatment.

Materials and methods

Study design

This study is part of a larger project [7, 8–10] and has received ethical approval from the local ethical comity (Comité Consultatif de Protection des Personnes en Recherche Biomédicale de Montpellier, Hôpital Saint Eloi, Montpellier, France). The study has been conducted according to the principles expressed in the Declaration of Helsinki. The protocol (Fig. 1) was explained to participants who provided written informed consent. The metabolic study included 12 healthy aerobically trained young men randomly assigned to either EPO (n=6, 26.8±4.1 years) or control group (n=6, 26.8±6.0 years). Participants first attended the laboratory for screening, and baseline measures of anthropometry, blood parameters, and maximal aerobic capacity ($\dot{V}O_{2max}$). Body composition was assessed with a multi-frequency (1, 5, 10, 50, 100 kHz) bioelectrical impedance-meter (HUMAN IM Scan, Dietosystem, Milan, Italy). Blood samples were collected at rest and further analyzed (Pentra 120 Retic Hematology Analyzer, France). Within 1 week, participants performed a 60-min steady state aerobic exercise test at a workload corresponding to 75 % of $\dot{V}O_{2max}$ (LOAD1-pre). They then received subcutaneous injections of EPO (Eprex®, France), 50 U/kg three times a week (EPO group) or NaCl 0.9 % (control group) for 4 weeks. Both groups also received a daily oral dose of iron sulfate (Ferograd®, Abbott Laboratories, France), vitamin B_9 (SpeciaFoldine®, Théraplix Aventis, France), and vitamin B_{12} (Gerda®, Gerda Laboratories, France) [8, 10]. Hematocrit (Hct), hemoglobin (Hb), and training characteristics were followed during the whole experiment. Hct was monitored three times weekly and, for ethical reasons, dose was adjusted to maintain Hct below 50 %. EPO treatment was stopped and injections done with saline when Hct reached 50 %. After the 4-week treatment period, body weight, percentage of fat and $\dot{V}O_{2max}$ were re-assessed. Constant load exercise was performed at 75 % of $\dot{V}O_{2max}$ pre- and post-treatment. All participants were physically active, trained four to five times a week and were asked to maintain their habitual physical activity during the study period. The training load was quantified weekly for each subject using a model that takes into account both the volume and the intensity of each training session [23]. For each participant, blood data were followed during a wash-out period of 3 weeks or until Hct, Hb, and reticulocytes count were back to baseline levels. The total follow-up for each individual averaged 63 days. Participants were not allowed to participate in any competitive or official sporting event before normalization of Hct and Hb (at least 61 days).

Exercise tests

All exercise tests were performed on an electrically braked cycle ergometer (Jaeger Ergoline 800S, Germany) and gas exchanges were continuously sampled and analyzed by an automated system (Jaeger Oxycon

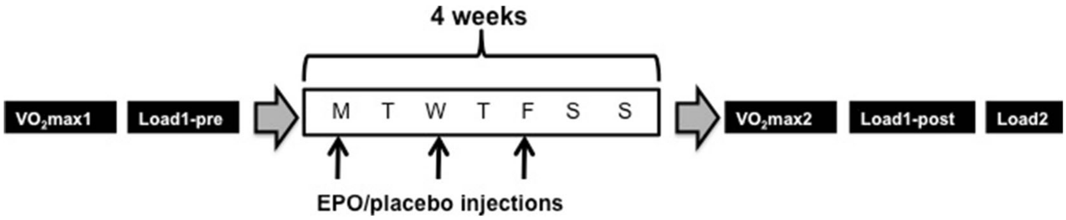

Fig. 1 Study design. Participants performed a VO_{2max} test ($\dot{V}O_{2max}$ 1) and a 1-h constant load exercise at 75 % of $\dot{V}O_{2max}$ before EPO treatment (LOAD1-pre). During the 4 weeks of the experiment, they received subcutaneous injections of either EPO (Eprex®, 50 U/kg) or saline (NaCl 0.9 %) three times a week. On week 5, participants performed the second VO_{2max} test ($\dot{V}O_{2max}$ 2) followed by two 1-h constant load exercises performed at 75 % of VO_{2max} 1 (LOAD1-post) and 75 % of $\dot{V}O_{2max}$ 2 (LOAD2). Each exercise was performed on separate days, with a 48-h recovery period between exercise tests

Alpha, Germany). $\dot{V}O_{2max}$ was determined by using a progressive exercise test as previously described [8]. The 60-min constant load aerobic exercises were performed between 8:00 a.m. and 10:00 a.m. after a 12-h overnight fast. Subjects were asked to repeat the same diet before all subsequent trials and to refrain from exercise the day before each exercise test. The target workload was calculated from the pre-treatment $\dot{V}O_{2max}$ test and each participant exercised at this load pre- (LOAD1-pre) and post-treatment (LOAD1-post). Since we expected an increase of $\dot{V}O_{2max}$ following treatment, participants also performed exercise at 75 % of post-treatment $\dot{V}O_{2max}$ (LOAD2). Carbohydrate and fat oxidation during exercise were assessed via indirect calorimetry. This method has been validated [26] against stable isotope technique during steady state exercise. Oxygen uptake $\dot{V}O_2$, CO_2 output $\dot{V}CO_2$, and respiratory exchange ratio ($RER = \dot{V}CO_2/\dot{V}O_2$) were recorded at rest and during exercise. After a period of 15 min in sitting position on the bicycle, resting data were collected for 10 min, as the subject was quietly seated in the same position with no bodily movement. During exercise, gas exchange data were collected for 5 min every 10 min. The data recorded during the first 10 min of exercise, as the subjects were still adjusting to the exercise intensity, were not taking into account in the analysis. Data were filtered for aberrant values and then averaged over each 5-min period. Calculation of carbohydrate and fat oxidation was made according to the table of non-protein respiratory exchange ratio (RER) [24]:

$$\text{Carbohydrate oxidation rate}\ \left(\mathrm{g.min}^{-1}\right) = 4.585 \times \dot{V}CO_2 - 3.226 \times \dot{V}O_2$$

$$\text{Fat oxidation rate}\ \left(\mathrm{g.min}^{-1}\right) = 1.695 \times \dot{V}O_2 - 1.701 \times \dot{V}CO_2$$

with $\dot{V}CO_2$ and $\dot{V}O_2$ expressed in liters per minute.

Statistical analysis

Data were tested for normality and equality of variance and analyzed using a two-way analysis of variance (ANOVA), i.e., groups (control versus EPO) with repeated measures, i.e., time (pre- versus post-treatment). Detailed analysis of fat oxidation during exercise was conducted using a two-way ANOVA with repeated measures including time (pre- versus post-treatment) and exercise (6 time points). Where relevant, specific effects were tested using a Bonferroni test for multiple comparisons. Correlations were investigated using the Pearson product-moment coefficient. The significance level for all comparisons was set at $P<0.05$. The statistical analysis was conducted with SPSS software (v. 20, IBM SPSS Statistics, Chicago, IL).

Results

Anthropometry and hematology

Participants were lean with an average body fat of 6.5 % (Table 1). Four weeks of EPO treatment led to a small non-significant change in percentage of body fat (interaction between time and group: $F\,(1,10)=4.33$, $P=0.076$, Table 1) with no modification of body weight ($P>0.05$, Table 1). Hct and Hb were within normal range at baseline and increased significantly following EPO treatment (interaction between time and group: $F\,(1,10)=18.7$, $P=0.002$ and $F\,(1,10)=9.78$, $P=0.011$ for Hct and Hb, respectively; Table 1). As a result, both Hct and Hb were significantly elevated in EPO group

Table 1 Anthropometry, blood, and submaximal exercise data

	Time	Control (n=6)	EPO (n=6)
Weight (kg)	Pre	69.5±6.7	69.8±8.4
	Post	69.9±6.9	69.8±8.3
Fat (%)	Pre	6.3±2.2	6.7±1.6
	Post	6.7±2.6	5.8±0.8
Hb ($g.dl^{-1}$)**	Pre	14.6±0.9	14.8±0.4
	Post	14.5±1.5	16.2±1.0*, ***
Hct (%)**	Pre	44.3±2.9	44.0±2.9
	Post	44.2±4.1	49.4±3.7*, ***
$\dot{V}O_{2max}$ ($ml.kg^{-1}.min^{-1}$)**	Pre	63.0±4.8	61.9±4.6
	Post	62.0±6.4	65.8±5.9***
Resting fat oxidation rate ($g.min^{-1}$)	Pre	0.10±0.04	0.10±0.05
	Post	0.10±0.03	0.16±0.07
Workload (W)*	Pre (LOAD1)	249±23	247±31
	Post (LOAD2)	250±23	269±31
RER, LOAD1**	Pre	0.91±0.02	0.92±0.04
	Post	0.92±0.02	0.89±0.02*, ***
Carbohydrate oxidation rate, LOAD1 ($g.min^{-1}$)**	Pre	3.52±0.37	3.99±0.31
	Post	3.72±0.36	3.26±0.37***

Exercise data for RER and carbohydrate oxidation are calculated over the last 20 min of exercise. Values are mean±SD

*$P<0.05$, EPO versus control (Bonferroni test)

**$P<0.05$, for interaction between time and group (two-way ANOVA with repeated measures)

***$P<0.05$, pre versus post within group (Bonferroni test)

following treatment compared to control and baseline ($P<0.05$).

Exercise tests and substrate utilization during aerobic exercise

$\dot{V}O_{2max}$ levels before treatment showed that participants were aerobically fit with an average $\dot{V}O_{2max}$ of 63.0±4.8 $ml.kg^{-1}.min^{-1}$ (4408±294 $ml.min^{-1}$) and 61.9 ±4.6 $ml.kg^{-1}.min^{-1}$ (4468±512 $ml.min^{-1}$) in control and EPO, respectively (Table 1, no difference between groups). The training load was maintained with no statistical difference between groups and over time during intervention ($P>0.05$, Fig. 2). $\dot{V}O_{2max}$ increased in EPO group following treatment (interaction between time and group: F (1,10)=5.28, P=0.044) and was unchanged in control. There was also a slight increase in maximal power output (+7.3 % P=0.07) in the EPO group. The average $\dot{V}O_2$ during LOAD1-pre exercise was 3.34±0.37 $l.min^{-1}$ and 3.42±0.31 $l.min^{-1}$ in control and EPO, respectively. After treatment, $\dot{V}O_2$ during LOAD1-post was 3.32±0.42 and 3.55±0.25 $l.min^{-1}$ in control and EPO groups, respectively (~75.5 of $\dot{V}O_{2max}$). Metabolic data averaged over the last 20-min of exercise (Table 1) shows that RER was significantly reduced in EPO compared to the control group for LOAD1-post (interaction between time and group: F (1,10)=8.89, P=0.014, Table 1) while carbohydrate oxidation rate was increased ($P<0.05$, Table 1). Accordingly, fat oxidation rate was elevated following EPO treatment during exercise at same absolute load (time× group F (1,10)=11.7, P=0.007, Fig. 3a) with no statistical difference between LOAD1-pre and LOAD2 (F (1,10)=1.78, P=0.211, Fig. 3a). Analysis of data during the course of exercise confirmed that EPO increased fat oxidation at same absolute load (time×group interaction F (1,10)=12.7, P=0.005, Fig. 3b, c), with a significant difference between groups from 30 min up to the end of exercise (ANOVA with repeated measures and post hoc comparison using Bonferroni correction, Fig. 3c). At no time point during exercise was fat oxidation at LOAD2 different between control and EPO (F (1,10)=0.52, P= 0.48, Fig. 3d). There was no significant relationship between Hct or Hb and fat oxidation rate during exercise at LOAD1 pre- or post-treatment ($P>0.05$).

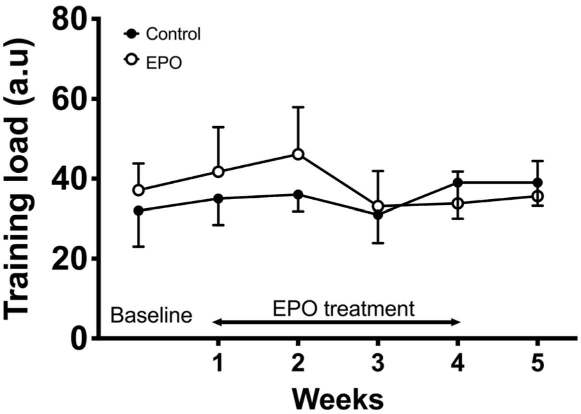

Fig. 2 Training load during 4 weeks of erythropoietin (EPO) treatment. Training load was evaluated a week before and during the whole treatment period in both groups. Training load was unchanged and did not differ between the control and EPO groups over the course of the study. Statistical analysis (two-way ANOVA with repeated measures) did not reveal statistical differences between groups or any interaction between time and groups. *Control:* control group, *EPO:* EPO-treated group, *a.u:* arbitrary units

Discussion

This study shows that a 4-week EPO treatment increased whole body fat oxidation during aerobic exercise in young healthy physically active males. The shift toward fat oxidation was not related to the enhanced aerobic capacity, or to Hb levels, suggesting a role for EPO in metabolism.

Studies conducted in ESKD patients suggested that long-term EPO treatment might alter metabolism in humans. In ESKD patients treated with EPO, plasma levels of triglycerides and total cholesterol [18, 21] were found reduced, while muscle glycogen content increased was associated with concomitant reduction in muscle fat [11]. In these patients with anemia, the metabolic changes observed following EPO treatment were thought to be mainly mediated by increased red blood cell count, Hb levels, and improved oxygen transport. In the present study, participants had normal Hb levels and the shift toward enhanced fat oxidation rate was not related to the changes in Hb. This suggests that EPO itself, potentially in synergy with exercise training, promoted fat oxidation. This study is unique in investigating the metabolic effect of EPO treatment during submaximal exercise. Results suggest that key pathways, either in adipose tissue (leading to increased fatty acid availability) or in skeletal muscle (leading to enhanced uptake and oxidation), could be activated. In resting condition, no significant change in the balance of substrate utilization could be detected, and this is in agreement with a previous study conducted in young healthy subjects showing a non-significant trend toward enhanced whole body resting fat oxidation following a single EPO injection [6]. A recent study did not provide evidence for a synergetic effect between aerobic training and darbepoietin-α treatment in previously sedentary subjects [5]. However, the study did not investigate substrate utilization during exercise. In addition, the dosage regimen of erythropoiesis-accelerating agent (ESA), as well as the participants training status, training load, and duration of the interventions differed largely between that study and ours, and this could explain some of the discrepancies. Longer study duration generally leads to a progressive decline in ESA dosage in order to maintain Hct and Hb within acceptable limits, and this may reduce the extra-hematopoietic effect of these agents.

The changes in RER and fat oxidation rate reported in the EPO group are in line with those reported following 5 to 12 weeks of aerobic training in previously untrained subjects [22, 25]. Previous research shows that whole body lipid oxidation during exercise changes with the same pattern as muscle fat oxidation rate, although active muscles used a lower percentage of lipids than the whole body [13]. The data reported here suggest that fat oxidation increased either in active muscle or inactive tissues following EPO treatment. Muscle fat metabolism during exercise is controlled by numerous factors including adipose tissue lipolysis, fatty acid delivery to the muscle, and movement across both the muscle and the mitochondrial membranes [4]. Training status, diet, and fasting state also contribute to modulate fat oxidation during exercise [14]. In the present study, the latter factors were minimized (although not controlled) since all participants were aerobically trained and were asked to maintain both habitual training regimen and diet throughout the study. Relative exercise intensity during the 60-min exercise was about 75 % of $\dot{V}O_{2max}$. At such exercise intensity, carbohydrate oxidation is the major source of energy; however, the capacity to oxidize fat plays a critical role in resistance to fatigue [1, 34]. Also, at that intensity, ~70 % of the fat oxidized comes from plasma long chain fatty acid [32], although intramuscular triglyceride can also be a significant source of fatty acid [22, 25]. The present study does not provide indications on whether the rise in total lipid oxidation is due to increased intramuscular or circulating fat oxidation.

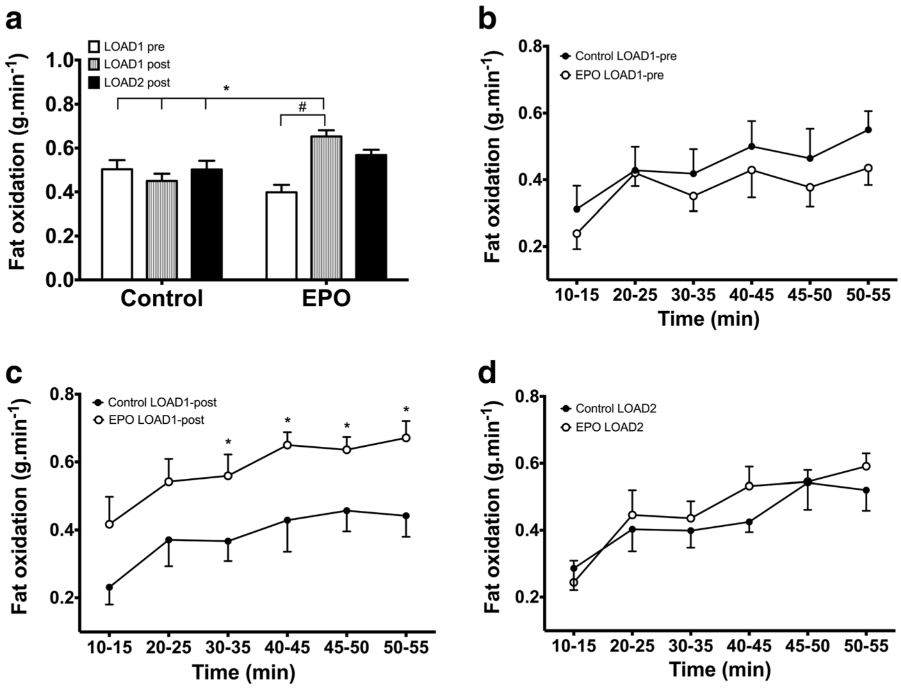

Fig. 3 Erythropoietin (EPO) treatment enhances fat oxidation rate during aerobic exercise. **a** Fat oxidation during the last 20 min of exercise at LOAD1-pre, LOAD1-post, and LOAD2 in both control and EPO groups. Statistical analysis (two-way ANOVA with repeated measures) indicated a significant interaction between time and groups and multiple comparisons with Bonferroni correction was used to assess specific effects between and within groups. *$P<0.05$ between groups, $^{\#}P<0.05$ within group as shown on figure. **b–d** Fat oxidation in control and EPO groups during 60 min of exercise performed at 75 % of pre-treatment maximal oxygen uptake ($\dot{V}O_{2max}$) **b** before (LOAD1-pre) and **c** after EPO treatment (LOAD1-post) and **d** 75 % of post-treatment $\dot{V}O_{2max}$ (LOAD2). Statistical analysis (two-way ANOVA with repeated measures) indicated a significant interaction between time and groups, and multiple comparisons with Bonferroni correction was used to assess specific effects between groups. *$P<0.05$ between groups as shown on figure

The current results show that fat oxidation rate was increased by EPO treatment at same absolute load and tended to be higher at same relative load. The lack of association between the increase in fat oxidation and the increase in $\dot{V}O_{2max}$ after EPO treatment suggests that the improvement of aerobic capacity was not a key factor in the metabolic adaptations induced by EPO injections. Another study investigating submaximal exercise, reported that 11 weeks of EPO treatment increased submaximal exercise performance (time to exhaustion measured at 80 % of $\dot{V}O_{2max}$) independent of the change in $\dot{V}O_{2max}$ [30].

In mice, muscle and systemic EPO over-expression via electro-transfer led to increased palmitate oxidation in vitro and to upregulation of genes involved in lipid metabolism while genes implicated in insulin and glucose metabolism were downregulated [15]. This led to the hypothesis that EPO can promote fat metabolism in muscle. There is less evidence of such an effect in humans. The question of the presence of the EPOR in human muscle has been raised, and studies have shown that EPOR is expressed in muscle tissue on sarcolemma and vascular cells [19]. It is interesting to note that one study showed that EPOR mRNA increased following exercise, suggesting a possible synergy between exercise and EPO [27]. It still remains to be established if EPOR is functional in human skeletal muscle. In young healthy untrained male subjects, EPO treatment has been reported to increase Hct, Hb, and $\dot{V}O_{2max}$ but does not seem to lead to major muscle adaptations [19]. In participants who did not train during the study, 15 weeks of EPO treatment (5000 U/injection, with injections once a week for the last 10 weeks) increased leg and pulmonary oxygen uptake but did not modify capillary density of muscle fibers cross-sectional area. The authors concluded that EPO exerted its main effect on aerobic capacity via increased oxygen transport and delivery, affecting $\dot{V}O_{2max}$ [19]. Other studies showed that expression of some muscle proteins, such as the alpha 2 subunit of the Na^{+}/K^{+} pump, were modified by

EPO treatment, although oxidative enzymes such as cytochrome c and hexokinase, and other major proteins involved in cross-membrane transport of metabolites, were not altered [16]. Acute EPO injection has also been studied in resting condition in human muscle and results did not demonstrate a significant effect on the activation of proteins involved in fat metabolism as ACC (acetyl-CoA-carboxylase) and AMPK (AMP-activated protein kinase) [6]. Increase fat availability via enhanced released of fatty acid from adipose tissue may be a key aspect of the modulation of fat oxidation during exercise.

In conclusion, this study shows for the first time that short-term EPO treatment has the potential to enhance fat oxidation during aerobic exercise. Although the understanding of the underlying mechanisms needs further investigations with a larger sample size, this raises the question of a role for native EPO in metabolism during exercise.

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