

## Injections of recombinant human erythropoietin increases lactate influx into erythrocytes

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**Connes, Philippe, Corinne Caillaud, Jacques Mercier, Didier Bouix, and Jean François Casties.** Injections of recombinant human erythropoietin increases lactate influx into erythrocytes. *J Appl Physiol* 97: 326–332, 2004. First published February 13, 2004; 10.1152/jappphysiol.00715.2003.—Previous studies showed that erythropoietin not only increases erythrocyte production but is also essential in both the synthesis and the good functioning of several erythrocyte membrane proteins, including band 3. It is still unknown whether anion and/or H<sup>+</sup> fluxes are modified by erythropoietin. This study aimed to evaluate the effect of recombinant human erythropoietin (rHuEPO) injections on lactate transport into erythrocytes via band 3 and H<sup>+</sup>-monocarboxylate transporter MCT-1, two proteins involved in lactate exchange. Nine athletes received subcutaneous rHuEPO (50 U/kg body mass 3 times a week for 4 wk), and seven athletes received a saline solution (placebo group). All subjects were also supplemented with oral iron and vitamins B<sub>9</sub> and B<sub>12</sub>. Lactate transport into erythrocytes was studied before and after the rHuEPO treatment at different lactate concentrations (1.6, 8.1, 41, and 81.1 mM). After treatment, MCT-1 lactate uptake was increased at 1.6, 41 ( $P < 0.01$ ), and 81.1 mM lactate concentration ( $P < 0.001$ ) although lactate uptake via band 3 and nonionic diffusion were unchanged. MCT-1 maximal velocity increased in the rHuEPO group ( $P < 0.05$ ), reaching higher values than in the placebo group ( $P < 0.05$ ) after treatment. Our results show that rHuEPO injections increased MCT-1 lactate influx at low and high lactate concentrations. The increase in MCT-1 maximal velocity suggests that rHuEPO may stimulate MCT-1 synthesis during erythrocyte formation in bone marrow.

lactate uptake; H<sup>+</sup>-monocarboxylate cotransporter; band 3

NEW DRUGS DEVELOPED FOR MEDICAL therapeutics have in some instances been misused to improve performance in competitive sports. Soon after recombinant human erythropoietin (rHuEPO) became available for the treatment of anemia, it was being used in endurance sports (13). Several years later, despite the setting up of different strategies of detection, this drug is still illegally used by some athletes. A better understanding of the physiological and biological effects of rHuEPO will help to establish preventive measures and to establish new strategies for doping detection.

It is now well known that rHuEPO administration increases aerobic power (2, 3), an effect mainly mediated by the rise in blood oxygen content (11). Erythropoietin (EPO) is a strong stimulator of erythropoiesis, which results in a significant increase in hematocrit (Hct) and total hemoglobin concentration (Hb) (18).

In addition to these effects on aerobic performance, several studies have also observed lower plasma lactate levels during

exercise in hemodialized patients or in rats treated with rHuEPO (28, 33). This may be related to different physiological or biological modifications in muscles, including metabolic adaptation or a change in lactate (and H<sup>+</sup>) fluxes. Some studies have reported changes in substrate oxidation, possibly resulting from higher oxygen delivery or indirect action of rHuEPO on muscle (23, 26, 28). The EPO receptor has indeed never been identified in muscle differentiated cells, and it is more likely that EPO mainly acts on its target cell, i.e., the erythrocyte. Two transporter proteins are involved in both lactate transport across the erythrocyte membrane and acid-base balance in blood: the band 3 (7, 10, 16, 20, 37) and the H<sup>+</sup>-monocarboxylate cotransporter (MCT-1) (7, 10, 31), which account for ~5–10% and 80–90% of total lactate influx, respectively. If rHuEPO improves uptake capacity for lactate (and H<sup>+</sup>), it will augment the ability of the erythrocyte to function as an important dilution space during intense exercise (19). In addition, the storage of lactate and H<sup>+</sup> in erythrocyte will reduce the levels of these ions in plasma, leading to greater gradients from muscle to plasma, and will potentially improve the rate of release from muscle (19).

It is known that the integration of band 3 into the erythrocyte membrane is EPO dependent (22). There are no data regarding the effect of EPO or rHuEPO on MCT-1 synthesis or activity in erythrocytes, but Juel et al. (19) recently observed a dramatic increase in MCT-1 and band 3 content in erythrocytes after exposure to chronic hypoxia. Although this was not investigated, EPO may be involved in such modifications because high altitude is well known to stimulate kidney production of EPO (24, 1). Skelton et al. (35, 36) showed that lactate transport into erythrocytes was quite variable among subjects and closely related to the level of aerobic fitness. In vitro studies showed that the total lactate influx into erythrocytes goes faster in highly trained athletes than in untrained subjects (36) at several lactate concentrations. These studies clearly indicate that lactate transport into erythrocytes may be modulated by training (36) or by hypoxia (19).

The aim of this study was to characterize the changes in erythrocyte lactate uptake capacity, particularly via the MCT-1-mediated transport, in athletes treated with rHuEPO during 4 wk. We hypothesized that repeated injections of rHuEPO would increase lactate influx into erythrocytes. In addition, plasma lactate level during incremental exhaustive exercise was measured in an attempt to verify whether it was reduced after treatment.

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## METHODS

### Subjects

Sixteen male endurance-trained athletes participated in this study after giving informed, written consent. The athletes were cyclists, runners, or triathletes. All had been involved in regular training for several years and carried on with physical activity during the study period, although they were not allowed to take part in any official competition during this period. Each subject underwent clinical and physical examinations before admission to the study. Major exclusion criteria were pulmonary, cardiovascular, muscle, metabolic, renal, and hematological diseases. Two groups were randomly constituted: the placebo group ( $n = 7$ ,  $24.3 \pm 1.8$  yr,  $177.6 \pm 2$  cm,  $68.8 \pm 2.4$  kg) and the rHuEPO group ( $n = 9$ ,  $25.4 \pm 1.4$  yr,  $178 \pm 1.6$  cm,  $71.2 \pm 2.3$  kg).

### Protocol

This study was approved by the local Ethics Committee (Comité Consultatif de Protection des Personnes en Recherche Biomédicale de Montpellier, Hôpital Saint Eloi, Montpellier, France). After inclusion in the study, the subjects performed an initial incremental exercise test conducted to maximal oxygen uptake ( $\dot{V}O_{2\max}$ ;  $\dot{V}O_{2\max-1}$ ) on cycloergometer; this day was referred to as *day 0* (D0). They then received either 50 U/kg rHuEPO (rHuEPO group) or 1 ml NaCl 0.9% (placebo group) subcutaneously three times weekly for 4 wk. At the same time, all subjects of both groups received a daily oral dose of 200 mg of iron sulfate (Ferrograd, Abbott Laboratories, Rungis, France), two tablets of vitamin B<sub>9</sub> (SpeciaFoldine, Théraplix Aventis, Paris, France), and two tablets of vitamin B<sub>12</sub> (Gerda, Gerda Laboratories, Lyon, France). Group assignment was by a double-blind procedure. During the experiment, neither the athletes nor the technicians and investigators engaged in blood sampling, analysis, and exercise testing were aware of the group assignment. The training volume was quantified using the training questionnaire of Millet et al. (29). Hematological parameters were regularly monitored. Two days after the last injection, referred as *day 25* (D25), all athletes performed another incremental exercise test conducted to  $\dot{V}O_{2\max}$  ( $\dot{V}O_{2\max-2}$ ). On both D0 and D25, blood samples were collected at rest to assess hematological parameters and intraerythrocyte lactate influx and during the exercise tests to assess plasma lactate concentrations. Blood samples were then drawn regularly from all subjects over the next 3 wk until the hematological parameters returned to basal values.

### Drug Administration

rHuEPO alpha (Eprex, Issy-les-Moulineaux, France) was supplied by Cilag. The drug was provided as a sterile buffered solution in a 1-ml ampoule containing an activity of 10,000 units/ml, which corresponds approximately to 8.4 ng rHuEPO/ml. The drug was stored at 4°C and was protected from light exposure. Before administration of rHuEPO, the solution was equilibrated to room temperature.

Each subject of the rHuEPO group received 50 U/kg 3 times a week for 4 wk. The Ethical Committee requested that we stop treatment if the Hct level rose above 50%. In two subjects, the last injection was done with NaCl 0.9% as their Hct reached this critical level. To assess tolerance, all subjects were instructed to report any abnormal events. Placebo-treated athletes received 1 ml of NaCl 0.9% three times weekly for 4 wk. All the injections were administered subcutaneously in the morning.

### Exercise Test

Each subject performed an incremental maximal exercise on cycle ergometer (Jaeger Ergoline 800S, Hoechst, Germany) before ( $\dot{V}O_{2\max-1}$ ) and after 4 wk of rHuEPO or placebo injections ( $\dot{V}O_{2\max-2}$ ).  $\dot{V}O_{2\max}$  and maximal power output ( $PO_{\max}$ ) were assessed as follows: after a 3-min warm-up at 60 W, the load was increased by steps of 30 W every minute until exhaustion. Criteria for  $\dot{V}O_{2\max}$  were

1) a respiratory exchange ratio greater than 1.10, 2) attainment of age-predicted maximal heart rate (HR) [ $210 - (0.65 \times \text{age}) \pm 10\%$ ], 3) an increase in oxygen uptake lower than 100 ml with the last increase in work rate, and 4) an inability to maintain pedaling frequency above 60 rpm despite maximum effort and verbal encouragement. During the exercise test, subjects wore a nose clip and breathed through a mouthpiece connected to a low-resistance turbine volume transducer. The expired gases were continuously sampled and analyzed by an automated system (Jaeger Oxycon Alpha, Hoechst, Germany) for breath-by-breath determination of metabolic and ventilatory variables. Oxygen uptake, carbon dioxide production, ventilation, and respiratory exchange ratio were averaged every 30 s. HR data were automatically recorded and calculated during exercise by using an integrated electrocardiograph.

During the two  $\dot{V}O_{2\max}$  exercise tests, venous blood samples were drawn from the antecubital vein of the nondominant arm through a catheter. To evaluate plasma lactate concentrations, 5 ml were collected in heparin tubes at rest, 150 W, 300 W, and  $PO_{\max}$ . During  $\dot{V}O_{2\max-2}$ , a sample was also taken at the intensity corresponding to the  $PO_{\max}$  reached during  $\dot{V}O_{2\max-1}$  ( $PO_{\max-1}$ ). Plasma was obtained via centrifugation at 2,000 g at 4°C for 5 min in a refrigerated centrifuge (Jouan, Saint Nazaire, France) and was then isolated and frozen at -80°C until assay. Plasma lactate concentration was determined by an enzymatic method (Roche Diagnostics kit, Mannheim, Germany).

### Hematological Parameters

Hct, Hb, and percentage of reticulocytes were measured before injection (D0), once weekly during the experiment for medical monitoring, and then at D25 (always at rest). For each sample, 2 ml of blood were collected from an antecubital vein in EDTA tubes and immediately analyzed with an automated cytoanalyzer and standard laboratory procedures.

### Lactate Influx Into Red Blood Cells

Transport of lactate across the erythrocyte membrane proceeds by three distinct pathways: 1) nonionic diffusion of the undissociated acid; 2) an inorganic anion-exchange system, referred to as the band 3 system; and 3) MCT-1-mediated transport. In the present study, we measured total lactate influx, MCT-1-mediated influx, and influx via band 3 and inorganic diffusion altogether.

For lactate influx into erythrocytes, 5 ml of blood was collected in heparin tubes (heparin, 0.2 units/ml) on D0 and D25 at rest before exercise testing. All samples (5 ml) were placed in ice and prepared before lactate influx measurements.

*Preparation of erythrocytes.* The techniques for erythrocyte preparation and lactate influx measurement were modified from previously published methods (35, 36). The initial Hct (pre-Hct) was determined for all blood samples. Half (2.5 ml) of each blood sample was transferred to a 50-ml conical tube, depleted of lactate, and washed according to the following procedure.

First, the erythrocytes were isolated by centrifugation at room temperature (25°C, 15 min, 2,000 g). Plasma and buffy coat were removed by aspiration. Thirty volumes of chloride buffer [150 mM NaCl and 10 mM sodium tricine, pH = 8.0, at 37°C, osmolality ~315 mosmol/kgH<sub>2</sub>O, volume (ml) =  $30 \times 2.5 \times \text{pre-Hct}$ ] were added to the pellet, which was mixed by inversion and incubated in a water bath for 30 min at 37°C to ensure complete removal of endogenous lactate (9, 35, 36). After incubation, the erythrocytes were sedimented at room temperature (25°C, 10 min, 2,000 g) and the supernatant was removed by aspiration. The cell pellet was then washed twice with chloride buffer and suspended in a volume of HEPES buffer (90 mM NaCl, 50 mM HEPES, pH 7.4, 37°C, osmolality ~267 mosmol/kgH<sub>2</sub>O) equivalent to a 30% Hct level (packed cell volume) to obtain the stock cell suspension for influx measurements. This suspension was divided into two tubes, each containing 1 ml. One of the two tubes contained no lactate transport blockers. The second tube con-

Table 1. Hematological parameters and physiological responses of rHuEPO and placebo subjects to maximal cycling exercise before and after 4 wk of treatment

|   | rHuEPO Group |            | Placebo Group |           |
|---|--------------|------------|---------------|-----------|
|   | D0           | D25        | D0            | D25       |
| Hct, %  | 44.4±0.8     | 48.1±1.0*  | 44.0±1.1      | 43.6±0.8  |
| Hb, g/dl  | 14.6±0.3     | 16.0±0.3*  | 14.5±0.3      | 14.5±0.3  |
| Ret, %  | 1.22±0.10    | 2.01±0.11* | 1.34±0.12     | 1.23±0.12 |
| $\dot{V}O_{2\max}$ , ml·min <sup>-1</sup> ·kg <sup>-1</sup> | 63.9±1.5     | 68.4±1.9*  | 64.8±2.0      | 64.6±2.0  |
| HR <sub>max</sub> , beats/min                               | 179±3        | 182±3      | 187±4         | 189±4     |
| PO <sub>max</sub> , W                                       | 402±12       | 431±15†    | 404±16        | 416±13    |

Values are means ± SE. Hct, hematocrit; Hb, hemoglobin; Ret, reticulocytes;  $\dot{V}O_{2\max}$ , maximal oxygen uptake; HR<sub>max</sub>, heart rate at  $\dot{V}O_{2\max}$ ; PO<sub>max</sub>, power output at  $\dot{V}O_{2\max}$ . Statistical difference in recombinant human erythropoietin (rHuEPO) group between before (D0) and after 4 wk of treatment (D25), and between rHuEPO group and placebo at D25: \**P* < 0.05. Statistical difference in rHuEPO group between D0 and D25: †*P* < 0.05.

tained 1 mM of *p*-chloromercuribenzenesulfonic acid (PCMBs), which is known to inhibit the monocarboxylate-specific carrier at this concentration (35, 36). The tubes were then incubated in a water bath for an additional 30 min at 37°C. Part of the stock cell suspension was used for Hct determination (post-Hct).

**Influx assay.** All samples were run in triplicate. At *t* = 0, a 25- $\mu$ l sample of stock cell suspension was added to a 13 × 100-mm test tube containing 75  $\mu$ l of HEPES influx buffer at 37°C. The HEPES influx buffer contained L-[U-<sup>14</sup>C]lactate (sodium salt, specific activity of 50  $\mu$ Ci/mmol) at four lactate concentrations of 2, 10, 50, and 100 mM. The HEPES influx buffer was adjusted to pH 7.4. Because of dilution with the stock cell solution, the actual lactate concentration values were 1.6, 8.1, 41, and 81.1 mM. The cells were exposed to the influx buffer and mixed at 37°C for 20 s (35, 36). At that time, 5 ml of an ice-cold stop solution [150 mM NaCl, 10 mM Na-2-(*N*-morpholino)ethanesulfonic acid, pH = 6.5] was then added to each test tube to stop influx. The sample was spun for 15 min at 2,000 *g* and 4°C, and the supernatant was removed by aspiration. An additional wash phase was conducted by adding 5 ml of stop solution to each sample. The cells were spun again, and the supernatant was removed. The erythrocyte pellet was lysed and deproteinized with 0.5 ml of 4.2% perchloric acid followed by centrifugation of the sample for 15 min (2,000 *g*) at 4°C. A 0.4-ml sample was then placed into scintillation vials containing 5 ml of aqueous counting fluid and counted in a liquid scintillation counter (Tri-Carb liquid scintillation analyzer, model 2200).

**Calculation of lactate influx.** Total lactate influx into the erythrocytes was determined at the four lactate concentration values. For total lactate influx determination, neither the stock cell solution nor the HEPES influx buffer contained any lactate transport blocker. Influx into PCMBs-treated erythrocytes was measured at four concentrations of lactate (1.6, 8.1, 41, and 81.1 mM). Because PCMBs treatment inhibits the monocarboxylate pathways, influx into the PCMBs-treated erythrocytes was the sum of lactate transport by the

band 3 pathway and nonionic diffusion and corresponded to the band 3 and diffusion-mediated lactate influx. Therefore, total lactate influx minus influx into the PCMBs-treated cells represents the MCT-1-mediated lactate influx.

The percentage of contribution from this pathway was calculated by dividing MCT-1-mediated lactate influx by the corresponding total lactate influx. Lactate influx was calculated in nanomoles of lactate per milliliter of cells (erythrocyte) per minute: the 25- $\mu$ l volume of stock solution was multiplied by its Hct fraction (0.30) to obtain a packed cell volume.  $K_{mLa}$  (Michaelis-Menten parameter for lactate uptake via MCT-1) and  $V_{maxLa}$  (maximal lactate transport capacity via MCT-1) were determined via a curve-fitting analysis and visually verified by using Lineweaver-Burk representation.

#### Quantification of Training Amount

During the injection period, each subject assessed the weekly amount of training using the questionnaire of Millet et al. (29). They reported their daily training in terms of volume and intensity for each sport (swimming, cycling, running, and miscellaneous). Volume was calculated in hours at each of the five following training intensities: aerobic, under the anaerobic threshold, above the anaerobic threshold,  $\dot{V}O_{2\max}$  intensity, and supramaximal intensity. A coefficient was attributed to each intensity, and volumes were then multiplied by the corresponding coefficients. The addition of these results gave us the training amount in arbitrary units.

#### Statistics

Values are presented as means ± SE. Data related to subject characteristics were compared between groups by use of an unpaired Student's *t*-test. The athletes' physiological responses to maximal cycling and hematological parameters were compared before and after treatment by a two-way ANOVA (analysis of variance). ANOVAs with repeated measures were used to compare plasma lactate concentrations: first, 2 groups × 2 times (before and after treatment) × 4 times (rest, 150 W, 300 W,  $\dot{V}O_{2\max-1}$ ), and second, 2 groups × 1 time (after treatment) × 5 times (rest, 150 W, 300 W, power output corresponding to  $\dot{V}O_{2\max-1}$  and  $\dot{V}O_{2\max-2}$ ). Lactate influxes into erythrocytes were compared with a three-way ANOVA with repeated measures.  $K_{mLa}$  and  $V_{maxLa}$  were compared between groups before and after treatment by a two-way ANOVA. Training volume was compared during the four assessment weeks by a two-way ANOVA with repeated measures. Pairwise contrasts were used when necessary to determine where significant differences occurred. Statistical significance was established at  $\alpha$  = 0.05.

## RESULTS

### Hematological Parameters and Exercise Tests

Before the administration of rHuEPO or NaCl, Hct, Hb, and percentage of reticulocytes were not significantly different between the two groups (Table 1). However, values of these

Table 2. Total lactate influx into erythrocytes before (D0) and after (D25) rHuEPO or placebo administration measured at 4 lactate concentrations

|               | 1.6 mM      | 8.1 mM        | 41 mM          | 81.1 mM        |
|---------------|-------------|---------------|----------------|----------------|
| rHuEPO group  |             |               |                |                |
| D0            | 343.8±20.0  | 1,283.1±36.4  | 2,328.2±107.2  | 3,198.2±125.4  |
| D25           | 439.6±27.4* | 1,376.4±136.3 | 2,745.1±162.0* | 3,799.0±150.0* |
| Placebo group |             |               |                |                |
| D0            | 394.5±38.8  | 1,532.9±94.8  | 2,667.3±237.6  | 3,399.4±278.9  |
| D25           | 409.7±37.8  | 1,360.1±125.3 | 2,542.1±130.4  | 3,204.0±120.6† |

Values are means ± SE (in nmol·ml<sup>-1</sup>·cells<sup>-1</sup>·min<sup>-1</sup>). Significant difference in the rHuEPO group between D0 and D25: \**P* < 0.05; significant difference between the two groups at D25: †*P* < 0.05.

Table 3. MCT-1-mediated lactate influx before (D0) and after (D25) rHuEPO or placebo administration measured at 4 lactate concentrations

|               | 1.6 mM      | 8.1 mM        | 41 mM          | 81.1 mM        |
|---------------|-------------|---------------|----------------|----------------|
| rHuEPO group  |             |               |                |                |
| D0            | 322.3±19.4  | 1,205.9±34.4  | 2,038.8±80.4   | 2,544.0±111.7  |
| D25           | 413.2±32.2* | 1,284.0±80.5  | 2,368.2±207.1* | 3,051.6±211.5* |
| Placebo group |             |               |                |                |
| D0            | 376.7±27.8  | 1,437.6±145.0 | 2,366.4±177.9  | 2,728.6±159.8  |
| D25           | 381.9±37.6  | 1,256.7±122.0 | 2,225.3±142.6  | 2,428.3±179.5† |

Values are means ± SE (in nmol·ml<sup>-1</sup>·cells<sup>-1</sup>·min<sup>-1</sup>). Significant difference in the rHuEPO group between D0 and D25: \**P* < 0.05; significant difference between the 2 groups at D25: †*P* < 0.05.

three parameters were higher in the rHuEPO group after treatment (D25) compared with baseline and placebo group values.

There was no group or treatment effect for maximal HR (Table 1). At D0,  $\dot{V}O_{2\max}$  and  $PO_{\max}$  were similar in both groups. At D25,  $\dot{V}O_{2\max}$  and  $PO_{\max}$  both increased in the rHuEPO group and were higher than for the placebo group.

#### Lactate Influx Into Erythrocytes

Values of total lactate influx into erythrocytes are presented in Table 2. After treatment, the total lactate influx was significantly increased in the rHuEPO group at 1.6, 41, and 81.1 mM of external lactate concentration. At 81.1 mM, there was also a difference between the two groups. At moderate external concentration, i.e., 8.1 mM, there was no significant difference in lactate flux after treatment in the rHuEPO group.

MCT-1-mediated lactate influxes between D0 and D25 are presented in Table 3. Significant differences were found after treatment in the rHuEPO group at 1.6, 41, and 81.1 mM. Moreover, at 81.1 mM of external lactate concentration, MCT-1-mediated lactate influx was also higher compared with placebo.

Fractional contribution of MCT-1 to total lactate influx decreased with external lactate concentrations (Table 4).

Band 3 and diffusion-mediated lactate influx are presented in Table 5. Whatever the external lactate concentrations, no statistical difference was observed between the two groups before or after treatment.

As shown in Fig. 1, the  $V_{\max\text{La}}$  for MCT-1 was significantly higher in the rHuEPO group after treatment (D25) compared with the baseline (D0) and the placebo group values. However, there was no statistical difference for  $K_{\text{mLa}}$ , which was identical for the two groups before and after treatment (Fig. 2).

Table 4. Fractional contribution of the MCT-1 to total lactate influx at 4 lactate concentrations

|               | 1.6 mM | 8.1 mM | 41 mM | 81.1 mM |
|---------------|--------|--------|-------|---------|
| rHuEPO group  |        |        |       |         |
| D0            | 94±1   | 94±1   | 88±1  | 80±2    |
| D25           | 95±1   | 93±1   | 89±2  | 80±1    |
| Placebo group |        |        |       |         |
| D0            | 94±1   | 93±2   | 86±1  | 80±2    |
| D25           | 93±1   | 92±1   | 87±2  | 75±3    |

Values are means ± SE (in %). No significant difference was observed between groups and between D0 and D25 for each concentration used.

#### Plasma Lactate Concentrations

Before treatment, at D0, lactate levels in the two groups were similar during the incremental exercise test (Fig. 3). No difference was observed between the D0 and D25 lactate levels measured at any exercise intensity in the placebo group. At D25, there was a significantly lower lactate level at the power output corresponding to the initial maximal aerobic power in the rHuEPO group (16 ± 3.5 and 11.3 ± 3 mM, respectively, before and after treatment at 402 ± 12 W). However, maximal lactate levels were unchanged after rHuEPO treatment.

#### Quantification of Training Amount

There was no significant difference in training amount between the two groups in any week (Table 6).

#### DISCUSSION

This study mainly showed that 4 wk of subcutaneous rHuEPO injection resulted in 1) higher lactate uptake via MCT-1 at different external lactate concentrations, leading to higher total lactate influx into erythrocytes; 2) increased  $V_{\max\text{La}}$  of MCT-1; and 3) lower plasma lactate level during high exercise intensity.

In the present study, repeated doses of 50 U/kg of rHuEPO alpha were administered to athletes subcutaneously for 4 wk. No illnesses requiring medication were noted in any of the subjects during the course of the study. The recombinant hormone was well tolerated, and no adverse effects such as hypertension, seizures, or vascular thrombolytic events were observed. We always performed blood samples at rest and in the same conditions to avoid the influence of body position and other factors on Hct (34). Even if plasma volume was affected by rHuEPO treatment, the strong increase of hematological

Table 5. Band 3 and diffusion-mediated lactate influx into erythrocytes before (D0) and after (D25) rHuEPO or placebo administration measured at 4 external lactate concentrations

|               | 1.6 mM   | 8.1 mM     | 41 mM      | 81.1 mM    |
|---------------|----------|------------|------------|------------|
| rHuEPO group  |          |            |            |            |
| D0            | 21.5±1.9 | 77.2±7.3   | 289.4±42.2 | 654.2±74   |
| D25           | 26.4±3.5 | 92.4±5.7   | 376.8±42.8 | 747.4±66.2 |
| Placebo group |          |            |            |            |
| D0            | 17.4±4.9 | 99.7±21.6  | 300.6±29.4 | 673.3±57.5 |
| D25           | 27.0±3.9 | 101.2±14.6 | 320.7±42.2 | 807.3±88.3 |

Values are means ± SE (in nmol·ml<sup>-1</sup>·cells<sup>-1</sup>·min<sup>-1</sup>). No significant difference was observed between groups and between D0 and D25.

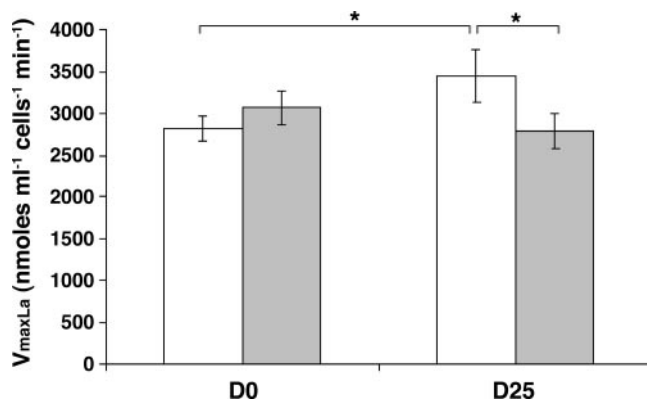


Fig. 1. Mean  $\pm$  SE level of  $H^+$ -monocarboxylate transporter (MCT-1) maximal lactate transport capacity via MCT-1 ( $V_{\max La}$ ) before (D0) and after administration (D25) in each group. Open bars, recombinant human erythropoietin (rHuEPO) group; shaded bars, placebo group. Significant difference between D0 and D25 in rHuEPO group and between two groups at D25: \* $P < 0.05$ .

indexes indicated the stimulation of erythropoiesis. In accordance with previous studies, we observed an improvement in aerobic fitness in the rHuEPO-treated subjects (2, 3, 33). This effect is generally attributed to the increase in arterial oxygen content due to the rise in erythrocyte mass and the rise in Hb concentration (11, 12). However, our results also suggested that other physiological and biological adaptations are involved in the improved physical performances of athletes illegally using rHuEPO.

The present study showed that rHuEPO significantly increased total lactate influx into erythrocytes at 1.6, 41, and 81.1 mM of external lactate concentration. This seems to be directly related to the changes in MCT-1-mediated lactate influx (+28% at 1.6 mM, +16% at 41 mM, and +20% at 81.1 mM) with no change in band 3 and diffusion-mediated lactate influx. It is interesting to note that Skelton et al. (36) have found a 22% difference in MCT-1-mediated influx between untrained subjects and cross-country runners ( $\dot{V}O_{2 \max} = 71 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) and a 30% difference between untrained subjects and aerobically trained subjects (exercising in running and cycling,  $\dot{V}O_{2 \max} = 58 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) at 1.6 mM of external lactate concentration. In the present study, the changes in MCT-1-

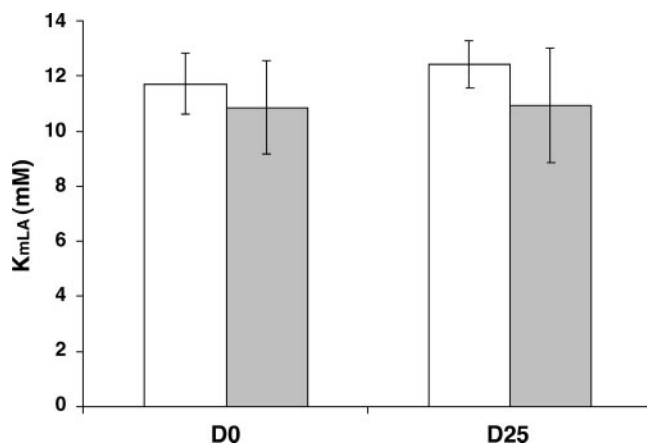


Fig. 2. Mean  $\pm$  SE level of MCT-1 Michaelis-Menten parameter for lactate uptake ( $K_{mLa}$ ) before (D0) and after administration (D25) in each group. Open bars, rHuEPO group; shaded bars, placebo group.

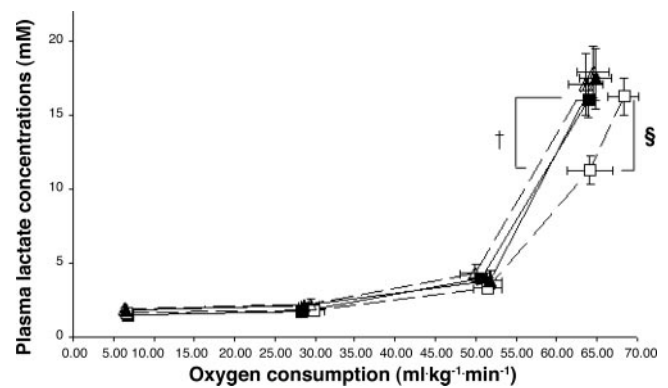


Fig. 3. Mean  $\pm$  SE level of plasma lactate concentration measured at different intensities during maximal exercise in each group before ( $\dot{V}O_{2 \max-1}$ ; D0) and after treatment ( $\dot{V}O_{2 \max-2}$ ; D25). ■ and ▲, rHuEPO group and placebo group, respectively, during  $\dot{V}O_{2 \max-1}$ ; □ and △, rHuEPO group and placebo group, respectively, during  $\dot{V}O_{2 \max-2}$ . Significant difference at the same absolute intensity in rHuEPO group between D0 and D25: † $P < 0.05$ . Significant difference in the rHuEPO group at D25 between the 2 highest intensity: § $P < 0.05$ .

mediated lactate influx induced by 4 wk of rHuEPO treatment are thus in the range of those observed between untrained and trained subjects. It still remains unclear why we found no significant difference at 8.1 mM of external lactate concentration, but it could be related to the higher variability in the individual results in both groups after 4 wk of treatment and training (36).

The band 3 pathway plus nonionic diffusion accounts for <10–20% of the total lactate influx into erythrocytes in endurance-trained subjects and aerobic-trained animal species (35). We did not separately study lactate influx via band 3 and via nonionic diffusion, because passive diffusion is thought to be relatively small and was not expected to change. We chose to focus on MCT-1 because this is the major pathway of lactate influx into erythrocytes (31, 35), a result confirmed in this study (Table 5).

During the 4-wk period, the athletes did not stop training, and the posttreatment results could have been related to the combination of training and rHuEPO treatment because previous studies have suggested that training status influences the rate of lactate transport into erythrocytes (35, 36, 39). We thus evaluated the training amount during the experimental period. The results showed a tendency for the rHuEPO group to train a little bit more, although the between-group difference was not significant. We thus think that the MCT-1 adaptations observed in the present study were mainly related to the rHuEPO injections.

The results also showed a rise in  $V_{\max La}$  in the rHuEPO group after treatment compared with both baseline and placebo group values. However, there was no change in  $K_{mLa}$ , which suggests that repeated injections of rHuEPO resulted in a

Table 6. Training amount during the 4 treatment weeks in each group

|               | Week 1         | Week 2         | Week 3         | Week 4         |
|---------------|----------------|----------------|----------------|----------------|
| rHuEPO group  | 38.9 $\pm$ 5.5 | 42.5 $\pm$ 8.7 | 43.9 $\pm$ 9.4 | 33.9 $\pm$ 6.8 |
| Placebo group | 26.5 $\pm$ 5.2 | 29.5 $\pm$ 4.5 | 35.8 $\pm$ 4.0 | 28 $\pm$ 5.6   |

Values are means  $\pm$  SE (in arbitrary units). No significant difference was observed between the 2 groups.

greater number of MCT-1 transporters in erythrocyte membranes (31, 35) but did not influence MCT-1 affinity to lactate anion. The direct effect of rHuEPO on MCT-1 expression needs to be confirmed by Western blot analysis. It is known that synthesis of several erythrocyte membrane proteins is EPO dependent (21, 38, 42), and Juel et al. (19) recently showed that chronic hypoxia resulted in a very important increase in MCT-1 expression on erythrocytes, indicating that this protein can be remarkably upregulated on erythrocyte membranes. The observed changes may result from an increase in protein density in mature erythrocytes or from a higher proportion of young erythrocytes (i.e., reticulocytes) or both. Indeed, it has been reported (14, 30) that protein density is related to the age of the erythrocytes and that reticulocytes had the higher protein density. Although we have found a greater percentage of reticulocytes after the rHuEPO treatment, it should be noted that the reticulocytes fraction is small: 1.2–2% of total erythrocytes. Juel et al. have shown elevated MCT-1 protein expression in erythrocyte membrane after 8 wk of altitude stay and also in Bolivian natives living permanently at high altitude. These results suggest that incorporation of more transporters during the maturation is possible.

Our study is the first to report an effect of rHuEPO on erythrocyte MCT-1 transporter in humans. The potential physiological effect of such changes is likely to be an increase in lactate and  $H^+$  fluxes from plasma to erythrocytes. During exercise, lactate and  $H^+$  accumulation can occur within the muscle, altering muscular function and subsequently causing fatigue (15, 17). The storage of lactate and  $H^+$  in erythrocyte reduces the levels of these ions in plasma, leading to greater gradients from interstitial fluid to plasma, and potentially improves the rate of release from muscle (8, 19, 32). This might lead to a greater total amount of lactate anion and hydrogen ions taken up by erythrocytes and to a better redistribution in different places in the body. This is particularly interesting in the light of the current concept of lactate exchange, which sets lactate as a metabolic intermediary (5). However, we did not measure lactate kinetics, and it is difficult to speculate on the potential effect of increased lactate uptake by erythrocytes on lactate metabolism during the incremental exercise.

During the incremental exercise test, rHuEPO seemed to result in lower plasma lactate concentration at high intensity. After treatment, lactatemia was significantly reduced ( $11.3 \pm 3$  vs.  $16 \pm 3.5$  mM) at the intensity corresponding to the pretreatment  $PO_{max}$  (intensity was then 93.9% of the new maximal power). However, the plasma lactate levels measured at maximal exercise during both  $\dot{V}O_{2\ max-1}$  and  $\dot{V}O_{2\ max-2}$  were similar in the two groups. Erythrocyte lactate influx may be involved in this downregulation of plasma lactate concentration during intense exercise (25). However, it is difficult to conclude because the only significant difference in plasma lactate concentration occurred at a level that was close to the lactate concentration for which no significant differences were found for the influx measurements. Other factors could have also contributed to the decrease of lactatemia. Several studies have suggested that rHuEPO may modify substrate oxidation and lactate metabolism at rest and during exercise (6, 23, 26, 28). Lavoie et al. (23) reported changes in substrate oxidation during exercise and a lower muscle lactate concentration at the

end of exercise in animal models after repeated injections of rHuEPO compared with placebo-treated rats.

Acute and chronic hypoxia exposure are known to induce several changes in erythrocyte, muscular, and enzyme activities (24), leading to modifications in the acid-base status and extracellular buffer capacity (4, 40, 41). Juel et al. (19) tested the hypothesis that the mechanisms underlying these alterations involve changes at the protein level of transporters in pH regulation and lactate transport in muscle and blood. Only 2 wk after high-altitude acclimatization, they observed an increase in MCT-1 and band 3 content in erythrocytes (+330% and +150%, respectively) and concluded that hypoxia had a direct impact on these proteins. It is known that hypoxia leads to endogenous EPO secretion and is the main stimulus of erythropoiesis (24). This last study, which focused on protein expression, and ours, which focused on lactate transport capacity via MCT-1, together suggest that EPO (or rHuEPO) has a strong effect on erythrocyte lactate transporter synthesis (mainly MCT-1).

In conclusion, our study is the first to report the effects of rHuEPO on erythrocyte MCT-1. Repeated injections of this hormone raise MCT-1-mediated lactate influx and seem to increase MCT-1 synthesis. These changes may influence acid-base regulation at rest and during moderate to intense exercise. These results should help us to understand the physiological adaptations observed in athletes misusing this drug so that we may find alternative solutions (such as new methods of training) and improve prevention and detection.

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