Effect of rhEPO administration on serum levels of sTfR and cycling performance

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ABSTRACT

BIRKELAND, K. I., J. STRAY-GUNDERSEN, P. HEMMERSBACH, J. HALLÉN, E. HAUG, and R. BAHR. Effect of rhEPO administration on serum levels of sTfR and cycling performance. *Med. Sci. Sports Exerc.,* Vol. 32, No. 7, pp. 1238–1243, 2000. **Purpose:** We assessed the possibility of using soluble transferrin receptor (sTfR) as an indicator of doping with recombinant erythropoietin (rhEPO). **Methods:** A double-blind, placebo-controlled study was conducted with the administration of 5000 U of rhEPO ($N = 10$) or placebo ($N = 10$) three times weekly (181–232 $U \cdot kg^{-1} \cdot wk^{-1}$) for 4 wk to male athletes. We measured hematocrit and the concentration of hemoglobin, sTfR, ferritin, EPO, and quantified the effects on performance by measuring time to exhaustion and maximal oxygen uptake ($\dot{V}O_{2max}$) on a cycle ergometer. **Results:** Hematocrit increased from $42.7 \pm 1.6\%$ to $50.8 \pm 2.0\%$ in the EPO group, and peaked 1 d after treatment was stopped. In the EPO group, there was an increase in sTfR (from 3.1 ± 0.9 to 6.3 ± 2.3 $mg \cdot L^{-1}$, $P < 0.001$) and in the ratio between sTfR and ferritin ($sTfR \cdot ferritin^{-1}$) (from 3.2 ± 1.6 to 11.8 ± 5.1, $P < 0.001$). The sTfR increase was significant after 1 wk of treatment and remained so for 1 wk posttreatment. Individual values for sTfR throughout the study period showed that 8 of 10 subjects receiving rhEPO, but none receiving placebo, had sTfR levels that exceeded the 95% confidence interval for all subjects at baseline (= 4.6 $mg \cdot L^{-1}$). $\dot{V}O_{2max}$ increased from 63.6 ± 4.5 $mL \cdot kg^{-1} \cdot min^{-1}$ before to 68.1 ± 5.4 $mL \cdot kg^{-1} \cdot min^{-1}$ 2 d post rhEPO administration (7% increase, $P = 0.001$) in the EPO group. Hematocrit, sTfR, $sTfR \cdot ferritin^{-1}$, and $\dot{V}O_{2max}$ did not change in the placebo group. **Conclusion:** Serum levels of sTfR may be used as an indirect marker of supranormal erythropoiesis up to 1 wk after the administration of rhEPO, but the effects on endurance performance outlast the increase in sTfR. **Key Words:** rhEPO, MAXIMAL OXYGEN UPTAKE, FERRITIN, HEMATOCRIT

New developments in pharmacology and medical therapeutics regularly lead to the misuse of these agents to improve performance in sport. Soon after recombinant human erythropoietin (rhEPO) became available for the treatment of anemia, rumors began to circulate that it was being misused in endurance sports (8). The peptide hormone has therefore been prohibited by the International Olympic Committee since 1990 (16), although no analytical method has been available to detect its misuse. Since then, many methods for the detection of rhEPO administration have been explored (4). These include direct proof of rhEPO administration with identification of rhEPO in blood or urine (20,21) and indirect parameters of stimulated erythropoiesis like the reticulocyte count and the number of hypochromic macrocytes (6) and the serum level of soluble transferrin receptor (sTfR) (1,2,7,13,14). Gareau and coworkers (13) showed previously that high-dose, short-term rhEPO treatment resulted in a significant increase in the serum concentration of sTfR and the ratio between sTfR and ferritin, and recently they confirmed this using lower doses during longer term (1). The authors suggested that an elevated serum level of sTfR or $sTfR \cdot ferritin^{-1}$ may be used to indicate doping with rhEPO.

An additional problem to the development of a test for rhEPO administration in athletes is the possibility that the duration of the effect on performance is greater than the duration of any hematological changes associated with rhEPO misuse. The probable mechanism for the improvement in performance from the use of rhEPO is a stimulation of erythropoiesis that increases circulating red cell mass, hemoglobin concentration, and arterial oxygen content (11,12). This increase in arterial oxygen content leads to an increase in maximal oxygen uptake in well-trained athletes (9,10,19). For athletic events dependent on maximal oxygen uptake, performance is enhanced (5,22). Once rhEPO administration is discontinued, red cell mass gradually returns to its original state, but this may take weeks (10). As a result, an "open window" may exist where there is no evidence of rhEPO misuse but where performance is enhanced. Furthermore, the enhanced red cell mass may allow the athlete to sustain a greater training stimulus, which could produce a subsequent improvement in performance potentially quite remote in time from when there is evidence of rhEPO misuse.

The aim of the present study was to document sTfR changes during 4 wk of administration of moderate doses of

rhEPO in healthy, well-trained, male athletes in a double-blind, placebo-controlled manner. We also wanted to quantify the effects of rhEPO administration on maximal oxygen uptake. Finally, we wanted to establish the time course of changes in maximal oxygen uptake and sTfR after rhEPO administration to determine at what time during the doping process sTfR can be used to reflect rhEPO misuse.

METHODS

Twenty healthy, well-trained male athletes from cycling, orienteering, running, triathlon, swimming, and cross-country skiing volunteered for the study. Inclusion criteria were: normal hematological parameters, including hematocrit between 38 and 45% and serum ferritin within the normal reference range of our laboratory (25–200 $\mu g \cdot L^{-1}$); no history of thromboembolic disease or hypertension; and no other known risk factor for cardiovascular disease. During the screening period before the study, all participants underwent a medical examination that included blood and urine sampling, a familiarization test, and a maximal exercise test. The tests were performed on a mechanically braked cycle ergometer (Monark Ergomedic 839E, Varberg, Sweden), and oxygen uptake and time to exhaustion were measured. The subjects were randomly assigned to the EPO group ($N = 10$; age: 23 ± 2 yr; height: 181 ± 6 cm; weight: 73 ± 6 kg; maximal oxygen uptake: 63.3 ± 3.9 mL·kg^{-1}·min^{-1}) or the placebo group ($N = 10$; age: 25 ± 4 yr; height: 182 ± 8 cm; weight: 75 ± 4 kg; maximal oxygen uptake: 60.7 ± 4.4 mL·kg^{-1}·min^{-1}) with pairwise matching for type of sport and training level.

Group assignment was blinded to the participants and to the technicians and investigators engaged in blood sampling, sample analyses, and exercise testing. The subjects received 5000 U (181–232 U·kg^{-1}·wk^{-1}) rhEPO (Recormon, Boehringer Mannheim GmbH, Mannheim, Germany) or placebo (1 mL NaCl 9 g·L^{-1}) subcutaneously three times weekly for 30 d, or until hematocrit $\geq 50\%$, when injections were stopped. They were thereafter followed for 4 wk with repeated blood sampling and exercise tests. All subjects, including placebo-treated individuals, were given oral iron supplementation with 270 mg·d^{-1} Fe^{2+} in liquid formula (Neo-Fer, Nycomed Pharma AS, Oslo, Norway). The subjects, including the placebo-treated athletes, were informed in writing and verbally, and gave written informed consent to participate. The study was approved by the Regional Ethics Committee of the Norwegian Research Council for Science and Humanities and the Norwegian Medicines Control Authority. The study was also approved by the Norwegian Olympic Committee and Confederation of Sports and the subjects did not participate in national competition for the duration of the study (i.e., for the duration of the rhEPO administration period and until at least 4 wk after the receiving the last injection). None of the subjects participated in international competition for 3 months after the study. No subjects became ill requiring medication during the study.

**Blood sampling.** Blood samples were obtained immediately before start of the treatment and thereafter three times weekly during the treatment period, each of the first 5 d after stopping the treatment and two times weekly during the remainder of the 4-wk follow-up period. The subjects reported to the laboratory before training between 7 a.m. and 9 a.m. in the morning and remained seated for 20 min until blood had been sampled from an antecubital vein after short stasis. Two mL of blood were collected in EDTA-tubes for measurement of hemoglobin concentration and hematocrit with a Sysmex K-100 Cell Counter (Toa Medical Electronic Comp. Ltd, Kobe, Japan) with interassay coefficients of variation (CV) $< 3\%$. Also, 5-mL blood samples were drawn and allowed to coagulate before serum was separated and frozen at $-20°C$ until analysis. Serum EPO concentration was measured with an immunochemoluminometric method (Nichols Inst. Diagnostics, San Juan Capistrano, CA) with intra- and inter-assay CVs 4–12%. Serum sTfR concentration was measured with an immunoenzymometric method (Orion Diagnostica, Espoo, Finland) and also with an enzyme immunoassay (Ramco Laboratories Inc., Houston, TX), both methods with interassay CV $<10\%$. All sTfR results presented in this paper have been obtained using the Orion method. Serum ferritin concentration was measured with a standard method using a Cobas Bio Analyzer (Basel, Switzerland) with interassay CV$<5\%$. All samples from one individual was assayed in one run.

Exercise test. After a 5- to 10-min warm-up period, the subjects cycled to exhaustion on an electrically braked ergometer. The initial workload was set at 100 W and increased by 50 W every 2 min until volitional exhaustion. The subjects were instructed to maintain cadence at 85–95 rpm, and if cadence fell below 75 rpm, the test was stopped and time to exhaustion was recorded. Expired gas was collected in three to five Douglas bags continuously over the last 3–4 min of the test. Volume was measured by spirometer (KL Engineering, Northridge, CA). Room air and expired gas concentrations were measured by mass spectrometry (Perkin-Elmer MGA 1100, St. Louis, MO). Bag temperature and pressure were recorded. The bag of at least 45-s duration with the highest value for oxygen uptake was designated as maximal oxygen uptake. Posttest analysis of respiratory quotient (>1.1) and heart rate (within 5% of age-predicted maximum) were used as additional criteria to confirm that maximal oxygen uptake had been reached. Additionally, the oxygen uptake-power output relationship was analyzed to determine whether there was a plateau in oxygen uptake.

Statistics. Values are presented as mean $\pm$ SD. An analysis of variance was performed on each variable of interest. *Post hoc t*-tests were applied where significant interactions were noted. For correlation analysis a Pearson's coefficient was calculated. Exact *P*-values are generally given and *P*-values <0.05 were considered significant.

RESULTS

Treatment was stopped before day 30 in two subjects in the EPO group (after 17 and 23 d) as they reached the predetermined hematocrit cut-off level of $\geq 50\%$. Injections to their
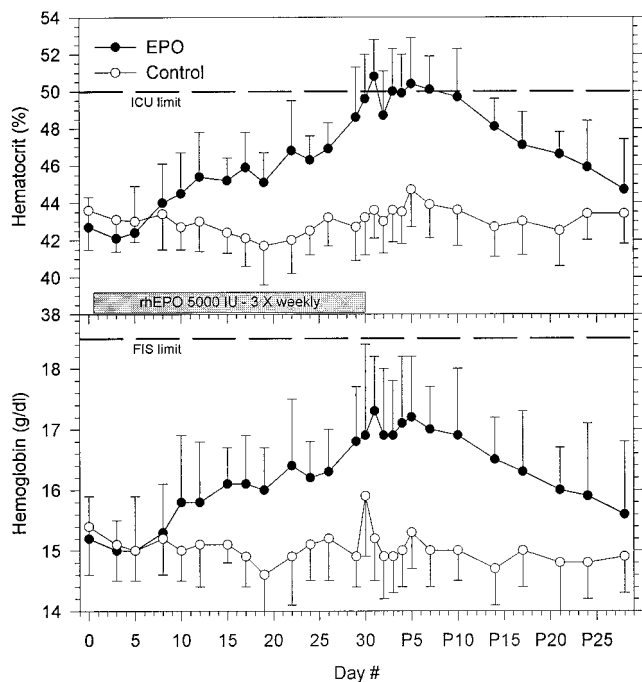
Figure 1—Mean (±SD) level of hematocrit (upper panel) and hemoglobin (lower panel) for the rhEPO treated subjects (*filled symbols*, *N* = 10) and control subjects (*open symbols*, *N* = 10) for the treatment (days 1–30) and posttreatment period (days P1–P28). *Dashed lines* show prerace limits of the International Cycling Union (ICU) for hematocrit (hematocrit = 50%) and of the International Skiing Federation (FIS) for hemoglobin (hemoglobin = 18.5 g·L^{-1}).
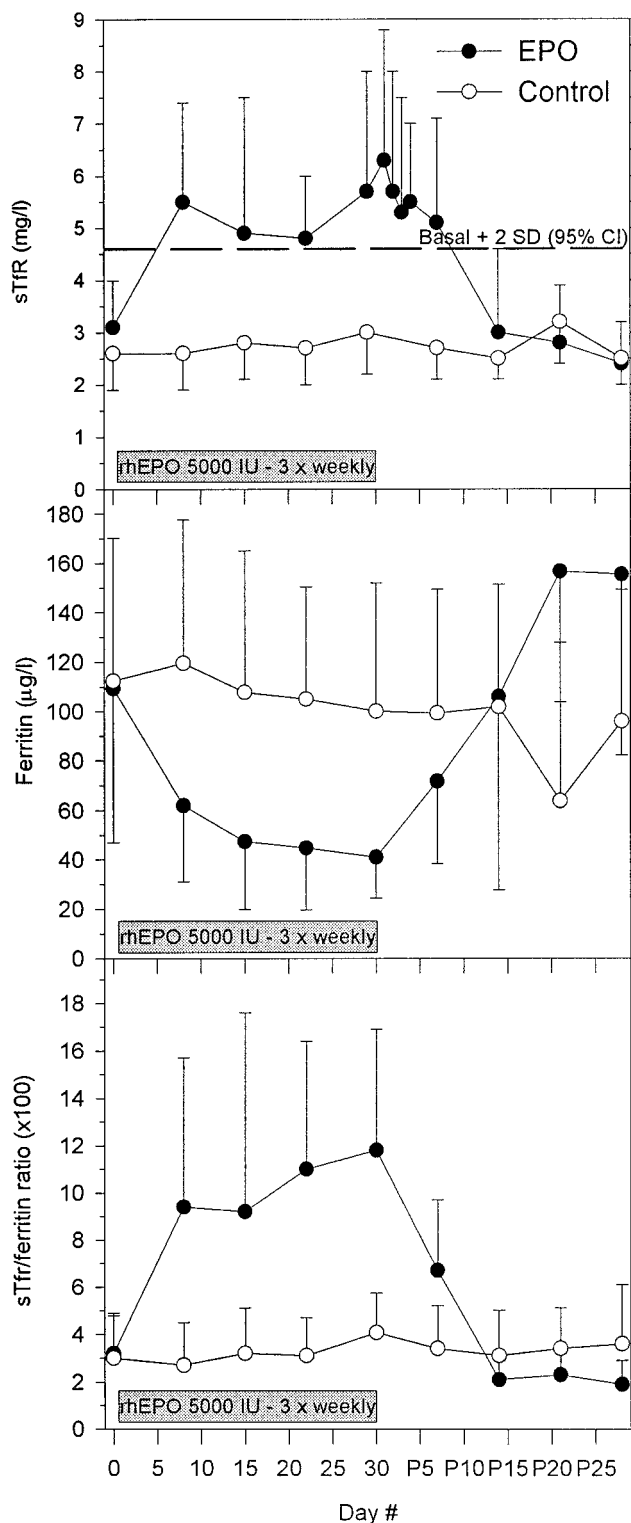
The change from baseline to 1 d after treatment was +4.9 ± 3.2 mL·kg^{-1}·min^{-1} in the EPO group and +0.6 ± 1.9 mL·kg^{-1}·min^{-1} in the placebo group ($P < 0.003$ vs the EPO

Figure 2—Mean (±SD) level of soluble transferrin receptor concentration (sTfR; upper panel), ferritin concentration (middle panel), and sTfR·ferritin^{-1} (×100; lower panel) for the rhEPO treated subjects (*filled symbols*, *N* = 10) and control subjects (*open symbols*, *N* = 10) for the treatment (days 1–30) and posttreatment period (days P1-P28). *Dashed line* in upper panel shows the mean +2 SD (95% CI) for all subjects at baseline (= 4.6 mg·L^{-1}).

matched pairs (control group) were also stopped. Hematocrit increased from 42.7 ± 1.6% before start to 50.8 ± 2.0% ($P < 0.001$) 1 d posttreatment in the EPO group (Fig. 1). All of the EPO treated subjects reached a hematocrit of ≥ 50% during treatment or during days 1–5 in the posttreatment period. Hematocrit did not change in the placebo group.

Serum levels of sTfR increased during treatment in the EPO group from 3.1 ± 0.9 to 6.3 ± 2.3 mg·L^{-1} ($P < 0.001$) and did not change in the placebo group (Fig. 2). The increase in sTfR was significant after 1 wk of treatment and lasted 2 wk after treatment was stopped. Ferritin levels were reduced during EPO treatment from 109 ± 62 to 41 ± 17 μg·L^{-1} ($P = 0.007$) despite iron supplementation. Ferritin levels did not change in the placebo group (Fig. 2). Hence, the ratio of sTfR to ferritin (×100) increased from 3.2 ± 1.6 to 11.8 ± 5.1 ($P < 0.001$) in the EPO-group and did not change in the placebo group (Fig. 2).
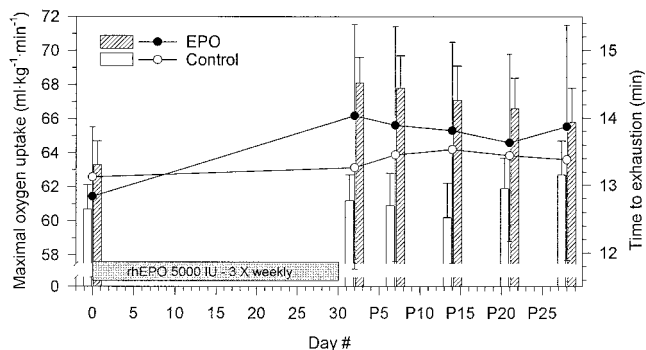
Individual values for sTfR throughout the study period show that 8 of 10 subjects acquired sTfR levels that exceeded the 95% confidence interval (CI) for all subjects at baseline (= 4.6 mg·L^{-1}) when treatment was stopped, and 7 of 10 exceeded the 99% CI (=5.4 mg·L^{-1}). None of the placebo treated subjects had values above these levels.

During treatment maximal oxygen uptake increased significantly in the EPO group, both when compared with the control group and to baseline values (Fig. 3). Maximal oxygen uptake at baseline in the EPO group was 63.3 ± 3.9 mL·kg^{-1}·min^{-1} and increased to 68.1 ± 5.4 mL·kg^{-1}·min^{-1} 1 d posttreatment ($P = 0.001$ vs baseline).

Figure 3—Mean (±SD) level of maximal oxygen uptake (*bars*) and time to exhaustion (*circles*) for the rhEPO-treated subjects (*hatched/filled symbols*, N = 10) and control subjects (*open symbols*, N = 10) before treatment (day 0) and in the posttreatment period (days P1–P28).

group). There was no change in body weight in either group. Maximal oxygen uptake showed a slight but not significant tendency to increase in the placebo group during the study period (Fig. 3). Time to exhaustion increased from 12.8 ± 1.0 min at baseline to 14.0 ± 1.4 min 1 d posttreatment in the EPO group ($P < 0.0001$) and from 13.1 ± 1.5 min to 13.3 ± 1.5 min in the control group ($P = 0.04$).

The baseline serum levels of EPO was 13.7 ± 7.7 $U \cdot L^{-1}$ in the EPO-group and 9.7 ± 6.2 $U \cdot L^{-1}$ in the control group (NS). Twenty-four hours after the last injection the serum EPO concentration was 41.8 ± 9.8 $U \cdot L^{-1}$ ($P < 0.001$ vs baseline), and it was reduced to pretreatment levels 48–72 h after the last injection (Fig. 4).

Because previously published results of sTfR measurements after rhEPO administration were obtained using a different immunoassay (1,13) and different immunoassays have been shown to perform differently (18), we also measured sTfR in our 198 serum samples with the Ramco immunoassay. Even though the results obtained with the two methods were significantly correlated (r = 0. 70, $P < 0.001$, $sTfR_{ORION} = 0.7 \times sTfR_{RAMCO} + 0.96$), considerable differences were found, and the limit for what is considered normal values for sTfR must be defined for each assay separately. However, the changes in serum levels of sTfR during treatment and the sensitivity to detect rhEPO administration did not differ between the two immunoassays.

DISCUSSION

The main objective of the present study was to evaluate the efficacy and time course of sTfR as a marker for rhEPO use in athletes and to relate that to the potential performance advantage. Serum sTfR was elevated after 1 wk of rhEPO treatment and the elevation persisted for the duration of treatment in 8 of 10 subjects. Further, in 5 of 10 subjects sTfR was elevated for 1 wk posttreatment.

Maximal oxygen uptake and time to exhaustion were significantly elevated for up to 3 wk postadministration, which confers a performance advantage in endurance sports. These data indicate a performance advantage for at least 2 wk longer than any indication of rhEPO use. A large "open

window" exists were rhEPO is undetectable by the sTfR method and endurance performance is greatly enhanced. Therefore, a normal value for sTfR does not rule out recent rhEPO use. However, an elevated sTfR value, if noted, is strong indirect evidence of rhEPO use. This shows that sTfR may be used as an indirect indicator for rhEPO doping, although the specificity of this method must be thoroughly investigated in large group of athletes under different physiological conditions, e.g., during altitude training.

Due to the decrease in ferritin levels, the changes in $sTfR \cdot ferritin^{-1}$ were even more pronounced than the sTfR levels themselves. However, the increase in $sTfR \cdot ferritin^{-1}$ was not as pronounced as in a previous study (13). In this study, Gareau et al. gave higher EPO doses and did not give iron supplementation, which may have produced a more pronounced reduction in serum ferritin and, consequently, a more dramatic difference between control and experimental groups. Athletes using rhEPO to gain a performance advantage are likely to use iron supplementation, and we suggest that sTfR levels alone might be a more robust parameter than the $sTfR \cdot ferritin^{-1}$ as an indication of rhEPO use. In our placebo-group of healthy athletes with normal ferritin levels, iron supplementation did not alter the mean levels of sTfR or ferritin during the study. However, we cannot exclude that larger doses of iron, especially if given intravenously, would result in different findings. Furthermore, no significant correlation was observed between baseline ferritin levels and the changes observed in sTfR during treatment with rhEPO.

Repeated injections of rhEPO lead to an increase in hematocrit and hemoglobin concentration in a dose- and time-dependent manner (12). This is the rationale for the use of rhEPO in clinical medicine as effective treatment for anemia caused by renal failure, inflammatory and malignant diseases, and to enhance the efficacy of preoperative auto-
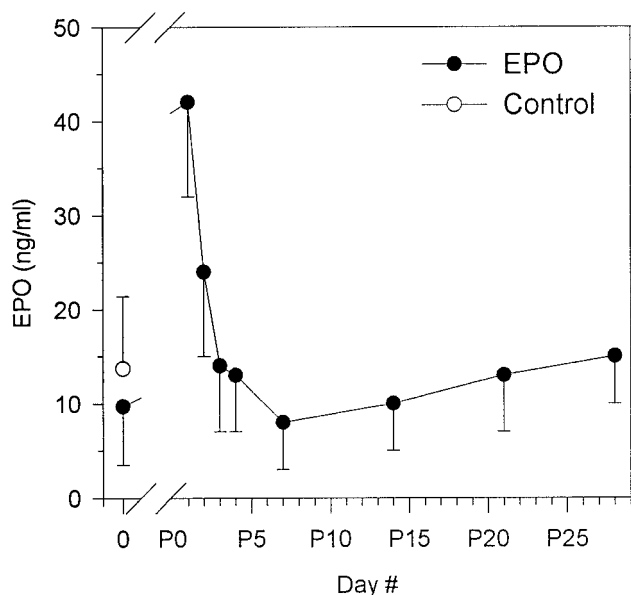
Figure 4—Mean (±SD) level of serum EPO for the rhEPO-treated subjects (*filled symbols*, N = 10) before treatment (day 0) and post-treatment period (days P1–P28), as well as the baseline value for the control subjects (*open symbols*, N = 10).

logous blood donation (3). This is also the background for its potential misuse as a doping agent to improve performance in endurance sport. The present study is the first randomized, placebo-controlled study to show that the administration of moderate doses of rhEPO for 4 wk significantly increases maximal oxygen uptake and time to exhaustion in healthy, well-trained endurance athletes. These data are similar to and confirm the findings from an uncontrolled, unblinded study (10). In a questionnaire administered 2 d after the end of the treatment period, when the effect on hematocrit and maximal oxygen uptake was maximal, only 5 of the 10 subjects in the EPO group responded that they thought they had received rhEPO, three reported that they believed they were in the placebo group, and two were uncertain. Also, there was an improvement in time to exhaustion in the control group, although not of the same order of magnitude as in the EPO group. This shows the importance of a blinded and placebo-controlled design.

As a measure to avoid potential health hazards and to combat doping, some international sports federations have excluded athletes with hematocrit or hemoglobin levels above certain limits from participation in competition. The International Skiing Federation (FIS) excludes male athletes with hemoglobin levels above 18.5 g·100 mL^{-1} (corresponds to a hematocrit of approximately 56%), whereas the International Cycling Federation (UCI) has set a limit at a hematocrit of 50% for participation in competition. Although the limits do provide a certain measure of safety for the athletes and limit the extent of the performance advantage, Fig. 3 shows that the subjects were at the UCI limit and well below the FIS limit after 4 wk of rhEPO use. Thus, a significant improvement in maximal oxygen uptake performance may be achieved despite hematocrit and hemoglobin levels within the present limits.

We did not include time trials in this experiment to determine whether the improvement in maximal oxygen uptake would translate into actual improvement in performance in the field and across different sporting disciplines. However, we did observe a 10% improvement in time to exhaustion on the cycle ergometer. In a previous study by one of the authors (15), an altitude-induced (4 wk at 2500 m) increase in hematocrit of less than half the magnitude observed in this study conferred a 1.5% improvement in 5000-m time trial performance lasting at least 3 wk after return from altitude. Even if the degree of improvement in performance were similar to the effect obtained from altitude exposure, it would clearly make the difference between winning a medal or not in international level competition.

Repeated injections of rhEPO result in several well defined changes in the body that can be monitored by analyzing a blood sample. In the minutes and hours after an injection of rhEPO, high levels of EPO can be measured in serum (17), and rhEPO may also be detected in the urine (21). However, as the half-life in blood after subcutaneous injections is relatively short and the interindividual variations are considerable, simply measuring EPO concentration is neither a specific nor a sensitive method for detection of rhEPO doping (17). A few days after the injection of rhEPO,

there is an increase in the reticulocyte count followed by an increase in the hemoglobin level, hematocrit, and the number of red blood cells (1). There also appears to be an increase in large erythrocytes with low hemoglobin content (6), and there is an increase in the sTfR level (2,7,14). It has been shown previously that high doses of rhEPO given to humans result in a significant increase in the serum concentration of sTfR measured by the ELISA technique (13). This is presently one of the most promising indirect parameter to indicate rhEPO use in athletes. However, our results show that the sensitivity of sTfR as an indicator of rhEPO-administration is limited. In two individuals, serum sTfR never did increase above the baseline mean +2 SD because of a low pretreatment level and a small increase during treatment (from 1.3 to 3.6 mg·L^{-1} and from 2.1 to 3.1 mg·L^{-1}, respectively). Although the mean sTfR for the group was elevated 7 d after treatment was stopped, when using the cut-off level of 4.6 mg·L^{-1}, only 5 of the 10 subjects exceeded this level at this time point. Further study is needed to refine the method before it can be applied to doping control. It will be necessary to define normal physiological variation in large populations and the variations due to the influence of high altitude, low pressure oxygen chambers, iron supplementation, training status, ethnicity, and other physiological and pathological states.

In addition to the limitations in sensitivity and specificity shown in the present study, the sTfR method is only an indirect test for rhEPO doping. Wide and Bengtsson (20) have presented a promising direct method for distinguishing between endogenous and recombinant EPO in 1990 and later provided data that it may be effective in detecting rhEPO misuse (21). However, further studies are necessary to explore the merits of the method of Wide and Bengtsson as a direct method. Even if this method is shown to be both accurate and practical, samples must be obtained within 48–72 h of the last injection, whereas the effects on performance last much longer.

In summary, our data show that administration of rhEPO sufficient to raise hematocrit from 42.7% to 50.8% in endurance athletes was detected by an increased sTfR in 80% of the athletes during the administration of rhEPO and in 50% of athletes a week after cessation of rhEPO administration. Further, maximal oxygen uptake was significantly improved for 3 wk postadministration, resulting in a large "open window" where detection by this method is unlikely and endurance performance is greatly enhanced. We conclude that detection of the use of rhEPO in athletes with this or other markers will only be possible with random, out-of-competition testing.

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