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Acute and chronic elevation of erythropoietin in the brain improves exercise performance in mice without inducing erythropoiesis

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ABSTRACT **Application of recombinant human erythropoietin (rhEpo) improves exercise capacity by stimulating red blood cell production that, in turn, enhances oxygen delivery and utilization. Apart from this, when applied at high doses, rhEpo crosses the blood-brain barrier, triggering protective neuronal effects. Here we show a fundamental new role by which the presence of Epo in the brain augments exercise performance without altering red blood cell production. Two different animal models, the transgenic mouse line Tg21, which constitutively overexpresses human Epo exclusively in the brain without affecting erythropoiesis, and wild-type mice treated with a single high dose of rhEpo, demonstrate an unexpected improvement in maximal exercise performance independent of changes in total hemoglobin mass, as well as in whole blood volume and cardiovascular parameters. This novel finding builds a more complete understanding regarding the central effects of endogenously produced and exogenously applied Epo on exercise performance.—Schuler, B., Vogel, J., Grenacher, B., Jacobs, R. A., Arras, M., Gassmann, M. Acute and chronic elevation of erythropoietin in the brain improves exercise performance in mice without inducing erythropoiesis. *FASEB J.* 26, 000–000 (2012). www.fasebj.org**

Key Words: Epo · cerebral · doping · neurotrophic effect · motivation

THE PRIMARY FUNCTION of the kidney-derived erythropoietic growth factor erythropoietin (Epo) is to promote red blood cell production by stimulating proliferation, differentiation, and maturation of erythroid progenitors in bone marrow (1). Accordingly, recombinant human erythropoietin (rhEpo) is commonly used for the treatment of various forms of anemia, including that due to kidney failure or following chemotherapy (2). Attendant to the increase in erythrocyte volume and total hemoglobin mass (tHb) following rhEpo treatment is an increase in oxygen (O_2) carrying capacity that improves maximal O_2 uptake ($\dot{V}O_{2max}$) and overall exercise performance (3, 4) at sea level. Accordingly, the manipulation of tHb *via* rhEpo has been embraced as an ergogenic aid in endurance sports.

The identification of extravascular Epo, such as that found in the brain (5, 6), has culminated in the identification of a large number of nonerythroid effects of Epo. Larger doses of systemically administered rhEpo have been shown to not only cross the blood-brain barrier but also to trigger neuroprotective and neurotrophic effects in animal models, as well as in patients presenting with central nerve damage or neurodegenerative diseases in the brain (7) and even in the eye (8–10). A few studies in humans, either patients or volunteers, have investigated the effect of rhEpo as a treatment for diseases associated with neuronal cell death, including stroke and depression (11, 12). Moreover, Miskowiak and colleagues (12–14) have recently shown that Epo activates areas of the brain that are involved in memory retention and fear processing in healthy subjects. In correlation with increased brain activity, humans treated with rhEpo have been claimed to develop enhanced memory (15) and mood improvement (13) as well as perceived elevated physical condition and strength scores (16). Notably, those subjects declared a boost in energy and an increase in overall self-esteem on receiving rhEpo, a fact that might have euphoric effects on self-perception. Consequently the latter may have influence exercise performance by modulating mechanisms associated with central fatigue (17). In contrast, a recent study claimed that elevated cerebral Epo level affects neither exercise performance nor cognition and voluntary activation in healthy sub-

Abbreviations: Epo, erythropoietin; Hb, hemoglobin; HR, heart rate; Htc: hematocrit; MAP: mean arterial blood pressure; PDGF, platelet-derived growth factor; POMC, proopiomelanocortin; RER, respiratory exchange ratio; rhEpo, recombinant human erythropoietin; RPP, rate pressure product; tHb, total hemoglobin mass; $\dot{V}CO_2$, carbon dioxide production; $\dot{V}O_2$, oxygen consumption; $\dot{V}O_{2max}$, maximal O_2 uptake; WT, wild type

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jects (18). As the neuroprotective effect of Epo occurs in a dose- and time-dependent manner (19, 20), however, the amount of rhEpo applied by Rasmussen *et al.* (18) might have been too low and/or not administered long enough to enhance exercise performance.

We hypothesized that chronic elevated levels of cerebral Epo, independent of changes in Epo plasma levels, hemoglobin (Hb) concentration, or hematocrit (Htc), enhances exercise performance. To test this notion, we made use of our transgenic mice, termed Tg21, which constitutively overexpress human Epo cDNA exclusively in the brain in an oxygen-independent manner (21). Moreover, to confirm that rhEpo indeed does cross the blood-brain barrier and to show a central effect of Epo on exercise performance, very high doses of rhEpo were acutely administered in wild-type (WT+rhEpo) mice. Both animal models showed elevated cerebral Epo levels, whereas tHb and blood volume remained unaltered. Metabolic and cardiovascular parameters were measured at rest in addition to an incremental and constant load exercise test.

MATERIALS AND METHODS

Experimental animals and setup

The transgenic mouse line TgN(PDGFBEPO)322ZbZ (Tg21) exhibits elevated Epo levels in the brain but not in the circulation (21). This line was generated by microinjection of the full-length human Epo cDNA, driven by the human platelet-derived growth factor (PDGF) B-chain promoter, into pronuclei of fertilized oocytes derived from B6C3 hybrid mice (22). Transgenic mice were bred to homozygosity (23). For experimentation, only homozygous Tg21 and WT male mice were used. All mice were housed in standard rodent cages (T3) with fixed temperature (21±1°C), free access to food and water, and a 12-h light-dark cycle. Mice were 12 wk of age at the first exercise test. Compared to the WT controls, we did not observe elevated spontaneous activity of the Tg21 mice, as determined by comparing the counts of spontaneous visits of the animals to the corners of the Intellicage (http://www.newbehavoiur.com), an automated home cage (data not shown). The experimental protocols were approved by the Kantonales Veterinäramt Zürich and were performed in accordance with the Swiss animal protection laws and institutional guidelines.

A diagram illustrating the experimental timeline is given in **Fig. 1**. When mice were 6 wk old, telemetric blood pressure transmitters were implanted in Tg21 (n=12) and WT (n=19) mice. Following sensor implantation, the animals were allowed to recover fully, using a 2-wk postoperative intensive care regime, as described previously (24), followed by an additional 2 wk of normal housing. At 10 wk of age, all animals were habituated to run on a rodent-specific motorized treadmill 1×/wk. About 4-6 h before performing a graded test, WT (WT+Epo, n=9) mice were i.v. (tail vein) injected with rhEpo (2000 IU; Eprex; Janssen-Cilag AG, Baar, Switzerland), whereas Tg21 mice and WT control animals (n=12 and 10, respectively) received an isovolemic dose of saline (0.9%). To avoid any possible influence of the circadian rhythm, all measurements were performed at the same time of day. Necropsy did not reveal any signs of thromboembolism, as tested in all available organs, as described earlier (25).

Implantation of telemetric transmitters

Blood pressure sensors were implanted as described previously (24). In short, inhalation anesthesia was induced and maintained with 3.5-4% sevoflurane. After shaving and disinfecting the neck, the left common carotid artery was localized and isolated. A catheter extending from the body of the transmitter was inserted into the artery and pushed forward until the tip just entered the thoracic aorta. The transmitter body was fixed under the skin on the mouse's upper dorsum. Transmitter implantations were carried out under aseptic conditions. Mice were allowed to recover for total 4 wk before the experiment was started. Cardiovascular measurements were performed using a TA11PA-C10 transmitter (DataSciences International, St. Paul, MN, USA). Data were reordered by the Dataquest A.R.T 3.0 software (DataSciences International).

Exercise performance

All animals performed a maximal incremental exercise test, followed by a maximal constant load exercise test on the next day, using a Columbus Instrument Simplex II metabolic rodent treadmill fitted with an Oxymax gas analyzer (Columbus Instruments, Columbus, OH, USA). This system measures $\dot{V}O_2$ and carbon dioxide production ($\dot{V}CO_2$). Respiratory exchange ratio (RER) is then calculated using $\dot{V}CO_2/\dot{V}O_2$. Prior to each exercise test, the gas analyzer was calibrated with a high-precision standard gas mixture. A mild electric shock (0.2 mA, pulse 200 ms, 1 Hz) was used to force mice to run on the belt until exhaustion.

All exercise tests were performed as described previously (26). Briefly, 10-μl blood samples were taken from the tail vein for determination of Htc before initiation of the incremental exercise test. Mice were then placed within individual treadmill lanes and closed off from room air for at least 1 h, allowing them to acclimatize and the air flux to equilibrate. The end of this period was identified once basal heart rate (HR), blood pressure, $\dot{V}O_2$, and $\dot{V}CO_2$ reached and maintained basal levels for ≥5 min. Workload for the incremental exercise test began with 2.5 m/min at 0° inclination for 10 min. Thereafter, the intensity was gradually increased by 2.5 m/min and 2.5° every 3 min until exhaustion. Exhaustion was defined when mice could no longer keep pace with tread speed. $\dot{V}O_{2max}$ was confirmed once $\dot{V}O_2$ ceased to increase despite increasing workload. Reported values for blood pres-

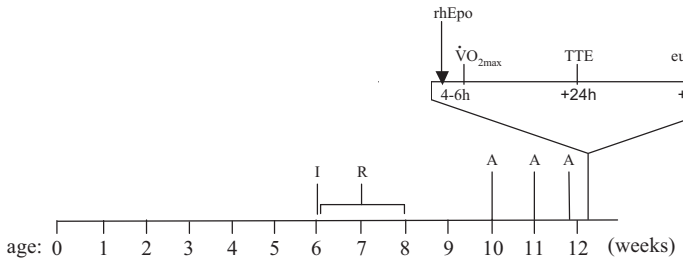

Figure 1. Timeline of animal age and experimental setup. I, implantation of the telemetric transmitter; R, recovery with postoperative care; A, habituation to motorized treadmill running, rhEpo: injection of rhEpo; $\dot{V}O_{2max}$, start of incremental exercise test; TTE, start of time to exhaustion (constant load) test; euthanasia, animal euthanasia, including blood and organ harvest.

sure and HR at exhaustion were based on a 1-min average taken during the last minute of exercise, or, for $\dot{V}O_{2max}$, the highest 1-min interval (26). From these values, the O_2 pulse and rate pressure product (RPP) were calculated to assess the stroke volume and myocardial $\dot{V}O_2$, respectively.

Following 24 h of rest, all mice performed a constant workload exercise test to exhaustion. The workload was set to 80% of the maximal workload attained during the incremental exercise test. Prior to this test, animals were allowed to become familiarized to the treadmill lane for ≥1 h. Mice initially warmed up for 10 min at 20%, followed by an additional 10 min at 40% of the maximal attained power output of the $\dot{V}O_{2max}$ test. Time to exhaustion at 80% maximal workload was measured, and exhaustion again was defined as the inability to continue regular treadmill running despite repeated electrical stimuli.

Blood analysis

One day after performing the constant workload test, mice were anesthetized with a subcutaneous injection containing 100 mg/kg ketamine (Ketasol-100; Dr. Graub, Bern, Switzerland), 20 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany), and 3 mg/kg acepromazine (Sedalin; Chassot, Belp, Bern, Switzerland). Catheters were then introduced into the left femoral artery and vein. A small sample of blood was taken for arterial acid-base status, and then Evans blue dye (10 μl) was injected into the femoral vein. After 20 min, animals were terminally bled, and the blood collected was used to determine plasma Epo concentration, Htc, and tHb, as well as plasma and blood volume. The brain was harvested for analysis of cerebral tissue Epo.

The Htc of heparinized blood was measured in duplicate using a microcentrifuge (Autokrit II; Pharmap, Geneva, Switzerland), and [Hb] was determined by use of Abbott Cell Dyn 3500 (Abbott Diagnostic Division, Santa Clara, CA, USA). tHb mass was calculated from the Hb concentration and blood volume (see below).

Quantification of blood volume has been described previously (27). Briefly, 10 μl Evans blue solution (1% in saline) was injected into a femoral vein catheter. The plasma concentration of Evans blue was measured photometrically. Plasma volume and Htc values allowed calculation of the blood volume.

Measurement of Epo in plasma and brain tissue

Epo in plasma and cerebral tissue was measured using a commercial kit (Epo-Trac 125I RIA kit; DiaSorin, Saluggia, Italy) as described previously (28).

Statistics

All data were analyzed using StatView 4.57 software (Abacus Concepts, Berkeley, CA, USA). Results are expressed as means ± SD. Comparisons between experimental groups were performed using ANOVA followed by *post hoc* Bonferroni to detect significant differences. Statistical difference was set at $P < 0.05$.

RESULTS

Elevated Epo levels in the brain of WT+rhEpo and Tg21 mice

Before starting the exercise performance tests, we compared several parameters of the different mouse groups. Male WT, WT+rhEpo (on acute intravenous injection of 2000 IU Eprex), and Tg21 mice (that constitutively express human Epo in brain only) showed no difference in age, body weight, Htc, basal mean arterial blood pressure (MAP), HR, $\dot{V}O_2$, or RER before performing the graded exercise test (**Table 1**). We determined Epo levels in blood and brain after exercise performance according to the timeline shown in Fig. 1. As expected, the plasma Epo level was elevated in the WT+rhEpo group but not in the WT control or in the transgenic group (**Fig. 2**). Also expected was the elevation of Epo levels in the brain of WT+rhEpo and Tg21 mice (Fig. 2).

Total Hb is not altered in either WT+rhEpo or Tg21 mice

It is well established that Epo enhances exercise performance by stimulating erythropoiesis, resulting in improved O_2 transport capacity due to elevation in tHb (26). Note, however, that the exercise performance tests were started 4-6 h after injection of rhEpo into WT mice (WT+rhEpo), thereby minimizing the time period in which Epo could stimulate red blood cell production in this mouse group. On the other hand, Tg21 mice were never observed to elevate their blood parameters, as these transgenic mice show elevation of human Epo exclusively in the brain. Thus, as expected, neither tHb nor plasma or blood volume was altered in all mice at the end of the experiment (**Fig. 3**).

Increased Epo levels in brain improve exercise performance

To characterize the effect of brain Epo on exercise performance, mice were subjected to incremental and constant load exercise tests. Both acutely (WT+rhEpo group) and chronically (Tg21 group) elevated levels of Epo in the brain markedly improved $\dot{V}O_{2max}$ and time to exhaustion (**Fig. 4**) compared to the control group. The maximal effort of the animals at $\dot{V}O_{2max}$ was assessed by the RER (29). Despite not reaching signif-

TABLE 1. *Basal parameters of WT, WT+rhEpo, and Tg21 mice before performing the graded exercise test*

Type	Weight (g)	Age (d)	Htc (%)	$\dot{V}O_2$ (ml/kg/min)	RER	MAP (mmHg)	HR (min^{-1})
WT	25.6 ± 2.3	87.3 ± 6.1	40.6 ± 2.2	50.5 ± 2.5	0.83 ± 0.01	100.7 ± 6.0	500.7 ± 42.2
WT+rhEpo	27.1 ± 2.8	85.6 ± 4.3	41.8 ± 1.9	50.8 ± 3.0	0.81 ± 0.01	101.0 ± 9.2	516.7 ± 36.5
Tg21	25.9 ± 1.2	86.9 ± 2.3	40.0 ± 0.6	50.1 ± 2.4	0.82 ± 0.01	103.6 ± 7.3	524.4 ± 46.2

Values represent means ± SD.

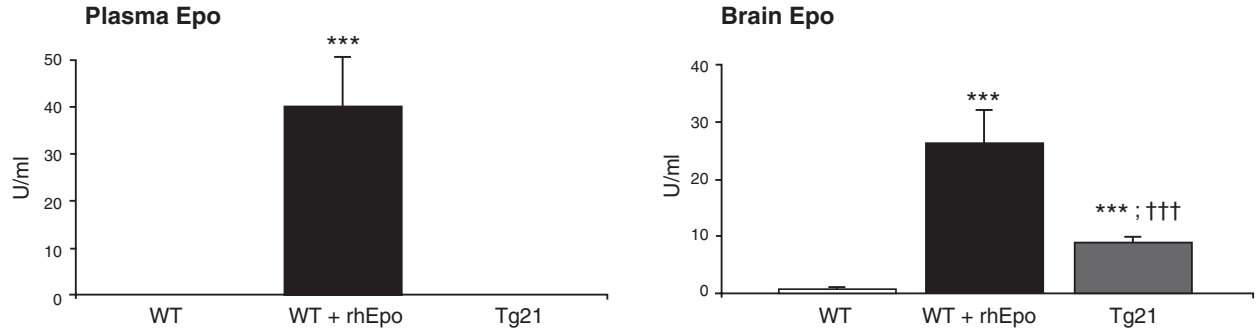

Figure 2. Plasma and cerebral Epo concentration in WT+rhEpo *vs.* WT as well as in Tg21 *vs.* WT animals, determined at the end of the experiment (*e.g.*, euthanasia according to Fig. 1). Data are means ± SD. ***$P < 0.001$ *vs.* WT, $^{\dagger\dagger\dagger}P < 0.001$ *vs.* WT+rhEpo.

icance, we interestingly observed a tendency of a higher RER in WT+rhEpo and Tg21 mice (Fig. 4).

No alterations of maximal MAP, HR, stroke volume, and myocardial $\dot{V}O_{2max}$ occur during exercise

MAP, HR, and stroke volume were quantified at $\dot{V}O_{2max}$. MAP and HR did not differ between WT, WT+rhEpo, or Tg21 mice (**Fig. 5**). As a previous study has identified a correlation between the stroke volume and O_2 pulse in exercising humans (30), we determined these parameters, too. Figure 5 also shows the O_2 pulse of WT, WT+rhEpo, and Tg21 mice, but there was no difference in O_2 pulse across the mouse groups. Finally, to analyze the effect of myocardial $\dot{V}O_2$ at $\dot{V}O_{2max}$, the RPP was calculated and was not found to differ between WT, WT+rhEpo, and Tg21 mice (Fig. 5).

DISCUSSION

This is the first report demonstrating that an elevated brain Epo level, observed in both WT mice treated with acute high doses (2000 IU) of rhEpo (WT+rhEpo) and transgenic mice that chronically overexpress human Epo solely in the brain (Tg21), enhances exercise capacity, as defined by an increase in maximal aerobic capacity ($\dot{V}O_{2max}$) and time to exhaustion. Notably, the marked elevation in exercise capacity occurs independently of changes in hematologic or cardiovascular (*e.g.*, MAP, HR, and stroke volume) parameters. Thus, this striking improvement in exercise performance suggests a direct, nonerythropoietic impact of Epo in the brain.

In the past, it was thought that the only function of Epo is the regulation of the red blood cell production. Very recently, however, Epo has been found to exert regulatory effects in different organs, such as liver, heart, kidney, spleen, lung, bone marrow, reproductive organs, and brain (10, 31). Of note, high plasma Epo levels facilitate the penetration of the blood-brain barrier. By doing so, Epo has been shown to exert potent neuroprotective as well as neurotrophic effects, especially in the damaged central nervous system (11). In healthy humans, Epo does affect brain areas related to

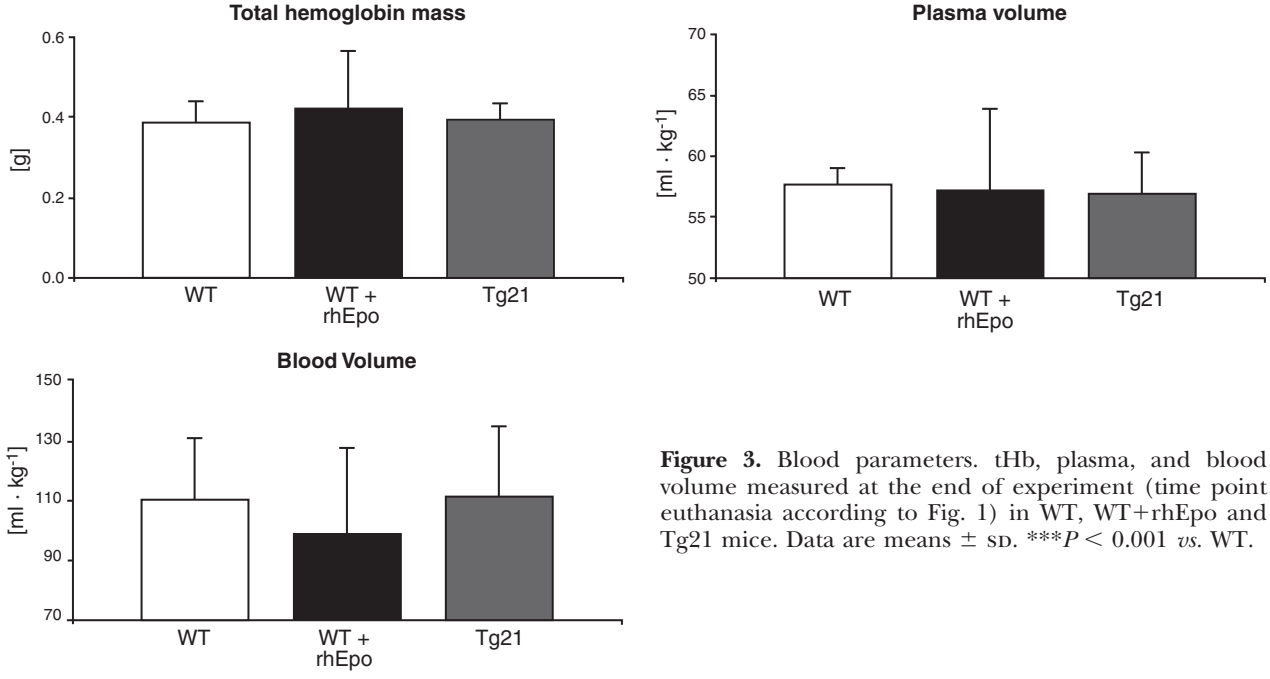

Figure 3. Blood parameters. tHb, plasma, and blood volume measured at the end of experiment (time point euthanasia according to Fig. 1) in WT, WT+rhEpo and Tg21 mice. Data are means ± SD. ***$P < 0.001$ *vs.* WT.

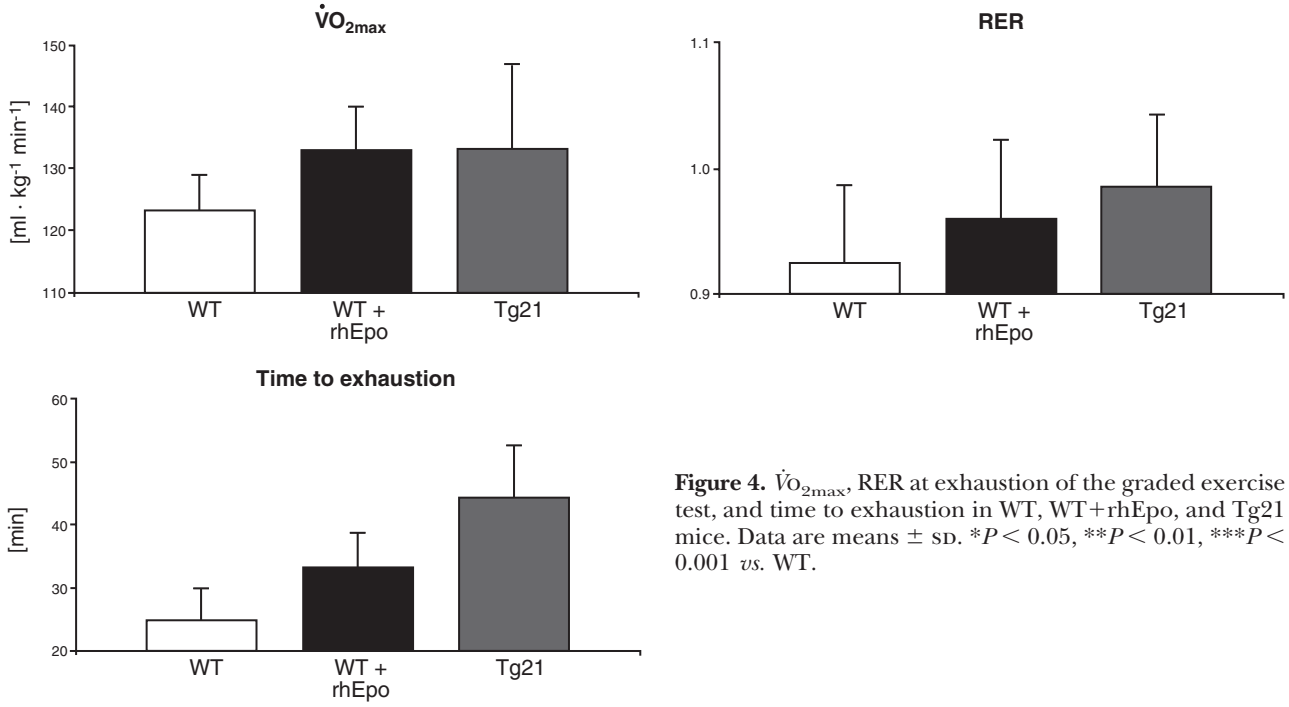

Figure 4. $\dot{V}o_{2max}$, RER at exhaustion of the graded exercise test, and time to exhaustion in WT, WT+rhEpo, and Tg21 mice. Data are means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* WT.

learning and emotional processing (12, 14, 32). Also, endurance athletes treated with rhEpo feel a significant increase in motivation (16). This marked emotive improvement with Epo administration could positively affect self-perception and motivation while reducing physical pain as well as mechanisms of central fatigue for a given workload.

The aforementioned observations, including ours, are in disagreement with recent data obtained by one group (18) reporting that exercise performance remains unaltered after administration of relatively high Epo doses during 3 consecutive days (3× 30,000 IU). As the amount of rhEpo reaching the brain's neurons is in the range of 1% only (10), high doses of rhEpo have to be administrated at once or within a short period of time (19, 20). In contrast to the human study, in which volunteers received 3× 400 IU rhEpo/kg body weight, our WT+rhEpo group of mice received a tremendously high rhEpo dose of ~77,000 IU/kg body weight at once. Therefore, it can be argued that the cerebral rhEpo level reached in the human study was not sufficient to induce changes in exercise performance.

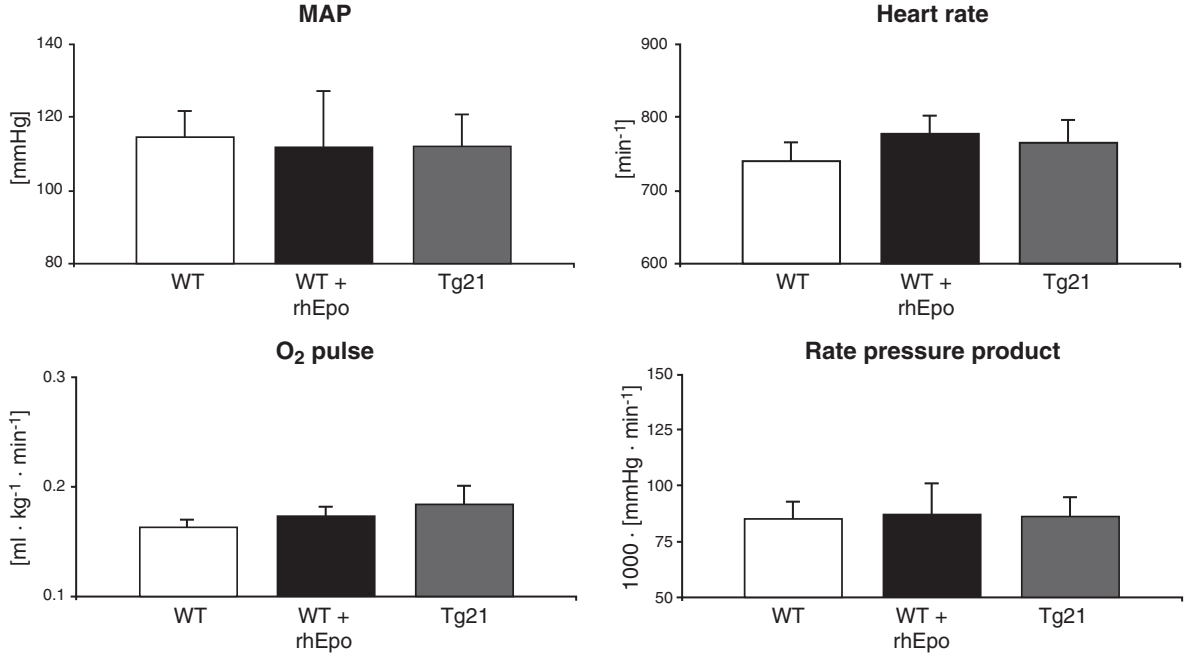

Figure 5. MAP, HR, O_2 pulse, and RPP at $\dot{V}o_{2max}$ in WT+rhEpo *vs.* WT, Tg21 *vs.* WT. Data are means ± SD.

There is an additional possible explanation for the discrepancy between human and animal exercise data: Several studies have provided evidence to support the hypothesis that the central nervous system is a limiting factor for exercise performance (33–35). Although there is no doubt that a given exercise performance is determined by metabolic power (35), voluntary exercise performance depends on the motoneuronal activity, which, in turn, is determined by the metabolic rate. Interestingly, the central fatigue hypothesis is supported by results of animal studies, whereas the experimental evidence in humans is not convincing (34). Accordingly, one might speculate that Epo's effect on central fatigue in relation to exercise limiting factor may be more pronounced in animals than in humans. The central fatigue hypothesis suggests that glucose, dopamine, and serotonine as well as noradrenaline levels in the brain play a key role in limiting maximal exercise capacity (36, 37). Interestingly, human brain areas, such as brain stem, cortex, telencephalon (hippocampus), diencephalon (hypothalamus), and mesencephalon, all postulated to be activated during central fatigue, were shown to bind Epo and to involve serotonine and dopamine during exhaustion at maximal exercise intensities (12). Moreover, compared to WT animals, Tg21 mice, even at rest, have a higher local cerebral glucose utilization in areas of the brain stem (potine gray, lateral lemniscus), mesencephalon (substantia nigra), telencephalon (hippocampus, pyriform cortex), and diencephalon (medial geniculate body) (38). Thus, the improvement in exercise capacity in WT+rhEpo and Tg21 animals may be explained, at least partially, by reduced central fatigue.

$\dot{V}O_{2max}$ is a well-established parameter to measure aerobic power of subjects. However, the definition of the plateau at $\dot{V}O_{2max}$ is highly contentious (39–41). In humans, as few as 17% (but up to 95%) of individuals may reach the $\dot{V}O_{2max}$ plateau (41, 42), and in animals this number may even be lower. To identify the plateau, additional criteria, such as RER $>$ 1.5, are typically included (40). Therefore, at first glance, our mice may not reach "true" $\dot{V}O_{2max}$ plateau, although they were completely working to full capacity at the end of the incremental exercise test. It has to be mentioned, however, that the measured parameters were in the range of other studies using similar methodology and conditions (43–45). Moreover, one must recognize the technical limitations in measuring $\dot{V}O_{2max}$ in small animals. Due to the volume ratio between the animal's tidal volume and the metabolic chamber, the metabolic measurements cannot reflect the immediate condition. This is particularly important to note, as it is known that the $\dot{V}CO_2$ increases to a greater extent compared to the $\dot{V}O_2$ by the end of the incremental exercise test (46). Indeed, we found a tendency of increased RER at $\dot{V}O_{2max}$ in WT+rhEpo and Tg21 mice that might indicate that these animals forced themselves to a higher metabolic rate at maximal exercise.

While the role of Epo on erythroid precursor cells is well established, the activity of Epo on nonerythroid tissue is poorly understood. Regarding central effects of Epo on metabolism and movement, Teng *et al.* (47) showed that the absence of the Epo receptor in brain and other organs, with exception of the hematopoietic tissue, led to decreased locomotor activity and energy expenditure. Moreover, the researchers provide evidence that Epo directly regulates expression of proopiomelanocortin (POMC) by binding the Epo receptor present on POMC neurons. Taken together, we postulate that the increase in cerebral Epo concentration facilitates its effects on exercise capacity by a complex response including elevated POMC expression, by means of enhanced ventilation during exercise-induced hypoxia (48, 49) and by minimizing central fatigue. FJ

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