Erythropoietin administration acutely stimulates resting energy expenditure in healthy young men

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Christensen B, Vendelbo MH, Krusenstjerna-Hafstrøm T, Madsen M, Pedersen SB, Jessen N, Møller N, Jørgensen JOL. Erythropoietin administration acutely stimulates resting energy expenditure in healthy young men. J Appl Physiol 112: 1114–1121, 2012. First published January 12, 2012; doi:10.1152/japplphysiol.01391.2011.—Treatment with recombinant human erythropoietin (rHuEpo) improves insulin sensitivity in patients with end-stage renal disease, and animal studies indicate that Epo increases fat oxidation. However, the metabolic effects of rHuEpo have never been experimentally studied in healthy humans. The aim was to investigate the effects of an acute rHuEpo bolus on substrate metabolism and insulin sensitivity in healthy young men. Ten healthy young men were studied in a single-blinded, randomized crossover design with a 2-wk washout period receiving 400 IU/kg rHuEpo or placebo. Substrate metabolism was evaluated by indirect calorimetry and tracer infusions, and insulin sensitivity by a hyperinsulinemic euglycemic clamp; and PCR and Western blotting measured protein expression and content, respectively. Resting energy expenditure (REE) increased significantly after rHuEpo [basal: 1,863.3 ± 67.2 (kcal/day) (placebo) vs. 2,041.6 ± 81.2 (rHuEpo), P < 0.001; clamp: 1,903.9 ± 68.3 (placebo) vs. 2,015.7 ± 114.4 (rHuEpo), P = 0.03], but the increase could not be explained by changes in mRNA levels of uncoupling protein 2 or 3. Fat oxidation in the basal state tended to be higher after rHuEpo but could not be explained by changes in mRNA levels of CPT1 and PPARα or AMPK and ACC protein phosphorylation. Insulin-stimulated glucose disposal, glucose metabolism, and whole body and forearm protein metabolism did not change significantly in response to rHuEpo. In conclusion, a single injection of rHuEpo acutely increases REE in healthy human subjects. This calorigenic effect is not accompanied by distinct alterations in the pattern of substrate metabolism or insulin sensitivity.

substrate metabolism; insulin sensitivity; skeletal muscle tissue; indirect calorimetry

ERYTHROPOIETIN (Epo), which is produced by the kidneys in response to low oxygen tension, stimulates the oxygen binding capacity of the blood by increasing hemoglobin mass and red blood cell volume. Anemia due to end-stage renal disease (ESRD) constitutes an approved indication for treatment with recombinant human erythropoietin (rHuEpo) (19).

Insulin resistance is a frequent occurrence in patients with moderate to severe chronic renal failure (3, 25, 30, 39, 41). It has been suggested that the primary site for insulin resistance in uremic patients is skeletal muscle due to postreceptor defect in insulin signaling and/or action (39). Treatment of ESRD patients with rHuEpo improves insulin sensitivity within 10 days in nondiabetic patients on hemodialysis in concomitance with a decrease in serum levels of triglycerides but prior to a detectable increase in the hematocrit value (39). Prolonged rHuEpo treatment (5 mo) of diabetic patients on hemodialysis also improves insulin sensitivity, which correlates positively with arterial oxygen tension (PO2) (3). Similar data have been obtained in a study in which rHuEpo treatment to hemodialysis patients improved insulin sensitivity, and it was concluded that the beneficial effects on insulin sensitivity were independent of the correction of anemia (41). Thus insulin sensitivity improves after treatment with rHuEpo to ESRD and/or diabetic patients.

Today, not much is known about the effects of rHuEpo treatment in the healthy state on insulin sensitivity and substrate metabolism, which is important in order to understand the molecular mechanisms for the changes seen in the patients. In cell lines of erythropoietin and/or megakaryocytic origin, treatment with recombinant Epo activates intracellular signaling pathways that are also activated by insulin stimulation (43). However, overexpression of Epo in mice models seems primarily to promote fat loss and fat oxidation rather than improving the ability of insulin to promote glucose disposal (23).

In addition, during the last decade the potential tissue-protective effects of Epo have been explored. It seems that a single bolus of high-dose rHuEpo can protect against damage induced by ischemia, ischemia-reperfusion, trauma, cytotoxicity, infection, and inflammation (4, 8, 16). In this regard, it is important to ascertain acute effects of a single high-dose bolus of rHuEpo on systemic and regional metabolism.

So far, the isolated effect of rHuEpo treatment on insulin sensitivity and substrate metabolism has not been experimentally studied in healthy humans. The aim of the present study was therefore to evaluate the acute effects of rHuEpo treatment on insulin sensitivity and substrate metabolism at the whole body level as well as in skeletal muscle.

METHODS

Subjects. Ten healthy young men [age 23 ± 0.7 yr, body mass index (BMI) 23.5 ± 0.4 kg/m2, hemoglobin 9.5 ± 0.2 mmol/l, hematocrit 44.8 ± 0.6%] were recruited after written informed consent and approval by the Local Human Ethical Committee of Central Denmark Region (M-2008–0016), in accordance with the Declaration of Helsinki. The study was registered at www.clinicaltrials.gov with the clinical trial number M-20080016.

Experimental design. The study had a single-blinded, randomized crossover design with a 14-day washout period. The subjects were screened beforehand with routine blood chemistry values and a clinical examination to rule out occult disease. The subjects were examined on two occasions separated by at least 2 wk, after intravenous administration at t = 0 min of rHuEpo [400 IU/kg Eprex (Epoietin alpha)] or placebo (saline); the order was randomized. The
subjects arrived at the clinical research unit after an overnight fast, where water consumption was allowed. The participants were instructed not to perform any physical exercise for 2 days prior to the experimental days. A total of three intravenous catheters were inserted: one in an antecubital vein for infusions, one in a heated dorsal hand vein to allow for sampling of arterialized blood, and one retrogradely inserted into the contralateral deep antecubital vein for sampling of venous blood. The experimental day was divided into a basal (t = 0–240 min) and a clamp period (t = 240–360 min). Unless otherwise stated, measurements referred to as “basal” represent the mean of three consecutive measurements between t = 210 and t = 240 min, and “clamp” measurements between t = 330 and t = 360 min. Urine and blood were collected before the start of the experiment (t = 0 min) and after the basal (t = 240 min) and the clamp period (t = 360 min). Skeletal muscle biopsies were obtained from the vastus lateralis muscle under local anesthesia at t = 60 min and t = 270 min and snap-frozen in liquid nitrogen. Subcutaneous fat tissue was obtained from the abdomen by liposuction under local anesthesia at t = 270 min and snap-frozen in liquid nitrogen. The experiments were conducted under thermoneutral conditions (21–23°C). A schematic overview of the experimental day is given in Fig. 1.

Indirect calorimetry. Resting energy expenditure (REE) and the respiratory exchange ratio (RER) were estimated by indirect calorimetry (Deltatrac monitor, Dantes Instrumentarium, Helsinki, Finland), which was performed for 30 min during both the basal (t = 170–200 min) and the clamp period (t = 300–330 min). The mean values of the last 25 min were used for calculations. Protein oxidation was calculated on the basis of urea nitrogen excretion. Protein, fat, and glucose oxidation rates were calculated as previously described (18).

The forearm model. Arterialized blood was drawn from the heated hand vein, and venous blood was obtained from the contralateral deep antecubital vein. Before deep venous sampling, a wrist cuff was inflated to a suprasystolic pressure in order to obtain venous blood from the hand vein, and venous blood was obtained from the contralateral deep antecubital vein. Before deep venous sampling, a wrist cuff was inflated to a suprasystolic pressure in order to obtain venous blood from the hand vein, and venous blood was obtained from the contralateral deep antecubital vein. The forearm model.

Glucose metabolism. To determine glucose turnover a priming dose of [3-3H]glucose [NEN Life Sciences Products, Boston, MA (20 mCi)] was given intravenously at t = 0, followed by a continuous infusion of [3-3H]glucose (12 mCi/h) for 6 h. Glucose rate of appearance (Ra) and disappearance (Rd) were estimated by Steele’s equation for non-steady state (14, 36, 40). Nonoxidative glucose disposal (NOGD) was calculated by subtracting oxidative glucose disposal as assessed by indirect calorimetry from whole body glucose disposal (Rd) (32). Basal endogenous glucose production (EGP) was estimated as the mean Ra calculated during the last 30 min of the basal period.

Insulin-stimulated glucose disposal was evaluated by a hyperinsulinemic euglycemic clamp. Insulin (1.0 mU·kg⁻¹·min⁻¹; Actrapid, Novo Nordisk A/S, Copenhagen, Denmark) was infused from t = 240 to 360 min and plasma glucose was clamped at ~5 mmol/l by adjusting the intravenous infusion rate of 20% glucose according to plasma glucose measurements every 10 min. Insulin sensitivity was estimated from the glucose infusion rate (GIR): GIR (mg·kg⁻¹·min⁻¹) = glucose infusion rate (ml/min) × glucose concentration (20% = 180 mg/ml)/total body wt (kg). Thus the amount of infused glucose required to maintain blood glucose at 5 mmol/l during the clamp, i.e., GIR, reflects peripheral insulin sensitivity. Endogenous glucose production (EGP) during the clamp period was estimated by subtracting the GIR from the Ra at the end of the clamp.

To minimize rapid dilution of the [3-3H]glucose administered throughout the study, [3-3H]glucose was also added to the infused glucose during the clamp (100 mCi [3-3H]glucose/500 ml 20% glucose) (10).

Protein turnover. After collection of baseline blood samples (t = 0 min), a primed dose of L-[15N]phenylalanine (0.7 mg/kg), L-[2H₄]tyrosine (0.5 mg/kg), L-[15N]tyrosine (0.3 mg/kg), and [13C]carbamide (390.6 mg) (Cambridge Isotope Laboratories, Andover, MA) was given followed by continuous infusions of L-[15N]phenylalanine (0.7 mg·kg⁻¹·h⁻¹), L-[2H₄]tyrosine (0.5 mg·kg⁻¹·h⁻¹), and [13C]carbamide (42 mg/h) for 4 h. All the tracers were prepared under sterile conditions and were tested free of bacteria and pyrogens before use. Phenylalanine, urea, and tyrosine kinetics were calculated as previously described (22).

PCR. Adipose tissue (100 mg) and skeletal muscle (20 mg) samples were homogenized in TriZol reagent (Gibco BRL, Life Technologies, Glenville, NY) and RNA was extracted by phenol/chloroform extraction and ethanol precipitation. RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Life Technologies, Glenville, NY) and the synthesized cDNA was subjected to two rounds of PCR amplification using hsp70 as internal control in a BIOMATE III thermocycler (Biometra, Göttingen, Germany). PCR primers for adiponectin (accession number AF202635) were 5’-CCATGGGAAAGCTCAAAAGT-3’ and 5’-ATGTTCTGGGAGACAGGTTG-3’. PCR products were identified by agarose gel electrophoresis, purified from agarose and sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Sequence data were analyzed using the Sequencher software (Gene Codes, Ann Arbor, MI). Each sample was measured in duplicate and each experiment was performed in triplicate.

The schematic overview of the experimental day is presented in Fig. 1.
Roskilde, Denmark) and total RNA was extracted following the manufacturer’s protocol. RNA was quantified by measuring absorbance at 260 and 280 nm using a NanoDrop 8000 (NanoDrop products, Becton, DE), and the inclusion criterion was a ratio ≥ 1.8. Finally, the integrity of the RNA was checked by visual inspection of the two ribosomal RNAs, 18S and 28S, on an agarose gel.

For real-time reverse transcriptase PCR, complementary DNA was constructed using random hexamer primers as described by the manufacturer (Verso cDNA kit, Abgene, Epsom, UK). Then KAPA SYBR FAST qPCR mastermix (Kapa Biosystems, Woburn, MA) and the primers listed in Table 1 were used. Real-time quantification of genes was performed using an iCycler from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). cDNA with specific primers amplified in the two ribosomal RNAs, 18S and 28S, on an agarose gel.

The phospho-specific (Ser79) antibody was from Mil- ler. All samples were amplified in duplicate. A similar set-up was used for negative controls, except that the reverse transcriptase was omitted and no PCR products were detected under these conditions. Western blotting. Proteins were purified from the biopsies (30–50 mg) by homogenization on ice with a polytron in homogenization buffer [20 mM Tris·HCl, 50 mM NaF, 5 mM tetrasodium pyrophosphate, 270 mM sucrose, 1% (vol/vol) Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM glycerophosphate, 2 mM DTT, 50 μg/ml soybean trypsin inhibitor, 4 μg/ml leupeptin, 100 μM benzamidine, and 500 μM PMSF]. The samples were then left on ice for 30 min and during this time occasionally vortexed, before being centrifuged at 14,000 g at 4°C for 20 min. The supernatant was collected, frozen in liquid nitrogen, and stored at −80°C until analyses were performed. Protein concentration was determined by the Bradford assay (Protein Assay, 500-0006, Bio-Rad Laboratories, Hercules, CA; albumin standard, Thermo Scientific, Waltham, MA; Vector 3, 1420 multilabel counter, Perkin Elmer).

The protein fraction was analyzed for phosphorylation of AMP-activated protein kinase (AMPK) and the downstream target acetyl CoA carboxylase (ACC), which are important regulators of fatty acid oxidation. The phospho-specific Ser(172)-ACC antibody was from Millipore (Billerica, MA, no. 07–303), the phospho-specific Akt- (Thr172) antibody was from Cell Signaling (Beverly, CA, no. 2531), total Akt antibody from Santa Cruz (Dallas, TX, no. sc-8312), total PPARδ antibody from Cell Signaling (Beverly, CA, no. 2800), and total β-actin antibody from Sigma (St. Louis, MO, no. A5441). Western blotting was, in short, performed as follows: 20 μg of protein was loaded onto a 4–12% SDS gel, followed by electroblotting onto a PVDF membrane. Membranes were blocked with blocking buffer (2% BSA, in TBS buffer) before primary antibody was added overnight at 4°C. Following several washes, the membrane was incubated with the secondary antibody for 60 min at room temperature. The protein of interest was then detected by a chemiluminescence detection system (Super Signal West Dura Extended duration substrate, Pierce, Thermo Scientific, Waltham, MA, cat. no. 34075) and visualized using an image system (EPI chemi II darkroom, UPV BioImaging systems). The PVDF membranes were stripped after visualization of the phospho-antibodies and reincubated with the total antibodies. Membranes were stripped (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 73.75 ml dH2O) for 1 h at 55°C.

Blood analysis. Plasma glucose was measured immediately after collection in duplicate on a Beckman Glucoanalyzer (Beckman Instruments, Palo Alto, CA) or on an YSI 2700 Select (YSI Life Sciences, Yellow Springs, OH). Additional serum samples were collected, frozen, and stored at −20°C for subsequent analysis. The specific activity of [3-3H]glucose was measured as previously described (31). Insulin and growth hormone (GH) were analyzed by commercial time-resolved immunofluorometric assays (TR-IFMA; Auto DELFIA, PerkinElmer, Turku, Finland) [insulin: intra-assay coefficient of variation (CV) 3.4% and interassay CV 3.8%; GH: intra-assay CV < 8% and interassay CV < 10%]. C-peptide was measured by a commercial ELISA kit (intra-assay CV 5.1% and interassay CV 4.2% at 200 pmol/l) (DakoCytomation, Cambridgeshire, UK). Free fatty acids (FFA) were analyzed by a commercially available kit (intra-assay CV 2–4% and interassay CV 3–6%, detection limit 0.02 mmol/l) (Wako Chemicals, Neuss, Germany). Urea was determined by a commercial method (intra-assay CV 1.3% and interassay CV 1.8%) (Cobas Integra 800, Roche, Mannheim, Germany). Glucagon was measured by an in-house radioimmunoassay (intra-assay CV < 5% and interassay CV < 16%) (34). Total IGF-I was measured in acid ethanol extracted serum, using an in-house TR-IFMA as previously described (intra-assay CV < 5% and interassay CV < 10%) (20). IGFFBP2 and adiponectin were measured by in-house TR-IFMAs based on commercial reagents as previously described (IGFBP2: intra-assay CV 5% and interassay CV 12%; adiponectin: intra-assay CV < 5% and interassay CV < 10%) (21, 26). Enrichments of [15N]-phenylalanine, [15N]-tyrosine, and [1-13C]-tyrosine were measured using gas chromatography-mass spectrometry (GC-MS) as their r-butyldimethylsilyl ether derivatives under electron ionization conditions, and concentrations of phenylalanine and tyrosine were measured (for calculation of regional amino acid kinetics) using l-[13C]phenylalanine and l-[1-13C]tyrosine as internal standards (33). Erythropoietin concentrations were measured by a commercially available ELISA kit (Quantikine IVD, Human Epo immunoassay, R&D systems, cat. no. DEPOO, Minneapolis, MN). The range of the assay was 2.5–200 mIU/ml with a sensitivity of 0.6 mIU/ml, and intra-assay precision (%CV) was between 2.18 and 7.82.

Statistics. A P value < 0.05 was considered significant. If not otherwise stated all results are represented as means ± SE. If data deviated from normality (Shapiro-Wilk) they were log transformed before further analysis. Differences between the two treatments (HuEpo vs. placebo) and between the basal and clamp period were assessed by a repeated-measures two-way ANOVA with Tukey’s post hoc test. Whenever data where only obtained during the basal or clamp period, a Student’s paired t-test was performed to evaluate treatment effects. If the data were not normally distributed after log transformation the Wilcoxon signed rank test was used. SigmaPlot 11.0 was used for statistical analysis.

RESULTS

Serum hormone concentrations. As expected serum Epo concentrations increased significantly after its administration (Table 2). Insulin levels during the clamp were not significantly different in the two treatment settings. Insulin infusion during the clamp was associated with predicted changes in the
levels of C-peptide and glucagon without any detectable effect of rHuEpo. However, the glucagon levels were higher during the basal period after treatment with rHuEpo (Table 2).

Indirect calorimetry. A higher resting energy expenditure (REE) was recorded after treatment with rHuEpo, both during the basal period and clamp period (Table 3). As expected, the RER increased during the hyperinsulinemic glucose clamp. The relative increase in RER during the clamp was higher during rHuEpo treatment compared with placebo. As expected the amount of carbohydrate oxidized increased significantly during the clamp period in both groups. This was paralleled by reduced fat oxidation. Fat oxidation during the clamp was higher during rHuEpo treatment compared with placebo. In the basal period after treatment with rHuEpo (Table 2). and NOGD increased significantly in both groups during the clamp period compared with basal period with no specific effect of rHuEpo. Endogenous glucose production (EGP) was significantly suppressed during the clamp in both groups without any difference between placebo and rHuEpo (Table 4).

Glucose turnover. Insulin-stimulated glucose uptake (GIR) was not significantly different at steady state (average of the 3 last measurements) between the placebo and rHuEpo group (Table 4). In addition, no significant difference was found when comparing the area under the curve (AUC) for GIR for rHuEpo and Placebo during the whole clamp period.

Glucose turnover (Ra and Rd) increased significantly within both groups during the clamp period compared with the basal period, with no differences between rHuEpo and placebo. As expected, NOGD increased significantly in both groups during the clamp period compared with basal period with no specific effect of rHuEpo. Endogenous glucose production (EGP) was significantly suppressed during the clamp in both groups without any difference between placebo and rHuEpo (Table 4).

Table 2. Serum hormone concentrations

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>rHuEpo</th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Basal</td>
<td>Clamp</td>
</tr>
<tr>
<td>Erythropoietin, mIU/ml</td>
<td>5.3 ± 0.7</td>
<td>6.7 ± 0.6†</td>
<td>7.3 ± 0.6†</td>
</tr>
<tr>
<td></td>
<td>30.8 ± 5.3</td>
<td>340.4 ± 21.1*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C-peptide, pmol/l</td>
<td>365.8 ± 47.6</td>
<td>176.7 ± 58.5*</td>
<td>0.002</td>
</tr>
<tr>
<td>Glucagon, pg/ml</td>
<td>55.4 ± 5.5</td>
<td>42.0 ± 7.0*</td>
<td>0.042</td>
</tr>
<tr>
<td>Adiponectin, mg/l</td>
<td>6.67 ± 0.75</td>
<td>6.48 ± 0.76</td>
<td>NS</td>
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</table>

Values are means ± SE. rHuEpo, recombinant human erythropoietin (rHuEpo). *Compared with basal period. †Compared with baseline. ‡Compared with placebo. §Interaction.

Table 3. Calorimetry results

<table>
<thead>
<tr>
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<th>Placebo</th>
<th>rHuEpo</th>
<th>P value</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>Basal</td>
<td>Clamp</td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>Basal</td>
<td>Clamp</td>
</tr>
<tr>
<td>REE, kcal/day</td>
<td>1,863.3 ± 67.2</td>
<td>1,903.9 ± 68.3</td>
<td>NS</td>
</tr>
<tr>
<td>Vco2, l/min</td>
<td>2.69 ± 0.97</td>
<td>2.73 ± 1.00</td>
<td>NS</td>
</tr>
<tr>
<td>RER</td>
<td>0.832 ± 0.006</td>
<td>0.875 ± 0.013*</td>
<td>0.001</td>
</tr>
<tr>
<td>Protein oxidation, mg·kg⁻¹·min⁻¹</td>
<td>0.96 ± 0.11</td>
<td>1.08 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>Carbohydrate oxidation, mg·kg⁻¹·min⁻¹</td>
<td>1.37 ± 0.07</td>
<td>1.97 ± 0.22*</td>
<td>0.017</td>
</tr>
<tr>
<td>Fat oxidation, mg·kg⁻¹·min⁻¹</td>
<td>0.71 ± 0.06</td>
<td>0.44 ± 0.07*</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Values are means ± SE REE, resting energy expenditure; RER, respiratory exchange ratio; Vco2, oxygen consumption; Vco2, carbon dioxide production; NS, not significant. *Compared with basal period. †Compared with placebo clamp. ‡Compared with placebo basal. §Interaction.
**Muscle CPT1 and PPARα mRNA levels.** Peroxisome proliferator-activated receptor alpha (PPARα) is a nuclear transcription factor acting as a fatty acid sensor by affecting the expression of genes involved in fat metabolism. The mRNA levels of PPARα in skeletal muscle were significantly increased (P < 0.001) 30 min into the clamp with no effect of the rHuEpo treatment. Carnitine palmitoyltransferase 1 (CPT1) is associated with the outer mitochondrial membrane and mediates the transport of long-chain fatty acids over the mitochondrial membrane. Skeletal muscle CPT1 mRNA levels were significantly (P < 0.001) upregulated 30 min into the clamp period; no effect of the treatment with rHuEpo was found (Fig. 3, C and D).

**DISCUSSION**

The present study was undertaken to assess the acute effects of rHuEpo on energy expenditure, substrate metabolism, and insulin sensitivity in healthy human subjects. The major effect was a significant increase in REE, which was accompanied by a tendency to increased fat oxidation following rHuEpo exposure. By contrast, acute rHuEpo exposure did not impact glucose or protein metabolism. Thus acute rHuEpo administration does not seem to induce major changes in insulin sensitivity or substrate metabolism.

In general, there are very limited data regarding the acute effects of rHuEpo administration on substrate metabolism and insulin sensitivity; therefore most of the findings in the present study have been discussed in relation to studies with long-term treatment with rHuEpo, both in patients and animals. However, it is important also to determine the acute effects of a single bout of high-dose rHuEpo, since such a treatment regimen is suggested to be useful in relation to stroke and thromboembolic events (8). In this regard, it could be considered beneficial that acute rHuEpo administration does not induce major perturbations in substrate metabolism and insulin sensitivity. Of note, however, a study by Ehrenreich et al. from 2009 showed increased mortality in patients with ischemic stroke after receiving high dose rHuEpo infusion (15), and data from the present experimental study should not be considered as potential salutary effects of rHuEpo treatment.

This is the first study to show an acute increase in REE in humans, but our observation is in agreement with a study by Carraway et al. (5), who recorded increased REE 7–10 days after administration of rHuEpo.

**Table 4. Whole body and forearm metabolism assessed by tracer infusions**

<table>
<thead>
<tr>
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<th>Placebo</th>
<th>rHuEpo</th>
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<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Clamp</td>
</tr>
<tr>
<td>Whole body metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbamide flux, μmol-kg⁻¹-h⁻¹</td>
<td>447.1 ± 66.8</td>
<td>413.6 ± 49.1</td>
</tr>
<tr>
<td>Phenylalanine flux, μmol-kg⁻¹-h⁻¹</td>
<td>41.7 ± 1.1</td>
<td>42.4 ± 1.1</td>
</tr>
<tr>
<td>Tyrosine flux, μmol-kg⁻¹-h⁻¹</td>
<td>28.9 ± 0.6</td>
<td>29.3 ± 0.8</td>
</tr>
<tr>
<td>Phenylalanine conversion to tyrosine, μmol-kg⁻¹-h⁻¹</td>
<td>4.05 ± 0.34</td>
<td>3.24 ± 0.21</td>
</tr>
<tr>
<td>Phenylalanine incorporation into protein, μmol-kg⁻¹-h⁻¹</td>
<td>37.7 ± 1.2</td>
<td>39.2 ± 1.0</td>
</tr>
<tr>
<td>Glucose (arterial), mmol/l</td>
<td>5.18 ± 0.11</td>
<td>5.16 ± 0.12</td>
</tr>
<tr>
<td>Glucose Ra, mg·kg⁻¹·TBW⁻¹·min⁻¹</td>
<td>1.51 ± 0.11</td>
<td>1.62 ± 0.08</td>
</tr>
<tr>
<td>Glucose Rd, mg·kg⁻¹·TBW⁻¹·min⁻¹</td>
<td>1.54 ± 0.06</td>
<td>1.63 ± 0.05</td>
</tr>
<tr>
<td>EGP, mg·kg⁻¹·TBW⁻¹·min⁻¹</td>
<td>1.51 ± 0.11</td>
<td>1.62 ± 0.08</td>
</tr>
<tr>
<td>NOGD, mg·kg⁻¹·TBW⁻¹·min⁻¹</td>
<td>0.18 ± 0.07</td>
<td>0.42 ± 0.24</td>
</tr>
<tr>
<td>GIR, mg·kg⁻¹·min⁻¹</td>
<td>0.37 ± 0.07</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>FFA (arterial), mmol/l</td>
<td>0.37 ± 0.07</td>
<td>0.05 ± 0.01*</td>
</tr>
<tr>
<td>Forearm metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine balance, μg·100 ml⁻¹·min⁻¹</td>
<td>−1.73 ± 0.27</td>
<td>−1.88 ± 0.43</td>
</tr>
<tr>
<td>Phenylalanine Ra, μg·100 ml⁻¹·min⁻¹</td>
<td>4.10 ± 0.45</td>
<td>4.14 ± 0.53</td>
</tr>
<tr>
<td>Phenylalanine Rd, μg·100 ml⁻¹·min⁻¹</td>
<td>2.37 ± 0.44</td>
<td>2.26 ± 0.26</td>
</tr>
<tr>
<td>FFA uptake, μg·100 ml⁻¹·min⁻¹</td>
<td>0.14 ± 0.11</td>
<td>0.03 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE. Ra, rate of appearance; Rd, rate of disappearance; EGP, endogenous glucose production; NOGD, nonoxidized glucose disposal; GIR, glucose infusion rate; FFA, free fatty acid. *Compared with basal period. No significant differences were found between placebo and rHuEpo groups (n = 10).
after three days of treatment with Epo in mice. However, in healthy human subjects it was found that both oxygen consumption (VO₂) and carbon dioxide production (VCO₂) (and hence REE) were unaltered after 15 wk of treatment with rHuEpo (28). However, low concentrations of rHuEpo due to only one weekly injection from weeks 4 until 15 and a half-life of rHuEpo of only 2–13 h (37) could be the explanation for the unaltered REE. In support of this, 3 days of very high rHuEpo injections (3 × 30,000 IU/day) to healthy young men led to significant increases in cerebral metabolic rate for oxygen (CMRO₂) during exercise (38). A long-term study with rHuEpo treatment to cancer patients for up to 30 mo also found significantly increased REE (11).

It has previously been shown that genes involved in thermogenesis are upregulated after recombinant Epo administration for 1 wk in mice (23), and that heat production in skeletal muscle from hemodialysis patients increases after rHuEpo administration (17). It remains to be further characterized whether this acute thermogenic effect, leading to increased REE, is caused by increased ATP utilization (i.e., futile cycling), reduced thermodynamic efficiency of energy utilization (i.e., uncoupling of oxidative phosphorylation), or increased erythropoiesis. Therefore, mRNA levels of UCP2 and UCP3 in skeletal muscle biopsies and UCP2 levels in subcutaneous fat biopsies were measured. These uncoupling proteins are located in the inner mitochondrial membrane, and are able to “uncouple” or discharge the proton gradient and thereby result in heat production rather than ATP generation. No effect of the treatment with rHuEpo was found at the mRNA levels of these proteins in either skeletal muscle or fat tissue. However, increased expression occurred in skeletal muscle tissue in relation to the hyperinsulinemic clamp, which supports a direct stimulatory effect of insulin on skeletal muscle UCP expression (35). In hemodialysis patients treated with rHuEpo, the levels of ATP decreased significantly, indicating that ATP consumption was greater than ATP production (17). UCP levels were not measured in these patients, and the mechanism behind this decrease in ATP levels remains unknown. Based on these data, it is not evident that a reduced efficacy of energy utilization in skeletal muscle or fat tissue is underlying our observation of increased REE found in the present study.

Skeletal muscle metabolism is positively associated with REE, possibly accounting for up to 30% of the total resting oxygen uptake (45). Changes in skeletal muscle blood flow due to rHuEpo treatment could be an explanation for the increase in REE observed in the present study. However, forearm blood flow did not change significantly throughout the study. Another mechanism underlying the increased REE could be an increase in pulmonary ventilation (V'E) and subsequent workload. Rasmussen et al. (38) found that V'E increased after treatment for 3 days with high levels of rHuEpo during low-intensity work. Unfortunately V'E was not measured during rest in either the present study or in the study by Rasmussen et al.

Fat oxidation tended to be higher after acute rHuEpo administration during the basal period and the relative degree of suppression of fat oxidation during the clamp was significantly higher in the rHuEpo group, mainly reflecting a higher level of fat oxidation during the basal period in the rHuEpo group. The mRNA levels of PPARα, a fatty acid sensor, were not affected by rHuEpo treatment. Neither were the mRNA levels of CPT1, an enzyme regulated by PPARα, which mediates the translocation of activated acyl-CoA esters across the mitochondrial membrane (42). Furthermore, we were not able to show any changes in the phosphorylation of the energy-sensing enzymes AMPK and ACC (regulated both by AMPK and PPARα) (42), indicating that the influx of fatty acids to the mitochondria was not altered. Data on acute effects of rHuEpo on fat metabolism
are very limited. One study found no changes in lipid and lipoprotein patterns after acute administration of rHuEpo to hemodialysis patients, when measured 16–20 h post-rHuEpo administration (1). Studies in mice overexpressing the Epo gene have recorded elevations in fat oxidation and reductions in the amount of total and abdominal fat mass (23, 24). Long-term studies in human subjects with renal disease have confirmed these positive effects on fat metabolism (1, 12, 25, 29, 30), although some of the studies found no changes in total body weight/fat mass (25, 29, 30). In addition, Cayla et al. (7) have shown that recombinant Epo administration to rats induces a shift in muscle fiber composition from fast glycolytic fibers to slower oxidative fibers. In support of this, Davenport et al. (13) found that muscle glycogen content was increased and muscle fat content decreased in patients with ESRD after treatment with rHuEpo. Both studies indicate that muscles rely more on fat than glucose oxidation after rHuEpo administration. Furthermore, there is evidence for Epo-stimulated mitochondrial biogenesis in cardiac muscle tissue (5); whether the same holds true for skeletal muscle tissue has to be determined.

Data regarding the acute effects of rHuEpo administration on glycemic control and insulin sensitivity are limited. Three days of injections with high-dose rHuEpo did not induce changes in the AV difference for glucose in healthy humans (38). In contradiction to the results in the present acute study, animal studies with prolonged Epo treatment have shown a reduction in basal resting plasma glucose levels (6, 9), improved glycemic control, and reduced body weight (24). However, in support of our data, Epo treatment in wild-type mice showed no changes in insulin sensitivity, although a glucose-lowering effect of the treatment was found (9). Furthermore, long-term treatment with rHuEpo in ESRD patients significantly improves insulin sensitivity (3, 29, 39, 41), although a complete correction of anemia in these patients may lead to increased insulin resistance (2). It is currently not known how prolonged rHuEpo treatment improves insulin sensitivity in these patients, but improved oxygen supplementation has been suggested (25). However, studies have demonstrated beneficial effects of rHuEpo treatment on insulin sensitivity in dialysis patients without alterations in hematological levels (39, 41). In the present study, we did not find any evidence that rHuEpo acutely and directly should impact insulin sensitivity in healthy humans. Neither whole body protein metabolism nor regional protein metabolism across the forearm were affected by rHuEpo treatment in the present study. Overexpression of Epo in mice has led to muscle hypertrophy through an activation of the Akt signaling pathway (23). However, treatment with rHuEpo for 14 wk did not affect either muscle fiber size in healthy humans (27) or body weight and mid-arm circumference in ESRD patients (12).

In summary, acute rHuEpo administration in healthy human subjects increases REE without any significant effect on insulin sensitivity, glucose or protein metabolism. Furthermore, a tendency to increased fat oxidation after treatment with rHuEpo was found. These observations could be of relevance when evaluating the clinical potential of short-term, high-dose rHuEpo administration.

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AUTHOR CONTRIBUTIONS
B.C., N.J., and J.O.L.J. conception and design of research; B.C., M.H.V., T.K.-H., and M.M. performed experiments; B.C. and B.P.; analyzed data; B.C., N.J., and J.O.L.J. interpreted results of experiments; B.C., prepared figures; B.C. drafted manuscript; B.C., M.H.V., T.K.-H., M.M., S.B.P., N.J., N.M., and J.O.L.J. edited and revised manuscript; B.C., N.J., and J.O.L.J. approved final version of manuscript.

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