Erythropoietin receptor in human skeletal muscle and the effects of acute and long-term injections with recombinant human erythropoietin on the skeletal muscle

Carsten Lundby,1–3 Ylva Hellsten,1,4 Mie B. F. Jensen,1,5 Anders S. Munch,1,5 and Henriette Pilegaard1,5
1Copenhagen Muscle Research Centre, 2Rigshospitalet section 7652, Copenhagen, 3Department of Sport Science, Århus, Denmark, 4Department for Exercise and Sport Science, University of Copenhagen; and 5Centre of Inflammation and Metabolism, Department of Molecular Biology, University of Copenhagen, Denmark

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Erythropoietin receptor in human skeletal muscle and the effects of acute and long-term injections with recombinant human erythropoietin on the skeletal muscle. J Appl Physiol 104: 1154–1160, 2008. First published January 24, 2008; doi:10.1152/japplphysiol.01211.2007.—The presence and potential physiological role of the erythropoietin receptor (Epo-R) were examined in human skeletal muscle. In this study we demonstrate that Epo-R is present in the endothelium, smooth muscle cells, and in fractions of the sarcot TEMS of skeletal muscle fibers. To study the potential effects of Epo in human skeletal muscle, two separate studies were conducted: one to study the acute effects of a single Epo injection on skeletal muscle gene expression and plasma hormones and another to study the effects of long-term (14 wk) Epo treatment on skeletal muscle structure. Subjects (n = 11) received a single Epo injection of 15,000 IU (double blinded, cross over, placebo). A single Epo injection reduced myoglobin and increased transferrin receptor and MRF-4 mRNA content within 10 h after injection. Plasma hormones remained unaltered. Capillarization and fiber hypertrophy was studied in subjects (n = 8) who received long-term Epo administration, and muscle biopsies were obtained before and after. Epo treatment did not alter mean fiber area (0.84 ± 0.2 vs. 0.72 ± 0.3 mm2), capillaries per fiber (4.3 ± 0.5 vs. 4.4 ± 1.3), or number of proliferating endothelial cells. In conclusion, the Epo-R is present in the vasculature and myocytes in human skeletal muscle, suggesting a role in both cell types. In accordance, a single injection of Epo regulates myoglobin, MRF-4, and transferrin receptor mRNA levels. However, in contrast to our hypothesis, prolonged Epo administration had no apparent effect on capillarization or muscle fiber hypertrophy.

angiogenesis; hypertrophy; vascular endothelial growth factor; rHuEpo; cancer; tumor

Erythropoietin (Epo) has for decades been known as the main regulator of erythropoiesis by stimulating growth, preventing apoptosis, and inducing differentiation of the red blood cell precursors (12). There are also several reports showing that activation of Epo-R has a pronounced angiogenic effect in part by enhancing the expression of several angiogenic growth factors (14) and by stimulating proliferation (10) and migration of endothelial cells (19, 20). Epo has been also shown to promote angiogenesis in various tumors, cardiac auricle, and in the healthy uterus (7, 11, 26, 36).

Interestingly, cell culture studies indicate that Epo-R is present in cardiomyocytes (35), suggesting that the Epo-R may also be present in skeletal muscle. Therefore, provided that Epo-R is present in skeletal muscle tissue, Epo may also be of importance for the regulation of angiogenesis and, hence, prolonged Epo treatment could result in an increased muscle capillarization.

Epo has been described as exerting effects similar to VEGF on the angiogenic process, and one of the mechanisms by which Epo appears to promote angiogenesis is by enhancing the level of VEGF in tissue. A close association between VEGF and Epo in angiogenesis has been proposed (1, 4) and Epo treatment has been found to enhance the release of VEGF from marrow stromal cells (37) and to increase levels of VEGF in brain (14, 32). Considering the importance of VEGF in skeletal muscle capillary growth (22), it is therefore plausible that one of the angiogenic effects of Epo is mediated by promoting VEGF levels in the muscle.

Another interesting potential physiological role of Epo in skeletal muscle is in muscle fiber growth. Epo-R activation stimulates the signal transducer and activator of transcription (STAT)5, which is known to modulate cell proliferation and differentiation (7). STAT5 also activates the PI3 kinase-Akt signaling pathway (24, 31), which is believed to result in activation of AKT and p70S6K, which in turn plays a role in transcription and cell-cycle progression. This pathway has been suggested to be critical in regulation of skeletal muscle hypertrophy (5, 29). On the basis of the above findings it appears plausible that Epo-R activation may contribute in regulating skeletal muscle fiber growth.

In addition, it may be speculated that Epo is regulating the expression of genes involved in iron homeostasis as recent findings demonstrate that 8 days of altitude exposure alters the level of several ion-regulating proteins including myoglobin (28).

In the present study we examined the presence and distribution of Epo-R in human skeletal muscle and we tested the hypothesis that prolonged treatment with rHuEpo in humans causes angiogenesis and hypertrophy in skeletal muscle. For this purpose eight subjects received rHuEpo for 14 wk where muscle biopsies were obtained before and after the treatment period. In addition, in another group of 11 subjects, the effect of acute injections of 15,000 IU rHuEpo on mRNA expression of genes related to angiogenesis and selected muscle characteristics in human skeletal muscle was investigated.

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Address for reprint requests and other correspondence: C. Lundby, Copenhagen Muscle Research Centre, Rigshospitalet, section 7652, Blegdamsvej 3, 2100 Copenhagen Ø, Denmark (e-mail: lundby@idraet.au.dk).
consecutive days, and during the third week three injections were given on three saline. For the first 2 wk one injection was administered every second of rHuEpo (NeoRecormon, Roche, Mannheim, Germany) in 0.3 ml 60 IU/kg body mass.

Subjects

METHODS

Subjects obtained after 20 min of supine rest. The specimen was mounted on laboratory between 0800 and 1000 in the morning and the biopsy was

Experimental Design

Table 1. DNA sequence for probes and primers for IGF-I, Epo, ferritin, and myoglobin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>TaqMan Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-IEa</td>
<td>X57025</td>
<td>5′-CAGCGCCACACCGACAT-3′</td>
<td>5′-AAGACCCAGAAGGAAGTACATTTGAAGAACGC-3′</td>
<td>5′-AAGACCCAGAAGGAAGTACATTTGAAGAACGC-3′</td>
</tr>
<tr>
<td>Epo-R</td>
<td>NM000121</td>
<td>5′-ACCAAGCACAAGATCCCCGTGAAGTACCT-3′</td>
<td>5′-ACCAAGCACAAGATCCCCGTGAAGTACCT-3′</td>
<td>5′-ACCAAGCACAAGATCCCCGTGAAGTACCT-3′</td>
</tr>
<tr>
<td>Ferroportin</td>
<td>NM014585</td>
<td>5′-TGCCACAACGGACGACTTC-3′</td>
<td>5′-CGGGTCCAGGTCTTCGAA-3′</td>
<td>5′-CGGGTCCAGGTCTTCGAA-3′</td>
</tr>
<tr>
<td>IGF-I</td>
<td>NM014585</td>
<td>5′-CAGCGCCACACCGACAT-3′</td>
<td>5′-AAGACCCAGAAGGAAGTACATTTGAAGAACGC-3′</td>
<td>5′-AAGACCCAGAAGGAAGTACATTTGAAGAACGC-3′</td>
</tr>
<tr>
<td>MRF4</td>
<td>XM006691</td>
<td>5′-GCTCGTGATAACGGCTAAGGAA-3′</td>
<td>5′-CGATGGAAGAAAGGCATCGA-3′</td>
<td>5′-CGATGGAAGAAAGGCATCGA-3′</td>
</tr>
<tr>
<td>HIF-1a</td>
<td>AB073325</td>
<td>5′-GCCCCAGATTCAGGATCAGA-3′</td>
<td>5′-TGGGACTATTAGGCTCAGGTGAAC-3′</td>
<td>5′-TGGGACTATTAGGCTCAGGTGAAC-3′</td>
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skeletal muscle samples. The sections were fixed in 2% formaldehyde for 2 min at room temperature and −20°C acetone for 30 s. The sections were rinsed with PBS containing 1% BSA and thereafter blocked with 1% PBS containing BSA. The sections were then incubated for 1 h at room temperature with a monoclonal antibody, mouse α-Epo-R (25 μg/ml; MAB 307, R&D Systems, Abingdon, UK) for the detection of Epo-R. Primary monoclonal antibody, mouse α-CD31 (50 μg/ml; Clone JC70A, DAKO A/S, Glostrup, Denmark) or Ulex Europeanus Agglutinin-I (UEA-1; Sigma Aldrich) was used for detection of endothelial cells. All sections, with the exception of those incubated with UEA-1, were rinsed and thereafter incubated with either a biotin coupled goat α-rabbit (E0432, DAKO A/S) or a biotin-coupled rabbit α-mouse antibody (E0354; DAKO A/S). UEA-1 was directly coupled to TRITC. Antibody binding was visualized with either streptavidin coupled to FITC (F0422, DAKO A/S) or ABC complex with alkaline phosphatase (ABC/Complex/AP, DAKO A/S). Negative controls were achieved with staining without the primary antibody. Immunoreactive cells were examined and photographed in a Zeiss Axioplan Microscope.

Immunohistochemical analysis of MHC I, MHC IIα, and capillaries. The distribution of muscle fibers containing MHC I or MHC IIα was determined by immunohistochemistry with the use of the antibodies and anti-human MHC I (M8421, Sigma, MO) or MHC IIα (N2.261, Developmental Studies Hybridoma Bank, University of Iowa). Capillaries were identified with the use of CD-31 (50 μg/ml; Clone JC70A, DAKO A/S) and proliferating endothelial cells were visualized using an antibody to human Ki67 (10 μg/ml; BD Pharmingen, Cat no. 5560003). Positive binding was visualized as described for immunohistochemical localization of Epo-R. The number of MHC I, MHC IIα-positive fibers, and their respective fiber area, and the number of capillaries were determined using the software program Tema version 1.04 by CheckVision. From the pre rHuEpo treatment biopsy a total of 122 (range: 70–165) fibers were included for the analysis and 120 (range: 89–149) from the post HuEpo specimen.

RNA isolation and reverse transcription. Total RNA was isolated from 20–25 mg of tissue. RNA was resuspended in 2 μl/mg original tissue in diethyl pyrocarbonate (DEPC)-treated H2O containing 0.1 mM EDTA. Reverse transcription (RT) of 2.9 μg total RNA of each sample was performed using the Superscript II RNase H-dt reaction, and verifying the efficiency of the amplification. PCR amplification was conducted determining optimal primer concentrations, probe concentration, and verifying the efficiency of the amplification. PCR amplification was performed (in triplicates) in a total reaction volume of 10 μl with 17 ng cDNA as previously described (17).

Determination of single-stranded cDNA content. The amount of single-stranded DNA (ssDNA) was determined in the RT samples using the OligGreen reagent (Molecular Probes). Samples were analyzed in a 96-well white microplate (Thermo LabSystems) with a total reaction volume of 200 μl in each well. Each sample was run in triplicate with 5 μl RNase-treated cDNA sample, 95 μl TE, and 100 μl of OligGreen reagent in each well. For further analytical details please see Ref. 16.

Determination of plasma hormones. Venous blood was centrifuged at 2,465 g for 15 min and stored at −40°C until analyzed. The plasma concentration of insulin, testosterone, growth hormone, and cortisol were determined in duplicates by ELISA (Electra-Box Diagnostica, Temesos, Sweden; IBL-Hamburg, Germany; BioSource, Nivelles, Belgium).

Statistical Analysis

In Study A, statistical differences were assessed by paired t-test, and in Study B two-way ANOVA for repeated measures with Student Newman-Keuls post hoc test to locate differences was applied. Statistical difference was set to \( P < 0.05 \). All values reported are means ± SE if not stated otherwise.

RESULTS

Immunohistochemical Localization of Epo-R

Staining for Epo-R was conducted with specific antibody that showed the receptor to be primarily localized in the region of capillaries, small arterioles, and venules as verified by colocalization of the Epo-R staining with CD-31 staining in serial sections and by double stain with UEA-1 on the same section as Epo-R (Fig. 1). Epo-R staining was also present in regions of the sarcolemma adjacent to endothelial cells as well as in some regions of the sarcolemma where no endothelial cells were located (Fig. 1). However, there was no continuous staining of the sarcolemma. Positive staining was also observed in the smooth muscle cells. Incubation of the rabbit Epo antibody with the matching peptide abolished the staining pattern and staining without primary antibody resulted in no visible staining.

Study A

Blood parameters. Hemoglobin concentration was 14.2 ± 0.6 before treatment and reached a peak of 17.1 ± 0.5 g/dl after 12 wk of rHuEpo administration (\( P < 0.05 \) from weeks 7 to 15). The hematocrit values followed a similar pattern, being increased from 42 ± 3 to 49 ± 3% at week 12 (\( P < 0.05 \) from weeks 6 to 15, Table 2).

Muscle morphological data. Morphological data are presented in Table 3 where it is shown that muscle fiber size, capillarization, and distribution were unaffected by 14 wk of rHuEpo treatment.

Immunohistochemical assessment of proliferating endothelial cells. In biopsies obtained before and after the 14 wk of rHuEpo treatment, no differences were found in the marker for proliferation of endothelial cells (ki67), the values being 0.02 ± 0.01 and 0.03 ± 0.02 pre and post, respectively.

Study B

Hormones. The single Epo injection had no effect on the plasma levels of either insulin, testosterone, cortisol, or growth hormone within 10 h after injection.

Content of mRNA. The Epo injection reduced the mRNA content of myoglobin to 68% of the prelevel 10 h after injection, the transferrin receptor mRNA content increased twofold 2 h after Epo injection, but had returned to pre levels already at the 4 h biopsy. In addition, Epo-R levels tended to change over time in the Epo group, and MRF4 mRNA levels were significantly elevated 6 h after the injection (Fig. 2). VEGF, HIF-1α, IGF-IIα, ferroportin, MyoD, and myogen were all unaffected at the mRNA level at the investigated time points following Epo injection.
DISCUSSION

The main findings of the present study are that the Epo-R is present within the skeletal muscle tissue with distribution both in the skeletal muscle sarcolemma and the vascular cells, suggesting a physiological role in skeletal muscle myocytes as well as in the blood vessels of the muscle tissue. Interestingly, a single Epo injection did indeed reduce the myoglobin transferrin receptor and augmented MRF4 mRNA content in skeletal muscle. However, acute Epo infusion did not affect the VEGF or HIF-1α mRNA levels in skeletal muscle, and even 14 wk of rHuEpo treatment did not increase angiogenic activity or muscle fiber hypertrophy. Combined these findings suggest that Epo administration may have effects within the human skeletal muscle, but that Epo alone cannot initiate angiogenesis or cause muscle fiber hypertrophy in the muscle tissue during basal conditions.

Until recently, the role for Epo was thought to be strictly related to erythropoiesis. However, within the last few years, new and very exciting functions of Epo have emerged (6). The current demonstration that the Epo-R is present in the sarcolemma as well as in endothelial and smooth muscle cells in

Table 2. Hematocrit, hemoglobin concentration, and arterial oxygen content before and after 14 wk of rHuEpo treatment

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<thead>
<tr>
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<th>Pre</th>
<th>Post</th>
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<tr>
<td>Htc, %</td>
<td>42±3</td>
<td>49±3*</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>14.2±0.6</td>
<td>17.1±0.5*</td>
</tr>
<tr>
<td>CaO₂, ml/dl</td>
<td>19.1±1.3</td>
<td>21.8±1.8*</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Htc, hematocrit; Hb, hemoglobin concentration; CaO₂, arterial oxygen content; Pre and Post, before and after, respectively, 14 wk of rHuEpo treatment. *P < 0.05 from Pre.
human skeletal muscle tissue supports the notion that Epo may also be involved in physiological processes in skeletal muscle. Within the skeletal muscle, three effects of Epo-R activation could be of interest: angiogenesis, hypertrophy, and iron homeostasis in human skeletal muscle.

In a previous study, we showed that altitude-induced erythropoiesis is associated with decreases in skeletal muscle transferrin receptor, ferritin, and myoglobin after 7–9 days of exposure to 4,559 m (27). On the basis of the decreased mRNA levels of myoglobin after 10 h of elevated rHuEpo in the present study, it may be speculated that the altitude-associated effects can be attributed directly to circulating Epo, whereas the transient increase in transferrin-receptor mRNA in the current study does not directly support that Epo mediates the altitude-induced decrease in this protein. In agreement with an effect on iron homeostasis-regulating proteins is a recent observation that prolonged rHuEpo treatment decreases muscle L-ferritin in healthy humans (G. Cairo, unpublished data). This combined would suggest that there is a functional role of Epo-R within the skeletal muscle.

The scientific evidence supporting a promoting role for Epo in angiogenesis in tissue undergoing growth or repair is quite clear (6). The Epo-R has been localized on endothelial cells in vivo and in vitro (2), and the activation of Epo-R on these cells has shown a response including mitogenesis, chemotaxis, and endothelin-1 release. Among the first investigations regarding the proliferation of vessels by Epo stimulation was the study by Anagnostou et al. (2). These investigators demonstrated a dose-dependent increase in proliferation of umbilical vein endothelial cells with rHuEpo added to the medium. In line with this, it was recently made clear that rHuEpo stimulates both the early invasive phase of the angiogenic process that leads to endothelial sprouting, as well as the late differentiation, and that VEGF and Epo amplify each others’ activities (25). The present observation that 14 wk of rHuEpo infusion did not induce capillarization is therefore not in agreement with the many previous observations on a role for Epo in angiogenesis. Nevertheless, the present observation does not exclude a role for Epo in promoting capillary growth in skeletal muscle, considering the fact that angiogenesis is a complex process involving many compounds. Thus it may be suggested that Epo cannot induce capillarization on its own when the tissue is in a normal maintenance state, as other angiogenic compounds need to be present in sufficient amounts.

Table 3. Morphologic skeletal muscle data obtained before (Pre) and after 14 wk of treatment with rHuEpo (Post)

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Pre</th>
<th>Post</th>
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<tr>
<td></td>
<td>Absolute number</td>
<td>Percent number</td>
</tr>
<tr>
<td>ST</td>
<td>85.3±27.3</td>
<td>82.9±15.4</td>
</tr>
<tr>
<td>FTa</td>
<td>34.7±9.7</td>
<td>35.0±12.9</td>
</tr>
<tr>
<td>FTb</td>
<td>27.3±10.2</td>
<td>69.1±8.9</td>
</tr>
<tr>
<td>FTc/x</td>
<td>9.7±2.3</td>
<td>66.6±8.9</td>
</tr>
<tr>
<td>Absol. Area (mm²)</td>
<td>0.56±0.01</td>
<td>0.52±0.02</td>
</tr>
<tr>
<td>Percent area</td>
<td>0.02±0.02</td>
<td>0.4±0.6</td>
</tr>
<tr>
<td>No. of cap to each fiber</td>
<td>0.8±2.0</td>
<td>0.5±0.8</td>
</tr>
<tr>
<td>Cap/fiber</td>
<td>4.3±0.5</td>
<td>4.4±1.3</td>
</tr>
<tr>
<td>Cap/mm²</td>
<td>628±116</td>
<td>693±84</td>
</tr>
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</table>

Values are means ± SD. Cap, capillarization; Absol, ??

Fig. 2. Effects of acute injections of saline (placebo, white bars; n = 9) or 15,000 IU rHuEpo (black bars, n = 11) on myoglobin (A), Epo-R (B), transferrin receptor (C), and MRF4 mRNA content (D) in skeletal muscle. Muscle biopsies were obtained before rHuEpo and saline injection (Pre) as well as 2, 4, 6, and 10 h after the injection. Values are means ± SE.
concomitantly with Epo. Previous studies demonstrating a role of Epo have all used either cell culture or models where the tissue is undergoing growth or repair and in which other angiogenic compounds necessary for capillary growth are likely to be present in an enhanced concentration relative to during maintenance. Thus it could be speculated that rHuEpo injections would be more effective in promoting angiogenesis if infused in combination with a growth factor such as VEGF, considering the proposed close association between these two factors (1, 4, 9).

On the basis of the fact that several previous studies have shown that Epo treatment can induce an enhanced expression of VEGF in different tissues (14, 32, 37), we hypothesized that Epo would promote the potential for angiogenesis in skeletal muscle tissue by increasing the content of VEGF mRNA. However, this single Epo injection did not affect VEGF or HIF-1α mRNA levels in muscle biopsies obtained from 2 to 10 h after injection, thus the proposition that Epo would enhance VEGF gene expression in skeletal muscle was not supported. Previous experiments from our laboratory have shown that VEGF and HIF-1α are rather easily induced genes that are increased between 1 and 6 h after a single endurance exercise bout (13, 15). Thus the timing of the obtained biopsies in the present study corresponds well with these previously observed induction patterns of both VEGF and HIF-1α mRNA and suggest that the lack of VEGF and HIF-1α mRNA responses was not a result of inadequate sampling. It rather appears that Epo may not be a stimulator of VEGF or HIF-1α gene expression in basal skeletal muscle tissue, unlike in other tissues (14).

It should be noted that the skeletal muscle is known as a tissue with a high level of plasticity. With endurance type of training for instance, it is well documented that the fiber composition is altered from Iib to Ila, and with strength training hypertrophy is universally observed, whereas inactivity studies, such as confinement to bed rest for weeks, clearly show a decrease in the cross-sectional area of the muscle fibers (30). The rHuEpo treatment regimen had the expected outcome on blood variables and was effective in increasing hematocrit from 42 to 49% (Table 1), which was the greatest increase possible considering the cardiovascular risks involved at high hematocrit levels. On the basis of the effects at the red blood cell level, it appears likely that the Epo dose given should have been sufficient to induce physiological effects. Nevertheless, as no effects were observed on capillarization or the muscle ultrastructure it cannot be excluded that the lack of effect on the muscle tissue was a consequence of a lower receptor density or a lower receptor sensitivity on the muscle cells compared with on erythrocyte progenitor cells, therefore requiring greater concentrations of Epo. In support of this possibility is that a single injection of Epo resulting in very high plasma Epo concentrations did increase MRF4 mRNA content in the hours after injection, suggesting a role in muscle fiber differentiation or growth as resistance exercise has been shown to increase MRF4 mRNA in human skeletal muscle as well (23).

In conclusion, the Epo-R is located in the sarcolemma and the vascular cells in muscle tissue, suggesting a physiological role of Epo in skeletal muscle myocytes as well as in the blood vessels. A single Epo injection regulates the mRNA expression of myoglobin, transferrin receptor, Epo-R, and MRF4, but prolonged Epo administration does not promote angiogenesis or stimulate muscle fiber growth in nongrowing skeletal muscle tissue. This indicates that Epo may contribute directly in regulating iron homeostasis, whereas an effect of Epo on capillary growth and ultrastructure may require concomitant stimuli from other angiogenic factors or tissue already undergoing angiogenesis.

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