Erythropoietin administration partially prevents adipose tissue loss in experimental cancer cachexia models

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Abstract
Cancer-associated cachexia is characterized, among other symptoms, by a dramatic loss of both muscle and fat. In addition, the cachetic syndrome is often associated with anemia. The object of the present investigation was to assess the effects of erythropoietin (EPO) treatment on experimental cancer cachexia models. The results clearly show that, in addition to the improvement of the hematocrit, EPO treatment promoted a partial preservation of adipose tissue while exerting negligible effects on muscle loss. Administration of EPO to tumor-bearing animals resulted in a significant increase of lipoprotein lipase (LPL) activity in adipose tissue, suggesting that the treatment favored triacylglycerol (TAG) accumulation in the adipose tissue. In vitro experiments using both adipose tissue slices and 3T3-L1 adipocytes suggests that EPO is able to increase the lipogenic rate through the activation of its specific receptor (EPOR). This metabolic pathway, in addition to TAG uptake by LPL, may contribute to the beneficial effects of EPO on fat preservation in cancer cachexia.


Supplementary key words anemia ● lipoprotein lipase ● lipogenesis ● lipolysis

Cancer patients frequently develop a condition of general wasting known as cachexia. This is a multifactorial syndrome that complicates patient management, increases morbidity and mortality rates, reduces the tolerance to antineoplastic therapies, and results in poor quality of life (1). Cachexia is characterized by wasting of muscle and adipose tissue, anemia, anorexia, and perturbations of the hormonal homeostasis. It occurs in 54–70% of newly diagnosed cancer patients (2), worsening their prognosis and clinical management and accounting for about 25% of cancer deaths (3). The pathogenetic mechanisms underlying cachexia are complex and only partially identified; therefore, effective therapeutic strategies are lacking.

Anemia is a frequent feature of patients with cancer cachexia, contributing to weight loss, reduced exercise capacity, and altered energy homeostasis (4). The incidence of anemia varies with tumor type and stage and with the patient’s age; up to one third of cancer patients are anemic at diagnosis (5), and chemotherapy frequently increases this number. Cancer-associated anemia can thus be considered a negative prognostic factor for survival (6). Anemia can lead to hypoxia in several tissues, increasing lactate production and thereby promoting a decrease in intracellular pH that enhances muscle protein degradation, possibly exacerbating tissue wasting (7). Nevertheless, mechanistic data explaining how anemia may contribute to the onset and progression of cachexia are still lacking.

Erythropoietin (EPO), a 34 kDa glycoprotein, is synthesized by the kidney in response to hypoxemia. Its main function is to stimulate erythropoiesis by activating the EPO receptor (EPOR) on erythroid progenitor cells. The binding of EPO to EPOR activates a positive-feedback loop mediated by the transcription factor GATA-1 (8). EPO signals through phosphatidylinositol-3-kinase (PI3K)/Akt, signal transducer and activator of transcription 5 (STAT5), mitogen-activated protein kinase (MAPK), and protein kinase C pathways, inducing erythroblast production and a consequent rise in red blood cell count evident 1–2 weeks after EPO administration (9). EPO has been used for the treatment of cancer wasting to fight the anemia associated with the cachectic syndrome. EPO treatment in unselected randomized cachectic cancer patients prevented the appearance of anemia and the loss of exercise capacity, showing

Abbreviations: aP2, activating protein 2; C26, Colon26 carcinoma; EPO, erythropoietin; EPOR, EPO receptor; FASN, fatty acid synthase; LLC, Lewis lung carcinoma; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein; TAG, triacylglycerol; TNF, tumor necrosis factor; WAT, white adipose tissue.

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a trend to significant improvement in general health (10). It has also been used in combination with cyclooxygenase (COX) inhibitors (11). Moreover, in anemic cancer patients with nonmyeloid malignancies, EPO treatment increased hemoglobin levels, decreased transfusion requirements, and improved both functional status and quality of life (12).

Recently, the discovery that EPOR is expressed on skeletal myoblasts suggests that the role of EPO may extend beyond erythropoiesis. The hypothesis is supported by observations showing that EPO enhances the proliferation and reduces the differentiation of both primary satellite cell cultures and C2C12 myoblasts (13). However, such action is likely useless in tumor hosts since the skeletal muscle of cachectic mice is characterized by a higher number of satellite cells with reduced differentiation capacity (14). Moreover, intramuscular EPO administration after a crush injury of rat skeletal muscle induces a faster and better regeneration and improved microcirculation (15). Besides the skeletal muscle, the adipose tissue, which is strongly affected during cachexia, has been recently suggested as target of EPO action (16). Indeed, EPO administration to nude mice previously transplanted with human fat tissue determined longer survival and stimulated angiogenesis of the grafts; these effects were comparable to those exerted by the proangiogenic protein vascular endothelial growth factor (VEGF) (16).

Considering the possible contribution of EPO signaling in the maintenance of both muscle and adipose tissue homeostasis, the aim of the present investigation was to examine, beyond the correction of anemia, the effects of EPO treatment on skeletal muscle or adipose tissue during experimental cancer cachexia.

MATERIALS AND METHODS

Experimental tumor models

C57BL/6 and Balb/C mice weighing about 20 g (Interfauna, Barcelona, Spain) and Wistar rats weighing about 150 g (Interfauna) were maintained on a regular dark-light cycle (light from 08:00 to 20:00), with free access to food and water during the whole experimental period. They were cared for in compliance with the Policy on Humane Care and Use of Laboratory Animals (ILAR 2011). The Bioethical Committee of the University of Barcelona approved the experimental protocol. All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals. On the day of sacrifices were made in accordance with the European Community guidelines for the use of laboratory animals (19). Animals were randomized and divided into two groups, namely controls (C) and tumor bearers (C26). C26 were divided into two subgroups: untreated and treated every three days with an intraperitoneal injection of recombinant human EPO (100 IU, Calbiochem), adopting the same protocol of a previous work (16). Mice were euthanized under anesthesia 14 days after tumor implantation.

Lewis lung carcinoma. Mice received an intramuscular (hind leg) inoculum of 5 × 10⁶ Lewis lung carcinoma cells obtained from previous tumor hosts. The Lewis lung carcinoma is a cachexia-inducing, rapidly growing murine tumor composed of poorly differentiated cells with a relatively short doubling time (17). Animals were randomized and divided into two groups, namely controls (C) and tumor bearers (LLC). LLC were divided into two subgroups: untreated and treated every three days with an intraperitoneal injection of EPO (100 IU). Mice were euthanized under anesthesia 14 days after tumor implantation.

AH-130 Yoshida ascites hepatoma. Male rats received an intraperitoneal inoculum of 10⁶ AH-130 Yoshida ascites hepatoma cells obtained from exponential tumors (18). On day 7 after tumor transplantation, the animals were euthanized, and the dorsal fat pads were excised for subsequent ex vivo experiments.

Hematocrit

Total blood was withdrawn from anesthetized mice by cardiac puncture and collected in heparinized tubes. A drop was used to fill hematocrit capillary tubes that were centrifuged in a hematocrit centrifuge for 5 min at 800 g. Hematocrit was calculated as percentage of packed cell volume of the total blood.

Lipoprotein lipase activity

White adipose tissue (WAT) samples were homogenized in a buffer containing 10 mM Hepes, 1 mM EDTA, 1 mM DTT, 250 mM Sucrose, 5 U/ml Heparin (pH 7.5) and used in an assay system containing [1-¹⁴C]triolein as substrate (19). [¹⁴C]fatty acids released after 30 min incubation period were extracted and quantified by the method of Nilsson-Ehle and Schotz (20).

Ex vivo lipogenic rate

After dissection, WAT from control and AH-130-bearing rats was weighed and sliced into fragments of about 15–20 mg. One hundred milligrams (4–5 pieces) were incubated in 4 ml Krebs-Henseleit buffer (pH 7.4) containing 2% fatty acid-free BSA, 5 mM D-glucose and 0.1 µCi/ml [¹¹C]acetate. Where indicated, the following substances were added: tumor necrosis factor (TNF; 5 ng/ml), EPO (10 U/ml) and insulin (4 µg/ml). Tissue slices were incubated for 1 h at 37°C in a shaking water bath under O₂/CO₂ (19:1) flow. Lipogenic rate was calculated by measuring [¹¹C]acetate incorporation into fatty acids in WAT pieces as previously described (21).

Ex vivo lipolytic rate

The lipolytic rate was determined as previously described by López-Soriano et al. (22). After dissection, WAT from control and AH-130-bearing rats was weighed and sliced into pieces of about 15–20 mg. One hundred milligrams (4–5 pieces) were incubated in 4 ml Krebs-Henseleit buffer (pH 7.4) containing 2% fatty acid-free BSA and 5 mM D-glucose. Where indicated, the following substances were added: TNF (5 ng/ml), EPO (10 U/ml), insulin (4 µg/ml), and isoproterenol (0.5 µM). Tissue slices were incubated for 1 h at 37°C in a shaking water bath under O₂/CO₂ (19:1) flow. Incubations were stopped by adding perchloric acid.
(final concentration 3%). Lipolytic rate was estimated as the glycerol released to the incubation medium as measured by the method of Hohorst et al. (23).

**Cell culture and adipocyte differentiation**

3T3-L1 preadipocytes (ATCC) were grown in DMEM supplemented with 10% (v/v) fetal calf serum (Invitrogen), 25 mM HEPES (pH 7.0), 1,000 U/ml penicillin, 1,000 U/ml streptomycin, and 25 µg/ml fungizone (BioWhittaker). To induce adipocyte differentiation, cells were grown for two days post confluence and then cultured in differentiation medium consisting of DMEM supplemented with 10% (v/v) FBS (Invitrogen), 25 mM HEPES (pH 7.0), 1,000 U/ml penicillin, 1,000 U/ml streptomycin, and 25 µg/ml fungizone, plus 0.5 mM isobutylmethyl-xanthine, 1µM dexamethasone, and 1 µg/ml insulin (MDI). After 48 h, MDI was replaced with insulin (1 µg/ml), and the medium changed every 2 days. Lipid accumulation in differentiating (day 4) 3T3-L1 cells was assessed by Oil Red O staining. Briefly, cells were fixed for 15 min in 3% paraformaldehyde, rinsed three times in PBS, and then incubated overnight with antibodies directed against EPOR (R&D systems, Minneapolis, MN). Peroxidase-conjugated IgG (Bio-Rad, Hercules, CA) was used as secondary antibody. Membrane-bound immune complexes were detected by an enhanced chemiluminescence system (Santa Cruz Biotechnology) on a photon-sensitive film. Protein loading was normalized according to GAPDH (Santa Cruz Biotechnology) expression. Band quantification was performed by densitometric analysis using specific detection was performed using a Cy3-conjugated goat IgG secondary antibody. Dye excess was washed away, and the cells were dried completely. Lipids were extracted (Sigma) dissolved in 65% isopropanol. Dye excess was washed away, and the cells were dried completely. Lipids were extracted (Sigma) dissolved in 65% isopropanol. Dye excess was washed away, and the cells were dried completely.

**Western blotting**

Cells were lysed in RIPA buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM PMSF) with freshly added protease and phosphatase inhibitor cocktails, sonicated, and centrifuged at 3,000 g for 5 min at 4°C, and then the supernatant was collected. Protein concentration was assayed by the method of Lowry (24) using BSA as working standard. Equal amounts of protein (30 µg) were heat-denatured in sample-loading buffer (50 mM Tris-HCl at pH 6.8, 100 mM DTT, 2% SDS, 0.1% Bromophenol blue, 10% glycerol), resolved by SDS-PAGE, and transferred to nitrocellulose membranes. The filters were blocked with TBS containing 0.05% Tween and 5% nonfat dry milk, and then incubated overnight with antibodies directed against EPOR (R&D systems, Minneapolis, MN), phosphorylated Akt (Cell Signaling, Beverly, MA), and total Akt (Santa Cruz Biotechnology). Chemiluminescence was detected with an enhanced chemiluminescence system (Santa Cruz Biotechnology) on a phosphor-sensitive film. Protein loading was normalized according to GAPDH (Santa Cruz Biotechnology) expression. Band quantification was performed by densitometric analysis using specific detection was performed using a Cy3-conjugated goat IgG secondary antibody. Dye excess was washed away, and the cells were dried completely.

**Results are mean ± SEM for the number of animals indicated in parenthesis. Tissue weights are expressed as milligrams per 100 grams of IBW. FBW excludes the tumor mass. FBW, final body weight; IBW, initial body weight. *P < 0.05 versus control; **P < 0.01 versus control; ***P < 0.001 versus control; $P < 0.05;  ^=P < 0.01 versus C26 tumor bearers.**
Real-time PCR

Total RNA was obtained using the TriPure reagent (Roche) following manufacturer’s instructions. RNA concentration was determined fluorometrically using the Ribogreen reagent (Invitrogen). Total mRNA was retro-transcribed using the i-Script cDNA synthesis kit (Bio-Rad). Transcript levels were determined by using the SsoFast EvaGreen Supermix and the MiniOpticon thermal cycler (Bio-Rad), normalizing the expression for both actin and calnexin levels. Primer sequences were as follows: peroxisome proliferator-activated receptor (PPAR)γ, CGGAAGCCCTTTGGTGACTT TGGGCTTCAGTTCAGCAAG; activating protein 2 (aP2), CAGAAGTGGGATGGAAATGTCGCACTGACTATTGTATGTTTTGA; sterol regulatory element-binding protein (SREBP)-1c, GATGTCGGAACGTGACACAG CATAAGCCCGGTCAACAG; fatty acid synthase (FASN), TCCACCTTTAAGTGCCCTG TCTGCTCTCGTCATGTCAAC; LPL, TCTGTACGGCACAGTCAACC; CTCTGTACGGCACAGTGAGGCCAGGAT; calnexin, GCAGCGACCTATGA TGACAACC GCTCCAAACCAATAGCACTGAAAGG.

Statistical analysis

Data were analyzed by ANOVA. Statistical significance of results is indicated by *$P<0.05$, **$P<0.01$, ***$P<0.001$. 

RESULTS AND DISCUSSION

To study the effects of EPO on tumor-induced wasting, we used two different murine experimental models: the Colon26 carcinoma (C26) and the Lewis lung carcinoma (LLC). As expected, tumor growth in both animal models resulted in important changes in body weight (Tables 1 and 2; C26, H11002 22%; LLC, H11002 22%) as well as in muscle (C26: GSN, H11002 23%, Tibialis, H11002 25%; LLC: GSN, H11002 29%, tibialis, H11002 32%) and white adipose tissue (WAT) mass (C26: dorsal WAT, H11002 85%, epididymal WAT, H11002 77%; LLC: dorsal WAT, H11002 95%, epididymal WAT, H11002 87%). In both models, tumor-bearing mice showed reduced hematocrit; the effect was more evident in the LLC (H11002 56%) than in the C26 (H11002 16%) hosts. EPO treatment did not modify body or muscle weight in any of the groups. By contrast, in the C26-bearing animals, EPO administration significantly increased both dorsal and epididymal WAT (+108% and +73%, respectively; Table 1) as compared with the untreated tumor-bearing mice. Similar, but quantitatively more marked, results were found in the LLC-bearing mice (dorsal WAT, +200%;
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The PI3K-Akt pathways. Since the latter signaling pathway regulates adipocyte growth and anabolism, we measured the levels of total and phosphorylated Akt, a serine-threonine kinase downstream of PI3K. The results presented in Fig. 1B show that the implantation of LLC led to a decrease of phosphorylated Akt, in agreement with decreased anabolic processes in the WAT of tumor-bearing animals. Treatment with EPO was able to partially prevent such a reduction, suggesting that the protective effects of EPO on adipose tissue against cancer cachexia are partially mediated through this intracellular signaling pathway.

To clarify the mechanisms underlying EPO-induced improvement of WAT depletion in tumor-bearing animals, the different pathways associated with fat accumulation have been investigated. Fatty acids (FA) enter adipose tissue through the action of lipoprotein lipase (LPL) on either VLDL (endogenous TAG) or chylomicra (exogenous TAG). This enzyme, located in the endothelial cells, breaks down TAG into FA and glycerol. The FA can then be esterified and incorporated into tissue TAG. Total LPL activity is markedly reduced in mice bearing the LLC tumor (Fig. 1C). This observation is in agreement with previous results from our laboratory (25). Treatment with EPO significantly increases total LPL activity in tumor-bearing mice, although it still remains lower than control values.
These data agree with the results obtained by Goto and coworkers in hemodialysis patients (26).

In addition to the entry of FA into adipose tissue through LPL, FA can be synthesized de novo through lipogenesis inside the adipose tissue. For this reason, the effects of EPO on the lipogenic rate were investigated by measuring the incorporation into lipids of [1-14C]acetate in rat WAT slices incubated in the presence of different stimuli. Fig. 2A shows the rates of lipogenesis in different conditions. As expected, the lipogenic rate is markedly induced by insulin and significantly reduced by incubation of WAT slices with TNF-α. Interestingly, although EPO alone had no significant lipogenic effects, it is able to abrogate TNF effects. In the attempt to compare experimental cachexia with direct TNF effects on lipogenesis, a rat cachexia model (AH-130 Yoshida ascites hepatoma) was used. As expected, the lipogenic rate in WAT slices from tumor-bearing rats was markedly reduced. Of interest, such reduction was significantly improved (+130%) by the addition of EPO in the incubation medium. These results suggest that, in addition to LPL, lipogenesis contributes to fat accretion observed as a result of EPO treatment in cachectic tumor-bearing animals.

Finally, the effects of EPO on adipose tissue could rely on decreased lipolytic rate. To test this hypothesis, we measured the glycerol release in WAT slices from control and AH-130-bearing rats in the presence of EPO. The results presented in Fig. 2B show that both isoproterenol and TNF increased the rate of glycerol release (+34% and +274%, respectively), while insulin, as expected, exerted an opposite effect (~43%). Unexpectedly, the glycerol release from AH-130 samples was lower than the respective controls, an effect likely resulting from the smaller amount of lipids present in the tissue of the AH-130 samples. No effects of EPO were observed on lipolysis in WAT from control or tumor-bearing animals or from TNF- or isoproterenol-treated samples, therefore excluding any EPO action on the regulation of lipolysis.

The effects exerted by EPO on the adipose tissue have been further investigated at the molecular level, taking advantage of an in vitro model system, namely, the 3T3-L1 preadipocytes, which can be induced to adipocyte differentiation when cultured in appropriate conditions (see Materials and Methods). As shown in Fig. 3, EPO administration to differentiating cells resulted in increased accumulation of lipid droplets as observed by both morphological appearance and quantification of Oil-Red-O uptake. Such effect is associated with increased EPOR staining (Fig. 3A). Another proof of the lipogenic action of EPO came from the transcript analyses of the above-mentioned cells (Fig. 3C), where EPO induced the expression and partially counteracted the TNF-mediated suppression of markers of adipogenesis (PPARγ, aP2, and LPL), though not of SREBP-1c or fatty acid synthase (FASN). Such data recapitulate the in vivo (LPL activation, Fig. 1C) and ex vivo (lipogenesis, Fig. 2A) results, showing the EPO ability to interfere with tumor- or TNF-induced lipid depletion.

The EPOR expression was then evaluated in a time-course experiment, from growing to fully differentiated cells. Fig. 4A shows that the presence of EPO increased the
survival during cancer (28). By contrast, no effects of EPO were observed in the preservation of skeletal muscle mass. These results are in contrast with a recent publication suggesting that preservation of adipose tissue during cachexia resulted in preservation of muscle mass, as if the muscle wasting were directly associated with the loss of adipose tissue (29). Concerning the metabolic pathways involved in the actions of EPO on fat mass, the results presented here suggest that the cytokine acts on both lipogenesis and LPL-mediated uptake of circulating TAG (summarized in Fig. 5).

In conclusion, EPO administration to cancer patients might be useful to counteract the anemia often present in cancer cachexia and to preserve adipose tissue homeostasis and fat stores. Moreover, in the last few years, adipose tissue has been recognized as an “endocrine tissue” due to the several cytokines and hormones released by adipocytes, thus highlighting the importance of its preservation in the design of future cancer cachexia treatments. Further studies are needed to better clarify the mechanisms of EPO action in nonhematopoietic cells. Furthermore, recent data showing that functional EPOR is absent in several human tumor cell lines (30) reduce the concerns regarding EPO safety for future clinical trials.

REFERENCES


