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Erythropoietin reduces the expression of myostatin in mdx dystrophic mice

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Abstract

Erythropoietin (EPO) has been well characterized as a renal glycoprotein hormone regulating red blood cell production by inhibiting apoptosis of erythrocyte progenitors in hematopoietic tissues. EPO exerts regulatory effects in cardiac and skeletal muscles. Duchenne muscular dystrophy is a lethal degenerative disorder of skeletal and cardiac muscle. In this study, we tested the possible therapeutic beneficial effect of recombinant EPO (rhEPO) in dystrophic muscles in mdx mice. Total strength was measured using a force transducer coupled to a computer. Gene expression for myostatin, transforming growth factor-β1 (TGF-β1), and tumor necrosis factor-α (TNF-α) was determined by quantitative real time polymerase chain reaction. Myostatin expression was significantly decreased in quadriceps from mdx mice treated with rhEPO (rhEPO = 0.60 ± 0.11, control = 1.07 ± 0.11). On the other hand, rhEPO had no significant effect on the expression of TGF-β1 (rhEPO = 0.95 ± 0.14, control = 1.05 ± 0.16) and TNF-α (rhEPO = 0.73 ± 0.20, control = 1.01 ± 0.09). These results may help to clarify some of the direct actions of EPO on skeletal muscle.

Key words: Muscular dystrophy; Erythropoietin; Myostatin; Skeletal muscle; Quadriceps

Introduction

Erythropoietin (EPO) has been well characterized as a renal glycoprotein hormone regulating red blood cell production by inhibiting apoptosis of erythrocyte progenitors in hematopoietic tissues (1). EPO receptors have been described in many different cells and tissues, including muscle, neurons, astrocytes, microglia, developing heart, cancer cell lines, Leydig cells, and gastric mucosal cells, suggesting other actions of this hormone (2). Indeed, many of these tissues are responsive to stimulation with recombinant human EPO (rhEPO) (2). The relationship between EPO receptors and skeletal muscle has been poorly investigated. It has recently been reported that EPO exerts regulatory effects on both cardiac and skeletal muscle (3).

Because mouse myoblasts express EPO receptors, administration of EPO can stimulate proliferation of myoblasts to expand the progenitor population during differentiation, resulting in a potential role in muscle development or repair (4). Mice lacking EPO or its receptors suffer from heart hypoplasia and have a reduced number of proliferating cardiac myocytes (5).

The effects of EPO on muscle cells have also attracted the attention of athletes. Because EPO increases red blood cell mass and exercise capacity in anemic patients, it might have the same effect in an athlete's body, thereby enhancing performance (6). In addition to the hematopoietic effects, EPO is capable of promoting angiogenesis in muscle cells (7), providing an additional route to increase the supply of oxygen to the working muscles. Furthermore, the possible involvement of EPO in muscle repair processes (4) can imply that athletes who abuse rhEPO have healthier muscles. With this reasoning, athletes began using rhEPO, and thus rhEPO has been on the International Olympic Committee's list of banned substances since 1990 (6).

Duchenne muscular dystrophy (DMD) is a lethal degenerative disorder of skeletal and cardiac muscles that affects 1 in 3500 male births (8). DMD patients characteristically display progressive muscle weakness, which begins in early childhood (9). Although DMD is present at birth, clinical symptoms are not evident until 3-5 years of age (10). Initial symptoms include leg weakness and increasing convex curvature of the spine muscles and results in progressive weakness, usually leaving DMD patients wheelchair bound by age 11 or 12 years (11).

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Affected individuals usually die due to respiratory or cardiac insufficiency by the second or third decade of life (12). So far, the only pharmacological treatment proven to be effective for DMD is steroids (13).

Dystrophin is considered a key structural element in muscle fiber, and the primary function of the dystrophin-associated protein complex is to stabilize the plasma membrane. The absence of dystrophin is followed by a sequence of events such as calcium influx, reactive oxygen species activity, and inflammation, leading to muscle injury (14).

This study tested the possible beneficial effect of recombinant EPO therapy in the degenerative process of dystrophic muscles in mdx mice. The absence of dystrophin in these mice produces a phenotype quite different from that of dystrophin deficiency in humans. Under normal conditions, mdx mice show little overt symptoms of weakness, but, if forced to engage, they show more pathological changes than when resting (15).

Material and Methods

Male mdx mice were maintained in the experimental laboratory of ABC Faculty of Medicine (FMABC) at constant temperature (20°C), with a 12:12-h light-dark cycle, and received diet and water *ad libitum*. The mice weighed 25-30 g. All procedures were conducted in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals, and were approved by the Ethics Committee of Faculdade de Medicina do ABC in 2006 (Protocol: #04/2006).

The experimental groups were as follows: 7 mdx mice were injected with rhEPO (1000 IU/kg *ip*) 3 times per week and 6 mdx mice received saline. All animals were submitted to exercise on an electric treadmill (5 days/week, 20 cm/s, 10 min).

Measurement of whole body strength

Whole body strength was measured weekly using a force transducer coupled to a computer (15). The tail of each mouse was connected through a nonflexible nylon tube to the transducer so that they were electrically stimulated to run, and the force required to pull the cable was continuously recorded. The force values were normalized to the weight of each animal.

Muscle preparation

After 12 weeks of treatment, a muscle biopsy was carried out on all mdx mice. Samples were collected from the left quadriceps, dissected, frozen in liquid nitrogen, and stored at –80°C. Total tissue RNA was extracted with Trizol reagent (Invitrogen Co., USA), according to the manufacturer's instructions, quantified by absorbance at 260 nm, and stored in diethylpyrocarbonate-treated water at –80°C. The integrity of RNA was routinely verified by agarose gel electrophoresis. Total RNA (2 μg) was used for first-strand cDNA synthesis (reverse transcriptase) using Moloney murine leukemia virus, and RNaseOUT was also added to protect the RNA during this process).

Quantitative real time polymerase chain reaction

Gene expression of myostatin, transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α) was determined by quantitative real time polymerase chain reaction (qRT-PCR) using SybrGreen Master Mix (Invitrogen). β-actin was used as an internal control. The reaction was carried out with 1 μL diluted cDNA (20 ng), 10 μL SybrGreen Master Mix (Invitrogen), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), and 8 μL RNAse-free water in a final volume of 20 μL/well. The thermocycle included an initial incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s, and 72°C for 15 s. The qRT-PCR was performed in triplicate. The primer sequences and their respective product length are reported in Table 1.

Relative gene expression fold change was calculated using the delta delta Ct method. The results were subjected to analysis of variance using the GBStat program (England), and $P<0.05$ was considered to be significant.

Results

Measurement of whole body strength

The muscle strength values were appropriate for the weight of the animal. There was no change in strength between the control animals and those treated with rhEPO during the 12 weeks of treatment. The total force of mdx mice is reported in Figure 1.

qRT-PCR

In order to determine whether recombinant EPO had an effect on the regeneration process of mdx dystrophic muscle in mice, we quantified expression of the myostatin gene, TGF-β1, and TNF-α by qRT-PCR. After 12 weeks of treatment with rhEPO, there was a significant decrease in myostatin gene expression (Figure 2). There was no difference in TGF-β1 and TNF-α gene expression between groups.

Discussion

This study evaluated the possible beneficial effect of recombinant EPO therapy in the degenerative process of dystrophic muscles in mdx mice, the most common experimental model used to study DMD (15). We observed no change in strength between the control animals and those treated with rhEPO during the 12 weeks of treatment. Myostatin expression was significantly decreased in quadriceps from mdx mice treated with rhEPO; however, no significant effect of rhEPO was seen in the expression of TGF-β1 and TNF-α.

Table 1. Forward and reverse primers for quantitative real time PCR analyses.

Primer	Sequence	Product length (pb)
TGF-β1 (accession No. M13177)		80
Forward	5′ CCCCACTGATACGCCTGAGT 3′	
Reverse	5′ AGCCCTGTATTCCGTCTCCTT 3′	
TNF-α (accession No. NM_013693.2)		275
Forward	5′ ATGAGCACAGAAAGCATGATC 3′	
Reverse	5′ TACAGGCTTGTCACTCGAATT 3′	
Myostatin (accession No. NM_010834.2)		83
Forward	5′ ACGCTACCACGGAAACAATC 3′	
Reverse	5′ AAAGCAACATTTGGGCTTTC 3′	

TGF-β1: transforming growth factor beta 1; TNF-α: tumor necrosis factor alpha.

DMD is the most common lethal genetic disease. Corticosteroids are currently the only available disease-modifying therapy for DMD, by prolonging independent ambulation and delaying the onset of secondary complications (16). However, the use of chronic high-dose corticosteroids for DMD is frequently associated with significant side effects and does not stop disease progression (16). An effective treatment for DMD requires a combination of therapies, including pharmacological agents, gene therapy, and target cells in an attempt to reach the process pathways involved in muscle degeneration and necrosis (17). EPO has several effects that could aid in the repair of skeletal muscle injury and prevention of fibrosis (1). In mdx mice, we did not observe any increase in muscle strength. An EPO analog, carbamylated EPO, was not demonstrated as an effective therapy for DMD in mdx mice (18).

We assessed the gene expression of cytokines involved in the disease and observed a direct action of the drug in spite of increased muscle oxygen delivery *in vitro*.

TGF-β1 is highly upregulated in dystrophic skeletal muscle (19), and the level of TGF-β1 protein is significantly elevated in the mdx diaphragm at 12 weeks of age (20). These cytokines are expressed by inflammatory cells such as macrophages (21) and have been shown to stimulate collagen synthesis (22). In our experiments, the results of TGF-β1 did not show any difference. Perhaps the EPO concentration we used was not able to activate this pathway.

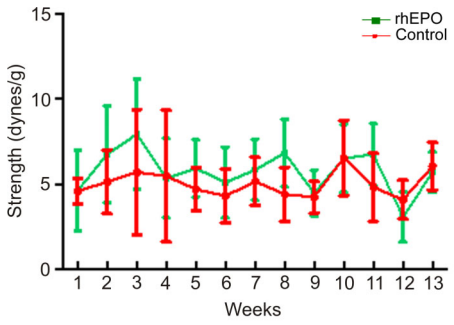

Figure 1. Strength of the entire body of mdx mice treated with recombinant human erythropoietin (rhEPO) or saline (control) for 12 weeks. P>0.05, GBStat test.

DMD patients have higher serum TNF-α levels, and TNF-α-positive fibers have been found by *in situ* hybridization and immunohistochemistry in muscles of dystrophic DMD subjects (23). Controversial results and muscle type dependent effects have been observed in TNF-α knockout mdx mice; and histopathological analysis has found that the absence of TNF-α *in vivo* resulted in equivocal findings as opposed to amelioration of muscle pathology as predicted (24), although long-term deletion of TNF-α appeared beneficial in older (12 months) mdx/TNF-α(–/–) mice (25). The pharmacological approach using weekly TNF-α antibody during early postnatal life clearly delayed and greatly reduced the breakdown of dystrophic muscle (26). However, no proof of functional benefit of this specific anti-TNF-α therapy has been provided. We did not observe a significant effect of EPO in expression of the TGF gene.

Myostatin, a member of the TGF family, is an important negative regulator of skeletal muscle mass (27). The deletion of the myostatin gene in the mdx mouse increases not only muscle mass but also muscle strength (as measured by grip strength). Remarkably, histological analysis of the diaphragm, one of the most severely affected muscles in the mdx mouse, showed a reduced dystrophic phenotype in myostatin/mdx double mutants (28). The injection of anti-myostatin monoclonal antibodies into mdx mice on a weekly basis for a period of 3 months resulted in muscle mass increases up to 35% in individual muscles after myostatin blockade (29). Conversely, transgenic mice that overexpress myostatin selectively in skeletal muscle have lower muscle mass (30). Finally, these observations indicate that myostatin negatively regulates skeletal muscle mass. For the first time, we report that a significant reduction in myostatin expression in mdx mice treated with rhEPO may signify a new direct mode of action of EPO in skeletal muscle.

The direct actions of EPO in skeletal muscles independent of its action in the hematopoietic system have been

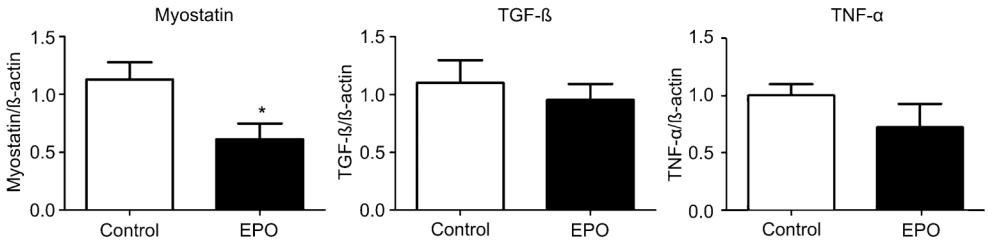

Figure 2. Myostatin, transforming growth factor beta (TGF-β), and tumor necrosis factor alpha (TNF-α) gene expression in the left quadriceps of treated (EPO) and control mdx mice. *P<0.05, GBStat test.

studied by several authors (31,32). EPO has been described as exerting effects similar to vascular endothelial growth factor (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 tissues. A close association between VEGF and EPO in angiogenesis has been proposed (33), and EPO treatment has been found to enhance the release of VEGF from marrow stromal cells (31) and to increase levels of VEGF in brain (32). Considering the importance of VEGF in skeletal muscle capillary growth (34), it is, therefore, plausible that one of the angiogenic effects of EPO is mediated by promoting VEGF levels in muscle. This effect can explain the decrease in myostatin in our experiments.

Human myoblasts treated with bupivacaine showed a dose-dependent decrease in mitochondrial membrane potential associated with unusual morphologies. Impairment of mitochondrial bioenergetics was prevented partially by the use of rhEPO co-administered with bupivacaine (35).

Another interesting potential physiological role of EPO in skeletal muscle is in muscle fiber growth. Erythropoietin receptor (EPO-R) activation stimulates the signal transducer and activator of transcription 5 (STAT5), which is known to modulate cell proliferation and differentiation (36). STAT5 also activates the phosphoinositide 3 (PI3)-kinase-protein kinase B (Akt) signaling pathway (37,38), 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 the regulation of skeletal muscle hypertrophy (38), On the basis of the above findings, it appears plausible that EPO-R activation may contribute to the regulation of skeletal muscle fiber growth, activating the STAT pathway and downregulating myostatin, as shown in our results.

We can speculate that EPO regulates the expression of various genes. Acute injections of rhEPO (15,000 IU) did not change mRNA levels of VEGF, hypoxia-inducible factor 1α (HIF-1α), insulin-like growth factor, ferroportin, myogenic differentiation 1 (MyoD), and myogen in biopsies obtained 2, 4, 6, and 10 h after injection of rhEPO, while small inductions of myoglobin, EPO-R, transferrin receptor, and myogenic regulatory factor (MRF4) were observed (39).

In our study, the control group was mdx mice treated with saline, because we wanted to observe the possible beneficial effects of recombinant EPO therapy in the degenerative process of dystrophic muscles. We adopted the same experimental model with mdx mice, as had been reported in a previous study from our group (40).

Studying the muscles of mice with muscular dystrophy, we observed no increase in muscle strength, but we found a significant reduction in myostatin. This result can help to clarify some of the mechanisms of the direct action of EPO on skeletal muscle.

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