Published in final edited form as:

Int J Biochem Cell Biol. 2013 July ; 45(7): 1155–1164. doi:10.1016/j.biocel.2013.03.007.

Erythropoietin contributes to slow oxidative muscle fiber specification via PGC-1 α and AMPK activation

Li Wang¹, Yi Jia¹, Heather Rogers¹, Norio Suzuki², Max Gassmann³, Qian Wang⁴, Alexandra C. McPherron⁴, Jeffery Kopp⁵, Masayuki Yamamoto², and Constance Tom Noguchi^{1,*}

¹Molecular Medicine Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD ²Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryō-cho, Aoba-ku, Sendai 980-8575, Japan ³Institute of Veterinary Physiology and Zurich center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland ⁴Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD ⁵Kidney Disease Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD

Abstract

Erythropoietin activity, required for erythropoiesis, is not restricted to the erythroid lineage. In light of reports on the metabolic effects of erythropoietin, we examined the effect of erythropoietin signaling on skeletal muscle fiber type development. Skeletal muscles that are rich in slow twitch fibers are associated with increased mitochondrial oxidative activity and corresponding expression of related genes compared to muscle rich in fast twitch fibers. Although erythropoietin receptor is expressed on muscle progenitor/precursor cells and is down regulated in mature muscle fibers, we found that skeletal muscles from mice with high erythropoietin production in vivo exhibit an increase in the proportion of slow twitch myofibers and increased mitochondrial activity. In comparison, skeletal muscle from wild type mice and mice with erythropoietin activity restricted to erythroid tissue have fewer slow twitch myofibers and reduced mitochondrial activity. PGC-1 α activates mitochondrial oxidative metabolism and converts the fast myofibers to slow myofibers when overexpressed in skeletal muscle and PGC-1 α was elevated by 2-fold in mice with high erythropoietin. In vitro erythropoietin treatment of primary skeletal myoblasts increased mitochondrial biogenesis gene expression including PGC-1 α by 2.6-fold, CytC by 2-fold, oxygen consumption rate by 2-fold, and citrate synthase activity by 58%. Erythropoietin also increases AMPK, which induces PGC-1 α and stimulates slow oxidative fiber formation. These data suggest that erythropoietin contributes to skeletal muscle fiber programming and metabolism, and increases PGC-1 α and AMPK activity during muscle development directly to affect the proportion of slow/fast twitch myofibers in mature skeletal muscle.

*Corresponding author C.T. Noguchi, Molecular Medicine Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bldg. 10, Rm. 9N319, 10 Center Dr., MSC 1822, Bethesda, MD 20892-1822. Tel.: 301-496-1163; Fax: 301-402-0101; connien@niddk.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Erythropoietin; slow twitch fiber; AMPK; PGC-1 α ; mitochondrial activity

Introduction

Skeletal muscles of vertebrates contain two types of myofibers, slow twitch (type I) and fast twitch (type II), that differ in function, mitochondrial density, and metabolic properties (Zierath and Hawley 2004). Slow twitch (ST) myofibers contain a high concentration of mitochondria and high oxidative capacity, and are associated with fatigue resistance and the ability of prolonged duration of muscle activity. In contrast, fast-twitch myofibers such as type IIB fibers, show low mitochondrial density and low oxidative metabolism (Zierath and Hawley 2004). The percentage of ST fibers is reduced in obese and type 2 diabetic patients, and within each fiber type, obese and type 2 diabetic patients have lower oxidative enzyme activity and a corresponding greater lipid content and smaller mitochondria in skeletal muscle (Gaster et al. 2000; Gaster et al. 2001; Szendroedi et al. 2011). Defective insulin signaling has been suggested to be associated with mitochondrial dysfunction (Hoeks et al. 2010; Sleight et al. 2011). Furthermore, mice engineered with increased type I muscle fibers exhibit resistance to obesity and improved metabolic profiles (Ryder et al. 2003; Wang et al. 2004).

A conversion of different fiber types can be found in adult skeletal muscle in response to chronic change in contractile demands (Oka et al. 2006). Some enzymes and regulatory factors have been demonstrated to be involved in maintaining specific fiber phenotypes. For example, PGC-1 α , which activates mitochondrial biogenesis and oxidative metabolism through its interaction with sirt1 (Gerhart-Hines et al. 2007), was reported to be a principal factor in regulation of fiber conversion to type I (Lin et al. 2002) and mediate increased GLUT4 expression in muscle (Michael et al. 2001), an insulin sensitive glucose transporter which is higher in slow-twitch fibers compared with fast-twitch muscle fibers and reduced in slow fibers from diabetic patients (Gaster et al. 2001). Some other factors have also been demonstrated to induce ST fibers; for example peroxisome proliferator-activated receptor δ (PPAR δ), and calcium signaling contribute to the control of type-I-fiber specific proteins (Ryder et al. 2003; Wang et al. 2004). In addition, chronic AMP-activated protein kinase (AMPK) activation has also been reported to evoke muscle plasticity and conversion to the slow oxidative myogenic program, possibly related to increased PGC-1 α expression and via cross talk with PPAR δ (Narkar et al. 2008; Ljubicic et al. 2011).

Erythropoietin (EPO) binds to its cell surface receptor, EpoR to promote early erythroid progenitor cell survival, proliferation and differentiation (Wu et al. 1995; Lin et al. 1996). However, EPO/EpoR signaling is not restricted to the erythroid lineage and can be found in many non-hematopoietic cells including endothelial, muscle, cardiovascular and renal tissues (Anagnostou et al. 1994; Wu et al. 1999a; Ogilvie et al. 2000). EPO stimulation of mitochondrial biogenesis in part by enhancement of PGC-1 α was suggested to mediate its cardioprotective activity (Carraway et al. 2010). We previously demonstrated that EpoR is readily detected in primary myoblasts or muscle progenitor/precursor cells isolated from skeletal muscle and is down regulated with myoblast differentiation (Ogilvie et al. 2000; Wang et al. 2012). EPO can stimulate proliferation of myoblasts in culture through binding to EpoR to expand the progenitor population during differentiation and may have a potential role in muscle maintenance or repair (Ogilvie et al. 2000; Jia et al. 2009). Recently, EPO/EpoR signaling has been shown to increase glucose tolerance and protect against diet induced obesity, and this activity has been associated with skeletal muscle response (Hojman et al. 2009; Teng et al. 2011). The association of EPO treatment in mice with

increased mitochondrial biogenesis and enhancement of PGC-1 α in cardiomyocytes raises the possibility that EPO may contribute directly to increased PGC-1 α and affect skeletal muscle development and the balance of slow and fast-twitch fiber determination.

Results

EPO/EPOR signaling contributes to slow twitch fiber formation

We previously determined the effect of EPO signaling in mice by examining muscle response to fatigue. Position holding time to an inverted wire grid for mice with overexpression of EPO (tg6) is about twice that of wild type (WT) mice (Jia et al. 2012). Considering that muscles with more ST fibers have elevated fatigue resistance compared with muscles with fewer ST fibers or more fast twitch (FT) fibers, we examined markers characteristic of ST and FT fibers in a mouse model of chronic exposure to elevated circulating EPO. Quantification of MHC gene expression showed a significant change with increased EPO signaling. In skeletal muscles of tg6 mice that chronically express high level of transgenic EPO, the gastrocnemius (GAS) muscle of tg6 mice showed an increase in slow MHC-I and myoglobin gene expression compared with WT mice and the mice with EPO receptor (EpoR) expression restricted to hematopoietic tissue (TgEpoR) (Figure 1A), suggesting an increase in the proportion of ST fibers compared with WT mice. Expression of MHC-IIA did not appear to be affected by the increase in EPO signaling although a mild reduction in MHC-IIB associated with FT muscle fibers and a trend toward decreased MHC-IID (Figure 1A). In the soleus (SOL) muscle of tg6 mice, like the GAS muscle, increases in slow MHC-I and myoglobin gene expression were observed as well as a modest and not significant decrease in MHC-IID expression (Figure 1B). In contrast to the GAS muscle, the SOL muscle of tg6 mice also exhibited a trend toward reduced expression of MHC-IIA and MHC-IID but no change in MHC-IIB expression was evident. Changes in MHC isoform expression in tg6 mice suggested a bias toward ST fiber formation in the GAS and SOL muscles with increased EPO signaling.

Hence, increasing or loss of EPO signaling can affect expression of markers characteristic of ST and FT muscle fibers. We did not observed any significant change in the expression of MHC I, IIA, IIB, and IID in the muscle of extensor digitorum longus (EDL), tibialis anterior (TA) and plantaris in either tg6 mice or TgEpoR mice compared with WT mice (data not shown). These data suggest that chronic changes in EPO signaling specially affect GAS and SOL skeletal muscle fiber type formation and that increased or decreased EPO signaling favors ST or FT muscle fibers, respectively.

Western blotting was used to assess the corresponding protein expression associated with muscle fiber type. As another marker for slow fiber formation, we examined protein level of TNNT1, the slow skeletal troponin T subunit that binds tropomyosin and contributes to regulation of calcium-sensitive contraction of striated muscle. TNNT1 protein was increased in tg6 GAS and SOL muscle and decreased in TgEpoR muscle (Figure 1C-D). Interestingly, myoglobin protein was found to be decreased only in TgEpoR mice compared to tg6 and WT mice (Figure 1C-D). These data provide further evidence that EPO signaling may promote the development of ST fiber formation in GAS and SOL skeletal muscle.

We performed immunohistochemistry to investigate the MHC-I level in muscles. The SOL and GAS muscles from tg6 mice both exhibited increased MHC-I fiber compared to WT mice and TgEpoR mice (Figure 2A). The total staining level of MHC-I was much less in EDL muscle compared to the SOL muscle. Although we found increased staining of MHC-I fiber in EDL muscle from tg6 mice compared to WT and TgEpoR mice (Figure 2A), an increased trend but not significant increase of MHC-I mRNA was found (Figure S1A). No staining was observed in TA muscle.

Using increased intensity of succinic dehydrogenase (SDH) staining in fibers as an indicator of ST oxidative fibers, we found that elevated EPO signaling corresponded with increase in the proportion of dark fiber staining. Examination of the GAS muscle from 4 week old tg6 mice revealed an increase in the percentage of type I oxidative (darkly stained) muscle fibers ($25.95 \pm 2.47\%$) compared with WT mice ($23.21 \pm 1.46\%$) (Figure 2B). The soleus muscle from 4 week old tg6 mice also exhibited an increase in the percentage of type I fibers ($66.21 \pm 1.11\%$) compared with WT mice ($44.15 \pm 2.55\%$). Exercise performance in young tg6 mice was comparable to WT mice (Schuler et al. 2010), suggesting the muscle fiber difference is not from increased exercise. In contrast, the percentage of SDH oxidative fibers in the GAS muscle of 4 week old TgEpoR mice was significantly reduced ($20.47 \pm 2.59\%$ SDH oxidative muscle) compared with WT and tg6 mice (Figure 2B). The percentage of SDH oxidative fibers in the SOL muscle of 4 week old TgEpoR mice was also significantly reduced ($27.42 \pm 2.22\%$) compared with WT and tg6 mice (Figure 2B). These data provide further evidence that chronic increased EPO signaling can promote ST fiber formation while reduced endogenous EPO signaling decreased ST fiber formation.

Endogenous EPO signaling contributes to mitochondrial gene expression

ST fibers are linked to high mitochondrial density and increased GLUT-4 expression (Gaster et al. 2000). To determine the contribution of endogenous EPO signaling to expression of genes involved in mitochondrial biogenesis, we compared real time quantitative RT-PCR analysis of the GAS and SOL muscles from TgEpoR mice that lack EpoR expression in non-hematopoietic tissue with that from WT mice. GAS and SOL muscles from TgEpoR mice exhibited decreased expression of genes related to mitochondrial biogenesis, isocitrate dehydrogenase 3 alpha (IDH3 α) that is responsible for the rate-limiting step of the tricarboxylic acid (TCA) cycle, and cytochrome C (CytC) (Figure 3A). These data are consistent with endogenous EPO signaling contributing to expression of genes related to ST fiber formation. We also observed reduced expression of GLUT-4 in TgEpoR muscle (Figure 3A), suggesting that endogenous EPO contributes to increased metabolic activity.

To investigate a direct effect of EPO on myoblasts as a possible early event during specification of ST/FT fiber type balance in skeletal muscle development, we isolated primary mouse skeletal muscle progenitor cells (embryonic 15 days) and subjected the corresponding cultures to EPO treatment. EPO stimulation for 24h increased expression of mitochondrial biogenesis genes CytC and IDH3 α in these primary myoblast cultures (Figure 3B). These data suggest that EPO affects gene expression related to mitochondrial biogenesis in skeletal muscle progenitor cells. Similarly, EPO treatment also increased CytC and IDH3 α expression in the skeletal myoblast C2C12 cell line (Fig 3C). To show the response of matured muscle fiber to EPO stimulation, we isolated mature muscle extensor digitorum longus (EDL) fiber of normal female 6 month mice and cultured the fiber with EPO treatment for 24 hours. Consistent with the down regulation of EpoR in muscle fibers (Ogilvie et al. 2000), we did not find increased expression of genes related mitochondrial biogenesis including CytC and IDH3 α (Figure S1B-C). These results are in contrast the direct EPO response of myoblasts to increase CytC and IDH3 α expression. These data indicate that in skeletal muscle, a direct EPO response is predominantly during development of skeletal muscle and not in the matured fiber. Such a direct response to EPO in skeletal myoblasts would combine with indirect activity including EPO regulated oxygen delivery through regulation of red blood cell production and/or vascular endothelial response to provide an overall EPO response. To address if increased mitochondrial biogenesis gene expression in skeletal myoblasts is reflected in increased protein and functional activity, CytC ELISA was used to evaluate the CytC activity. We observed that CytC protein level was greater in GAS and SOL muscles of tg6 mice compared to WT mice and CytC protein level was lower in TgEpoR GAS and Sol muscles (Figure 3D). Correspondingly, in vitro

primary myoblast culture treated with EPO showed a higher level of CytC protein compared to control cultures (Figure 3D).

EPO increased mitochondrial activity

We assessed mitochondrial DNA (mtDNA) content as an indicator of mitochondrial number. EPO treatment increased mtDNA level in primary myoblasts and C2C12 myoblasts (Figure 4A). These data indicate that EPO may promote mitochondrial biogenesis in myoblasts. Citrate synthase (CS) activity, a key component of the TCA cycle and is localized in mitochondria. In cultures of C2C12 myoblasts, EPO treatment increased CS activity (Fig 4B), indicating a direct myoblast EPO response and suggesting a resultant increase in mature skeletal muscle, in accordance with elevated IDH3 α expression with EPO signaling (Figure 3). We also observed the highest CS activity in GAS and SOL muscles from tg6 mice compared to WT and TgEpoR mice (Figure 4C), providing further evidence that direct myoblast EPO response may contribute to determination of skeletal muscle mitochondrial biogenesis. To determine if increased expression of genes related to mitochondrial biogenesis in the myoblasts was also reflected in increased mitochondrial function, we measured mitochondrial respiration via oxygen consumption rate without and with EPO treatment. We assessed the oxygen consumption rate (OCR) using different respiration inhibitors such as mitochondrial inhibitor oligomycin, uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and glucose analog 2-deoxyglucose (2-DG). EPO increased basal C2C12 myoblast oxygen consumption rate and especially under stress when treated with FCCP and 2-DG in C2C12 myoblast (Figure 4D), suggesting that EPO contributes to mitochondrial oxidative capacity and providing evidence for EPO promoting mitochondrial activity in muscle.

EPO enhances PGC-1 α expression and AMPK activity

Although our above data showed that EPO can increase mitochondrial oxidative activity in myoblasts, we can not distinguish that if the increased expression of genes related to mitochondrial biogenesis and enhanced mitochondrial respiration contribute to determination of slow oxidative fiber specification or if they are the resultant consequence of increased tendency for oxidative fiber formation. EPO treatment has previously been associated with enhanced expression of cardiac PGC-1 α (Carraway et al. 2010). To investigate possible molecular mechanism for EPO affecting slow fiber formation, we quantified the expression of PGC-1 α in skeletal muscle, a master regulator of mitochondrial biogenesis and reported to be sufficient to convert to type I fiber (Lin et al. 2002; Lin et al. 2005; Finck and Kelly 2006). Expression of PGC-1 α in the GAS and SOL muscle was increased in tg6 mice and decreased in TgEpoR mice compared with WT mice (Figure 5A). Protein levels of PGC-1 α determined by Western blotting were consistent with the mRNA expression pattern (Figure 5B), indicating increased PGC-1 α with elevated EPO signaling in muscle from tg6 mice and decreased PGC-1 α in muscle from TgEpoR mice with loss of EPO signaling. To address if EPO can directly increase PGC-1 α expression in skeletal myoblasts and, hence, possibly during skeletal muscle development, we examined cultures of primary myoblasts. EPO treatment significantly increased PGC-1 α expression in primary myoblasts cultures (Figure 5C) but not in the mature muscle fibers (Figure S1D). Western blotting also showed that EPO stimulation in cultured myoblasts increased the corresponding protein level of PGC-1 α (Figure 5D). EPO also increased PGC-1 α expression in C2C12 myoblasts (Figure 5E). PGC-1 α contributes to conversion to ST fiber and mitochondrial activity, and changes in PGC-1 α may mediate EPO activity in promoting ST fiber formation indirectly or directly during muscle development, as also suggested by increased CytC and citrate synthase activity. Matured skeletal muscle does not express EpoR. However, here we observed increased PGC-1 α in mature muscle with increased EPO signaling. We speculate that the increased PGC-1 α in adult muscle tissue could be the

consequence of continued high level of transgenic EPO production during development since we used transgenic mice because we also observed increased PGC-1 α level in the fetal muscle isolated from the TG6 compared to WT and TgEpoR mice (Figure S1E). We did not observe increase of PGC-1 α with ex vivo EPO treatment on isolated muscle fiber (Figure S1D). Therefore, it appeared that EPO solicits a response during skeletal muscle development in vivo but not in adult muscle with ex vivo EPO treatment. However, we could not exclude possible indirect and systemic effects of high EPO on the whole animal. Continued high level of EPO could increase oxygen delivery, which may have some positive effect on muscle fiber type and PGC-1 α expression.

AMPK has been reported to enhance PGC-1 α expression and AMPK activation is linked to slow and oxidative muscle fiber. We assessed the potential for AMPK activation by EPO stimulation. EPO treatment in C2C12 cells increased AMPK α phosphorylation. The increase in AMPK α activity was evident at 15 min after EPO treatment and continued to be elevated up to 48 hrs (Figure 6A). Importantly, phosphorylation of AMPK α was higher in the GAS and SOL muscle from tg6 mice compared to WT mice and reduced in TgEpoR mice (Figure 6B). Importantly, increased CytC, IDH3 α and PGCA-1 α by EPO treatment in C2C12 myoblasts were impaired by AMPK activity inhibitor Compound C (Figure 6C), and increased citrate synthase activity by EPO, the mitochondrial enzymatic activity marker, was also attenuated by inhibition of AMPK activity (Figure 6D), indicating that EPO effects on mitochondrial biogenesis and function may be dependent on AMPK. Since no detectable EpoR was found in matured muscle, we observed high level of AMPK phosphorylation in matured muscle, suggesting this could be the resultant effect of high EPO during development. However, we can not exclude that indirect and systemic effect of high EPO could also contribute to increased AMPK activity in matured muscle. Taken together, these data suggests that EPO can directly and/or indirectly increase AMPK α activity to induce PGC-1 α expression in myoblasts and skeletal muscle, and collectively provides a possible mechanism for the increase and decrease of ST fiber formation and mitochondrial activity associated with skeletal muscle in tg6 and TgEpoR mice respectively.

Discussion

Here we demonstrate that increased or decreased EPO signaling via chronic EPO treatment or disruption of endogenous EPO signaling beyond erythroid tissue, respectively, influences the proportion of oxidative muscle fibers that is accompanied by changes in PGC-1 α expression and AMPK activity in GAS and SOL skeletal muscles in mice. Our findings are the first to suggest a link between EPO stimulation and AMPK activity and imply that together they contribute to the slow, oxidative muscle fiber phenotype. Assessment of skeletal muscle fiber composition from mouse models with transgenic EPO expression or disrupted EpoR expression in conjunction with EPO stimulation of myoblast cultures provide evidence for direct and/or indirect action of EPO on fiber type specification.

EPO is known for its role in regulating red blood cell production. EPO activity in animal models beyond erythroid tissue include prevention from obesity, neuroprotection, myoblast proliferation, cardiovascular protection and anti-inflammatory reaction (Ogilvie et al. 2000; Yu et al. 2002; Jia et al. 2009; Carraway et al. 2010; Nairz et al. 2011). EPO injection into muscle promotes slow muscle protein expression (Cayla et al. 2008), although no detailed phenotype or mechanisms were suggested. Here we demonstrated that mice with chronic high level of EPO showed more slow-twitch fiber protein expression compared to WT mice including slow myosin heavy chain (I-MHC), TnnT1 and myoglobin. Conversely, mice with EpoR expression restricted to hematopoietic tissue exhibited reduced expression of these slow fiber proteins. Importantly, skeletal muscle mitochondrial activity was observed to be elevated in mice with high level of EPO as indicated by increased mitochondrial activity

marker CytC protein level and citrate synthase activity in mouse skeletal muscle while and mitochondrial activity was reduced by deletion of EpoR expression in non-hematopoietic tissue. That EPO signaling in myoblasts may contribute directly to this phenotype was raised by the increased oxygen consumption in C2C12 myoblasts treated with EPO. EPO treatment also stimulated the CytC and citrate synthase activity in primary myoblasts, which is consistent with increased slow oxidative fiber phenotype in adult muscle with increased EPO signaling, suggesting that EPO can contribute to early oxidative events in myogenic development before fiber type determination, although it cannot be ruled out that activation of oxidative related genes is not sufficient to promote slow oxidative fiber formation. Slow fibers preferentially express genes involved in mitochondrial respiration and fatty acid oxidation. Importantly, skeletal muscle rich in ST fiber protects from obesity and related metabolic disorders such as insulin resistance (Wang et al. 2004; Koves et al. 2005). Hence, the reported metabolic effects of EPO in improving insulin sensitivity and glucose tolerance, and reducing high fat diet induced obesity may be affected by EPO activity in promoting increased ST fiber in mice (Foskett et al. 2011; Teng et al. 2011).

PGC-1 α is closely linked to regulation of energy metabolism and promotes the slow and oxidative fiber specification (Wu et al. 1999b; Vega et al. 2000; Gerhart-Hines et al. 2007). Studies presented here in tg6 mice with increased EPO signaling due to transgenic EPO production and TgEpoR mice with decreased EPO signaling due to EpoR restricted to hematopoietic tissue identify EPO signaling as a positive modifier of PGC-1 α expression in skeletal muscle. It is reasonable to suggest that EPO stimulated increase in slow and oxidative muscle fibers are due in part to the increases in PGC-1 α content. Although further research is necessary to substantiate this assertion, our data showing the elevation of PGC-1 α content in GAS and SOL skeletal muscle by EPO stimulation suggest a direct effect to specify fiber type during fiber development since EpoR is expressed in muscle progenitor/precursor cells and/or an indirect effect since EpoR is down regulated in mature muscle fiber. Specifically, EPO stimulation in primary myoblasts and C2C12 cells was associated with the augmented expression of downstream targets, such as including cytochrome c and IDH3a. EPO stimulation of erythropoiesis and improved oxygen delivery may provide an indirect effect as the high level of EPO in tg6 mice results in high hematocrit >80%; however, erythropoiesis and hematocrit are comparable in WT and TgEpoR mice that exhibit decreased proportions of slow twitch muscle fibers, indicating that the bias toward slow twitch muscle fibers with increased EPO signaling may be independent of its erythropoietic activity. TgEpoR mice exhibit decreased energy expenditure and locomotor activity and develop obesity while EPO treatment in WT mice increases energy expenditure and locomotor activity and reduces fat mass accumulation (Teng et al. 2011); these metabolic associated activities of EPO may relate to an indirect effect of EPO on fiber specification.

AMPK is key regulator of energy metabolism. Importantly, AMPK stimulates PGC-1 α and they both synergistically increase their downstream target substrate to contribute to skeletal muscle fiber reprogramming (Bronner et al. 2004; Narkar et al. 2008). Here we also demonstrated high level EPO increased AMPK activity and non-erythroid specific deletion of EpoR impaired AMPK activity in mice. In myoblasts, EPO treatment also enhances AMPK activity. AMPK is an energy sensor. The activator of AMPK, AICAR can induce slow MHC expression in skeletal muscle and another activator of AMPK, metformin, has been thought to activate AMPK activity to lower blood glucose level and as a potential drug for type II diabetes (Narkar et al. 2008; Ljubicic et al. 2011). Our data in vivo and in vitro are the first to link EPO to AMPK activity and indicate that EPO could mediate AMPK function in energy metabolism. Moreover AMPK activity may also contribute to the effect of EPO on formation of slow, oxidative fibers and on its activated downstream targets such as PGC-1 α . Notably, the indirect and systemic effect of high EPO level in the transgenic

mice may also contribute to the increased PGC-1 α and AMPK activity as observed in our study. Continued high level of EPO in the tg6 transgenic mice has been associated with increased oxygen delivery and increased angiogenesis, which may also contribute to the activity of PGC-1 α and AMPK. Understanding the effects of EPO, AMPK and PGC-1 α and cross-talk among these factors during skeletal muscle development and in metabolism of muscle or extended to some other tissues may clarify their therapeutic potential in skeletal muscle disease and metabolic disorders such as obesity and diabetes.

MATERIALS AND METHODS

Animals and SDH staining

Hemizygous mice carrying a human EPO cDNA transgene driven by the PDGF- β promoter (tg6 mice) (Ruschitzka et al. 2000), WT littermates (control mice) and mice with an Epo-R transgene driven by the erythroid GATA-1 promoter (TgEpoR_E) on an EpoR-null background (EpoR^{-/-} TgEpoR_E) referred to as TgEpoR (Suzuki et al. 2002) were used. Mice were on a C57BL/6 background and four-week old to avoid age-related muscle degeneration observed in older (5 months or more) tg6 mice (Heinicke et al. 2006). Indeed, exercise performance in young tg6 mice was comparable to WT mice (Schuler et al., 2010). Mice were anesthetized and the GAS muscles were harvested and frozen sectioned for succinate dehydrogenase (SDH) staining. Frozen sections were incubated with the SDH solution (0.1 M succinate, 0.1M PBS pH 7.6, NitroBlue Tetrazolium) for 30 minutes at 37°C, rinsed in distilled water and mounted. Purple-blue (dark) stained muscle fiber means it uptakes succinate and is type I muscle fiber and oxidative, light stained fibers means is type IIB muscle fiber. All animal studies were carried out according to guidelines of the NIDDK Animal Care and Use Committee.

Immunohistochemistry

Frozen muscle cross sections were air dried, fixed with acetone and then quenched by incubating the sections in 0.3% H₂O₂ for 30 minutes. After washing with PBS and blocking with blocking reagent (Vector Laboratories Inc) for one hour, the sections were incubated with primary antibody for MHC-I (1:50) (Vector Laboratories Inc) overnight at 4°C. After washing with PBS, the sections were incubated with VECTASTAIN ABC reagent for 5 minutes and washed with PBS for two times. Preparing and applying peroxidase substrate solution according to substrate kit instructions (Vector Laboratories Inc). Sections were mounted using vector mounting medium and images were captured using a PerkinElmer image system.

Primary Myoblasts Isolation and Cell Culture

Skeletal muscle primary myoblasts were isolated from the muscles of embryonic 15 days as described (Jia et al. 2012). C2C12 cells (ATCC, CRL-1772, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% FBS, 0.5 units/ml penicillin/streptomycin, and 1 mM L-glutamine (Invitrogen) (C2C12 growth medium) at 37°C and 5% CO₂. Cells were treated with EPO (5U/mL) or as indicated or vehicle (PBS) as implied in the text. Media containing EPO or vehicle was replaced every 24 h.

Measurement of Oxygen Consumption

A Seahorse Bioscience XF24-3 Extracellular Flux Analyzer was used to measure the oxygen consumption rate. The C2C12 myoblasts were seeded in XF24-well microplates at 2.5×10⁴ cells per well. Respiration was measured under basal conditions, in the presence of the mitochondrial inhibitor oligomycin (0.25 μ M), mitochondrial uncoupler FCCP (5 μ M), and glucose analog 2-DG (0.1M).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding is provided by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases.

Abbreviations

EPO	erythropoietin
EpoR	erythropoietin receptor
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1 alpha
AMPK	AMP-activated protein kinase
SDH	succinic dehydrogenase
MHC	myosin heavy chain
WT	wild type
IDH3α	dehydrogenase 3 alpha
TCA	tricarboxylic acid cycle
CytC	cytochrome C

References

- Anagnostou A, Liu Z, Steiner M, Chin K, Lee ES, et al. Erythropoietin receptor mRNA expression in human endothelial cells. *Proc Natl Acad Sci U S A*. 1994; 91(9):3974–3978. [PubMed: 8171022]
- Bronner M, Hertz R, Bar-Tana J. Kinase-independent transcriptional co-activation of peroxisome proliferator-activated receptor alpha by AMP-activated protein kinase. *Biochem J*. 2004; 384(Pt 2): 295–305. [PubMed: 15312046]
- Carraway MS, Suliman HB, Jones WS, Chen CW, Babiker A, et al. Erythropoietin activates mitochondrial biogenesis and couples red cell mass to mitochondrial mass in the heart. *Circ Res*. 2010; 106(11):1722–1730. [PubMed: 20395592]
- Cayla JL, Maire P, Duvallet A, Wahrmann JP. Erythropoietin induces a shift of muscle phenotype from fast glycolytic to slow oxidative. *Int J Sports Med*. 2008; 29(6):460–465. [PubMed: 18080952]
- Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest*. 2006; 116(3):615–622. [PubMed: 16511594]
- Foskett A, Alnaeeli M, Wang L, Teng R, Noguchi CT. The Effects of Erythropoietin Dose Titration during High-Fat Diet-Induced Obesity. *J Biomed Biotechnol*. 2011; 2011:373781. [PubMed: 21541227]
- Gaster M, Poulsen P, Handberg A, Schroder HD, Beck-Nielsen H. Direct evidence of fiber type-dependent GLUT-4 expression in human skeletal muscle. *Am J Physiol Endocrinol Metab*. 2000; 278(5):E910–E916. [PubMed: 10780948]
- Gaster M, Staehr P, Beck-Nielsen H, Schroder HD, Handberg A. GLUT4 is reduced in slow muscle fibers of type 2 diabetic patients: is insulin resistance in type 2 diabetes a slow, type 1 fiber disease? *Diabetes*. 2001; 50(6):1324–1329. [PubMed: 11375332]
- Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, et al. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. *Embo J*. 2007; 26(7): 1913–1923. [PubMed: 17347648]

- Heinicke K, Baum O, Ogunshola OO, Vogel J, Stallmach T, et al. Excessive erythrocytosis in adult mice overexpressing erythropoietin leads to hepatic, renal, neuronal, and muscular degeneration. *Am J Physiol Regul Integr Comp Physiol*. 2006; 291(4):R947–R956. [PubMed: 16690772]
- Hoeks J, van Herpen NA, Mensink M, Moonen-Kornips E, van Beurden D, et al. Prolonged fasting identifies skeletal muscle mitochondrial dysfunction as consequence rather than cause of human insulin resistance. *Diabetes*. 2010; 59(9):2117–2125. [PubMed: 20573749]
- Hojman P, Brolin C, Gissel H, Brandt C, Zerahm B, et al. Erythropoietin over-expression protects against diet-induced obesity in mice through increased fat oxidation in muscles. *PLoS One*. 2009; 4(6):e5894. [PubMed: 19521513]
- Jia Y, Warin R, Yu X, Epstein R, Noguchi CT. Erythropoietin signaling promotes transplanted progenitor cell survival. *Faseb J*. 2009; 23(9):3089–3099. [PubMed: 19417086]
- Jia Y, Suzuki N, Yamamoto M, Gassmann M, Noguchi CT. Endogenous erythropoietin signaling facilitates skeletal muscle repair and recovery following pharmacologically induced damage. *Faseb J*. 2012
- Koves TR, Li P, An J, Akimoto T, Slentz D, et al. Peroxisome proliferator-activated receptor-gamma co-activator 1 α -mediated metabolic remodeling of skeletal myocytes mimics exercise training and reverses lipid-induced mitochondrial inefficiency. *J Biol Chem*. 2005; 280(39):33588–33598. [PubMed: 16079133]
- Lin CS, Lim SK, D'Agati V, Costantini F. Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. *Genes Dev*. 1996; 10(2):154–164. [PubMed: 8566749]
- Lin J, Handschin C, Spiegelman BM. Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metab*. 2005; 1(6):361–370. [PubMed: 16054085]
- Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, et al. Transcriptional co-activator PGC-1 α drives the formation of slow-twitch muscle fibres. *Nature*. 2002; 418(6899):797–801. [PubMed: 12181572]
- Ljubcic V, Miura P, Burt M, Boudreault L, Khogali S, et al. Chronic AMPK activation evokes the slow, oxidative myogenic program and triggers beneficial adaptations in mdx mouse skeletal muscle. *Hum Mol Genet*. 2011; 20(17):3478–3493. [PubMed: 21659335]
- Michael LF, Wu Z, Cheatham RB, Puigserver P, Adelman G, et al. Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proc Natl Acad Sci U S A*. 2001; 98(7):3820–3825. [PubMed: 11274399]
- Nairz M, Schroll A, Moschen AR, Sonnweber T, Theurl M, et al. Erythropoietin contrastingly affects bacterial infection and experimental colitis by inhibiting nuclear factor-kappaB-inducible immune pathways. *Immunity*. 2011; 34(1):61–74. [PubMed: 21256055]
- Narkar VA, Downes M, Yu RT, Embler E, Wang YX, et al. AMPK and PPAR δ agonists are exercise mimetics. *Cell*. 2008; 134(3):405–415. [PubMed: 18674809]
- Ogilvie M, Yu X, Nicolas-Metral V, Pulido SM, Liu C, et al. Erythropoietin stimulates proliferation and interferes with differentiation of myoblasts. *J Biol Chem*. 2000; 275(50):39754–39761. [PubMed: 10995753]
- Oka T, Maillat M, Watt AJ, Schwartz RJ, Aronow BJ, et al. Cardiac-specific deletion of Gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability. *Circ Res*. 2006; 98(6):837–845. [PubMed: 16514068]
- Ruschitzka FT, Wenger RH, Stallmach T, Quaschnig T, de Wit C, et al. Nitric oxide prevents cardiovascular disease and determines survival in polyglobulic mice overexpressing erythropoietin. *Proc Natl Acad Sci U S A*. 2000; 97(21):11609–11613. [PubMed: 11027359]
- Ryder JW, Bassel-Duby R, Olson EN, Zierath JR. Skeletal muscle reprogramming by activation of calcineurin improves insulin action on metabolic pathways. *J Biol Chem*. 2003; 278(45):44298–44304. [PubMed: 12941959]
- Schuler B, Arras M, Keller S, Rettich A, Lundby C, et al. Optimal hematocrit for maximal exercise performance in acute and chronic erythropoietin-treated mice. *Proc Natl Acad Sci U S A*. 2010; 107(1):419–423. [PubMed: 19966291]
- Sleigh A, Raymond-Barker P, Thackray K, Porter D, Hatunic M, et al. Mitochondrial dysfunction in patients with primary congenital insulin resistance. *J Clin Invest*. 2011; 121(6):2457–2461. [PubMed: 21555852]

- Suzuki N, Ohneda O, Takahashi S, Higuchi M, Mukai HY, et al. Erythroid-specific expression of the erythropoietin receptor rescued its null mutant mice from lethality. *Blood*. 2002; 100(7):2279–2288. [PubMed: 12239135]
- Szendroedi J, Phielix E, Roden M. The role of mitochondria in insulin resistance and type 2 diabetes mellitus. *Nat Rev Endocrinol*. 2011
- Teng R, Gavrilova O, Suzuki N, Chanturiya T, Schimel D, et al. Disrupted erythropoietin signalling promotes obesity and alters hypothalamus proopiomelanocortin production. *Nat Commun*. 2011; 2:520. [PubMed: 22044999]
- Vega RB, Huss JM, Kelly DP. The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol*. 2000; 20(5):1868–1876. [PubMed: 10669761]
- Wang L, Jia Y, Rogers H, Wu YP, Huang S, et al. GATA binding protein 4 (GATA-4) and T-cell acute leukemia 1 (TAL1) regulate myogenic differentiation and erythropoietin response via cross talk with Sirtuin1 (Sirt1). *J Biol Chem*. 2012
- Wang YX, Zhang CL, Yu RT, Cho HK, Nelson MC, et al. Regulation of muscle fiber type and running endurance by PPARdelta. *PLoS Biol*. 2004; 2(10):e294. [PubMed: 15328533]
- Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell*. 1995; 83(1):59–67. [PubMed: 7553874]
- Wu H, Lee SH, Gao J, Liu X, Iruela-Arispe ML. Inactivation of erythropoietin leads to defects in cardiac morphogenesis. *Development*. 1999a; 126(16):3597–3605. [PubMed: 10409505]
- Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell*. 1999b; 98(1):115–124. [PubMed: 10412986]
- Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, et al. Erythropoietin receptor signalling is required for normal brain development. *Development*. 2002; 129(2):505–516. [PubMed: 11807041]
- Zierath JR, Hawley JA. Skeletal muscle fiber type: influence on contractile and metabolic properties. *PLoS Biol*. 2004; 2(10):e348. [PubMed: 15486583]

Highlights

Erythropoietin contributes to slow twitch myofibers program.

Erythropoietin increases mitochondrial activity of myoblast and skeletal muscle.

Erythropoietin mediated increase in PGC-1 α expression and AMPK activity may contribute to slow twitch fiber specification.

Figure 1. EPO increases slow twitch fiber gene expression and SDH staining

A-B. Gene expression was assessed by quantitative real-time RT-PCR for MHC-I, myoglobin and MHC-IIA, IIB and IID in the GAS (A) and SOL (B) muscles from tg6, WT and TgEpoR mice (4-week of age, n=5). C-D. TNNT1 and myoglobin protein levels were assessed in GAS (C) and SOL (D) muscles from tg6, WT and TgEpoR mice (4-week of age, n=5) and β -actin was used as a loading control. One-way ANOVA was used and error bars represent $\pm$ s.e.m; * indicates $p < 0.05$ and ** indicates $p < 0.01$.

Figure 2. EPO contributes to slow twitch fiber protein production

A. Immunohistochemistry was performed to determine the MHC-I level in GAS, SOL, EDL and TA muscles from tg6, WT and TgEpoR mice (4-week of age, n=5). B. Muscle fibers from the GAS muscles and SOL muscle from tg6, WT and TgEpoR mice (4-week of age, n=5) were stained for succinate dehydrogenase (SDH) staining and quantified. One-way ANOVA was used and error bars represent $\pm$ s.e.m; * indicates $p < 0.05$. μ m.

Figure 3. EPO stimulates mitochondrial activity related gene expression

A. The GAS and SOL muscles from WT and TgEpoR mice were harvested and assessed for expression of cytochrome C (CytC), isocitrate dehydrogenase 3 α (IDH3 α) and GLUT4 using quantitative real-time RT-PCR. B-C. Cultures of primary skeletal myoblasts (B) and C2C12 cells (C) were assessed for expression of CytC and IDH3 α by quantitative real-time RT-PCR. S16 was used as internal control. D. Cytochrome C protein level was determined by ELISA for the GAS (left panel) and SOL (center panel) muscles in tg6, WT and TgEpoR mice (4-week of age, n=5) and in cultures of C2C12 myoblasts without (CTRL) and with EPO (5U/ml) treatment (right panel). Statistics were performed using Student's t-test, and

bar graphs are mean $\pm$ s.e.m. In vitro data are means of three independent experiments. *P < 0.05; **P < 0.01.

Figure 4. EPO increases mitochondrial activity

A. The ratio of mitochondrial DNA to nuclear DNA was determined by qPCR in primary myoblasts and C2C12 myoblasts. B-C. Citrate synthase (CS) activity was determined for C2C12 myoblasts with EPO treatment and for the GAS and SOL muscles from tg6, WT and TgEpoR mice. D. Oxygen consumption rate (OCR) was determined in C2C12 myoblasts with EPO treatment for 48 hours using XF-24 instrument. Statistics were performed using one-way ANOVA and Student's t-test, and bar graphs are mean±s.e.m. In vitro data are means of three independent experiments. *P < 0.05; **P < 0.01.

Figure 5. EPO induces PGC-1 α expression

A. PGC-1 α mRNA level was assessed in the GAS and SOL muscles from in tg6, WT and TgEpoR mice using quantitative RT-PCR. B PGC-1 α protein was assessed by Western blotting for GAS muscles from tg6, WT and TgEpoR mice. C-D PGC-1 α mRNA level and protein level were determined in muscle primary myoblasts treated without (CTRL) and with EPO using quantitative RT-PCR and Western blotting. E PGC-1 α mRNA level was determined in C2C12 myoblasts treated without (CTRL) and with EPO using quantitative RT-PCR. β -actin was used as a loading control for Western blotting. Statistics were performed using one-way ANOVA and Student's t-test, and bar graphs are mean $\pm$ s.e.m. In vitro data are means of three independent experiments. *P < 0.05; **P < 0.01.

Figure 6. EPO increases AMPK activity

A. AMPK α phosphorylation was detected in C2C12 myoblasts with EPO treatment at times indicated using Western blotting. B. AMPK α phosphorylation was assessed for GAS and SOL muscles from in tg6, WT and TgEpoR mice (4 week age, n=5) using Western blotting (P-AMPK α , AMPK α phosphorylation; T-AMPK α , total AMPK). C-D. CytC, IDH3 α and PGC-1 α expression (C) and Citrate synthase (CS) activity (D) were determined in C2C12 myoblasts without or with EPO treatment or with AMPK activity inhibitor Compound C. Statistics were performed using one-way ANOVA and Student's t-test, and bar graphs are mean $\pm$ s.e.m. In vitro data are means of three independent experiments. *P < 0.05; **P < 0.01.