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Skeletal muscle HIF-1 α expression is dependent on muscle fiber type

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Running head: HIF-1 α and muscle fiber types

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ABSTRACT

Oxygen homeostasis is an essential regulation system for cell energy production and survival. The oxygen-sensitive subunit α of the HIF-1 complex is a key protein of this system. In this work, we analyzed rodent HIF-1 α protein and mRNA expression in parallel to energetic metabolism variations within skeletal muscle. Two physiological situations were studied. First, we compared HIF-1 α expression between the predominantly oxidative soleus muscle and three predominantly glycolytic muscles. Second, HIF-1 α expression was assessed in an energy-metabolism switch model which was based on muscle disuse. Our results gave rise to the hypothesis that the oxygen homeostasis regulation system depends on the fiber type, with higher constitutive HIF-1 α expression in glycolytic muscle fibers.

Key words: HIF-1, skeletal muscle, oxidative fiber, glycolytic fiber, atrophy.

INTRODUCTION

Eukaryotic cells sense oxygen and adapt to hypoxia by regulating a certain number of genes. This was shown many years ago for various mammalian cell types, including C₂C₁₂ myoblasts (Webster, 1987). The HIF-1 (hypoxia inducible factor-1) transcription factor is a key element of this pleiotropic response. Wang and Semenza observed, more than a decade ago, that HIF-1 DNA binding activity is specifically present under low oxygen conditions for different cell lines, including muscle cells (Wang and Semenza, 1993). Activated HIF-1 comprises two subunits, i.e. HIF-1 α and HIF-1 β . HIF-1 α plays a central role as it is subjected to drastic O₂-dependent proteasomal control. At normoxia, HIF-1 α and HIF-1 β are constitutively expressed but HIF-1 α is mainly ubiquitinated and degraded by the proteasome (Jaakkola et al., 2001). In severe hypoxia, the HIF-1 α protein level increases by relaxing its degradation, and HIF-1 α can form an active complex with HIF-1 β . Activated HIF-1 induces the expression of genes involved, for example, in angiogenesis, erythropoiesis, glucose uptake and energy metabolism (Semenza, 2000; Wenger, 2000). At the RNA level, no HIF-1 α upregulation has been described in response to short-term hypoxia or other stimuli, e.g. CoCl₂, CO or insulin (Ladoux and Frelin, 1997). However, several studies have reported an increase of HIF-1 α mRNA in chronic hypoxia conditions, such as adaptation to altitude (Hoppeler and Vogt, 2001a, b) or high glucose concentration situations (Marfella et al., 2002), without any clear explanations. Moreover, a recent study involving a global analysis of DNA arrays of genes altered in chronic critical and acute-on-chronic human skeletal muscle ischemia conditions, demonstrated upregulation of genes involved in the HIF-1 system, including HIF1 itself (Tuomisto et al., 2004). These observations show that HIF-1 α could also be regulated at the RNA level in several conditions.

HIF-1 studies in skeletal muscle are difficult due to the presence of a number of energy metabolism systems which include different O₂ supplies and homeostasis, with different

proportions of oxidative, glycolytic, and intermediate fibers. Indeed, it has been shown that skeletal muscle presents a continuum of mechanical and metabolic properties from the slow contractile speed type I fibers, which have a low fatigability, to the fast contractile speed type IIb fibers, which have a high fatigability. Type I fiber energy is mainly sustained by an oxidative metabolism, while type IIb energy is mainly generated by a glycolytic metabolism (Booth and Baldwin, 1996). Moreover, this metabolic/mechanical status is not fixed, but adapts to environmental changes such as chronic activity modifications. For instance, slow-twitch muscles modify their phenotype from a predominantly oxidative slow- to a glycolytic fast-type metabolism under different physiological or experimental conditions of muscle atrophy induced by restriction of muscle activity (Diffie et al., 1991; Ohira et al., 1992; Cros et al., 2001).

In this study, we investigated HIF-1 α mRNA and protein expression in relation with muscular energy metabolism types. Our observations strongly suggest that HIF-1 α expression is fiber-type dependent.

MATERIALS AND METHODS

Animals and tissues. All procedures were approved by the local Centre National de la Recherche Scientifique ethics committee. Skeletal muscles were quickly excised from anesthetized (intra-peritoneal injection of pentobarbital, 1 μ L/g of body mass) 10 week-old Ico: OF1 (Caw) mice (Charles Rivers Laboratories), then cleaned and immediately frozen in liquid nitrogen for RNA and protein immunoblotting analysis. Rat gastrocnemius and soleus muscles atrophied by 2 weeks of hindlimb suspension and control muscles were derived from a previous study (Cros et al., 2001).

Cell culture. C₂C₁₂ mouse skeletal muscle cells were obtained from the American Type Culture Collection (ATCC n° CRL-1772), and grown in Dulbecco's modified Eagle's medium with 4.5 g/L glucose and 10% fetal calf serum. Myotube C₂C₁₂ differentiation was induced by withdrawing fetal calf serum and adding 10 μ g/ml insulin, 5 μ g/ml transferrin and 2% horse serum. Cells were stressed by adding 200 μ M CoCl₂ to the culture medium.

RNA analysis. Skeletal muscle RNAs were prepared from nine-rat pool muscles to eliminate individual variations or from three cell culture dishes. Semi-quantitative RT-PCR (reverse transcriptase and polymerase chain reaction) cDNA amplification and quantification were performed as described previously (Pisani et al., 2004). HIF-1 α cDNA fragments were amplified as described by Marfella *et al.* (Marfella et al., 2002) and 18S rRNA fragments were amplified as described by Pisani *et al.* (Pisani et al., 2004).

Protein analysis. For immunoblotting analysis, total soluble proteins were prepared from fresh cell cultures or frozen skeletal muscles as previously described (Yeow et al., 2002).

Equal amounts of proteins were separated by SDS-PAGE on 6% acrylamide gels. Proteins were transferred to Hybond-C extra membranes (Amersham) and stained with ponceau red to check whether the transfers were uniform. Blots were then incubated for 4 h at room temperature in TBS with 0.1% Tween-20 and 5% nonfat powdered milk. Membranes were probed overnight at 4°C with anti-HIF-1 α polyclonal antibody (kindly provided by Dr. Christiane Brahimini-Horn and Dr. Jacques Pouysségur), at 1:2000 dilution in TBS, 0.1% Tween-20, 1% nonfat powdered milk, and then for 1 h at room temperature with an HRP-conjugated swine anti-rabbit IgG (Dako) used at 1:2000 dilution in TBS, 0.1% Tween-20 and 5% nonfat powdered milk. After each incubation, membranes were washed three times in TBS, 0.1% Tween-20, for 10 min per wash. Proteins were then revealed using the Amersham ECL system.

Statistical analysis. *t*-tests were performed using SigmaStat software to compare differences between two groups of values. Statistical differences were noted * ($p < 0.05$).

RESULTS

Difference in HIF-1 α expression between oxidative- and glycolytic-energy metabolism muscle fibers. We compared HIF-1 α expression between a predominantly oxidative muscle (soleus) and three predominantly glycolytic muscles (gastrocnemius, tibialis anterior, and quadriceps). An analysis was performed on mouse muscles, which were immediately snap-frozen in liquid nitrogen after excision to avoid any modifications induced by tissue hypoxia. With the same goal, the first step of protein or RNA preparations were carefully carried out directly using frozen tissues. First, we investigated the HIF-1 α protein level by Western blotting (Fig. 1A). A specific 120 kDa signal was detected with a monoclonal anti-HIF-1 α antibody on nuclear protein extracts. HIF-1 α quantities were reproducibly found to be 8- to 10-fold higher in glycolytic muscles than in the oxidative soleus muscle. Despite the care taken in tissue storage and protein preparation for all muscles, we could not rule out that the drastic difference noted between glycolytic muscles and the oxidative muscle was caused by some more hypoxic conditions for glycolytic muscle sample preparations. Therefore, we compared HIF-1 α mRNA quantities in the oxidative soleus muscle with gastrocnemius and quadriceps glycolytic muscles by semi-quantitative RT-PCR, since no mRNA variations were expected under short-term hypoxia. HIF-1 α mRNA expression was found to be three-fold higher in glycolytic muscles than in the oxidative muscle (Fig. 1B). The higher HIF-1 α protein quantity observed in glycolytic muscles was thus found to be associated with a higher HIF-1 α mRNA content.

CoCl₂ stress induces an HIF-1 α protein increase but no mRNA modifications in differentiated C₂C₁₂ muscle cells. We wondered whether HIF-1 α regulation by hypoxia would also involve mRNA regulation in skeletal muscle, unlike other tissues. CoCl₂ is known to mimic acute hypoxia upon *in vitro* HIF-1 activity induction (Wang et al., 1995). It inhibits

HIF-1 α ubiquitination and consequently its degradation by proteasomes, thus allowing formation of the active HIF-1 α /HIF-1 β complex. Myotube differentiated C₂C₁₂ cells were stressed for 3 h with 200 μ M CoCl₂ treatments. As expected (Wang et al., 1995), Western blot analysis revealed an increase in HIF-1 α protein (Fig. 2A), but no variations in mRNA contents were detected by semi-quantitative RT-PCR (Fig. 2B-C). This was in agreement with the hypothesis that the increase in HIF-1 α protein was only due to a decrease in HIF-1 α degradation, as very well documented in other tissues (Wenger, 2000).

Variations in HIF-1 α mRNA in a model of muscle energy metabolism switch. To further test the hypothesis that the HIF-1 α protein level differences reported above between oxidative and glycolytic muscles was due to constitutive differences in HIF-1 α mRNA quantities between the two kinds of muscles and not to artefactual hypoxic conditions, we analysed another physiological situation. Instead of comparing different muscles, we used a procedure which induced an energy-metabolism switch within the same muscle. Thus, HIF-1 α mRNA expression was analysed by semi-quantitative RT-PCR in rat soleus and gastrocnemius muscles submitted to 2 weeks of activity restriction by hindlimb suspension (Diffie et al., 1991). The anti-gravity oxidative soleus muscles were more than 40% atrophied and approximately 20% of the oxidative fibers were replaced by glycolytic fibers, as assessed via the myosin isoform contents (Cros et al., 1999). In this situation of oxidative- to glycolytic-energy metabolism transition within soleus muscle, HIF-1 α mRNA was found to be threefold upregulated (Fig. 3). Conversely, the glycolytic gastrocnemius muscles, which underwent only 25% atrophy, showed no variation in energy metabolism (Jiang et al., 1992; Ohira et al., 1994) and the HIF-1 α mRNA content was not significantly modified (Fig.3).

DISCUSSION

Energy for muscle contraction is provided by oxidative and glycolytic metabolisms. Skeletal muscles are made of fibers that differ according to the relative involvement of each of these energy metabolisms. These metabolism differences are associated with different mechanical properties that allow muscles to meet various functional demands. As HIF-1 is a critical sensor of cellular oxygen, we investigated expression of the regulatory HIF-1 α subunit in accordance with the oxidative/glycolytic energy balance in skeletal muscle. Variations in this balance naturally occur in different muscles or can be experimentally forced by modifying the mechanical constraints. In this hypoxia-free study, we have shown: (i) higher HIF-1 α expression in predominantly glycolytic muscles than in the predominantly oxidative soleus muscle, and (ii) an HIF-1 α expression increase in the oxidative- to glycolytic-energy transition noted in soleus muscle activity reduction imposed by hindlimb suspension. These variations were noted at the protein level but also at the mRNA level. The existence of HIF-1 α mRNA variations ruled out the possibility of simple hypoxia artifacts in RNA preparations from glycolytic muscles. Indeed, as confirmed by the experimental CoCl₂ treatment of C₂C₁₂ myoblasts, muscle cells sense oxygen like other cells by regulating HIF-1 α protein and not HIF-1 α mRNA.

It was not surprising to detect HIF-1 α expression in normoxic skeletal muscle. Indeed, contrary to most tissues, it has been shown that HIF-1 α is present under normoxic conditions in a few mouse tissues, including brain and skeletal muscle, where its level increases in response to systemic hypoxia (Stroka et al., 2001). In the present work, we confirmed these observations, suggesting that HIF-1 could have a potential function in muscle oxygen homeostasis under normoxia. In addition, our results highlighted that HIF-1 expression was

constitutively higher in glycolytic fibers than in oxidative fibers. This would explain previous observations regarding HIF-1 α mRNA increases in human adaptation to altitude (Hoppeler and Vogt, 2001a, b), or under high glucose concentrations (Marfella et al., 2002).

Variations in HIF-1 α content according to skeletal muscle fiber types in normoxia conditions should be compared to the myoglobin content. According to our results, oxidative fibers have a low HIF-1 α content and are known to express a high level of myoglobin (Mb) (Lin et al., 2002), which is responsible for their red color. Mb is a cytoplasmic hemoprotein able to link O₂ in the interstitial compartment to carry it inside the fiber (Brunori, 2001). Mb forms an intracellular oxygen reserve which to a certain extent enables muscular fibers to buffer variations in blood O₂ supply (*i.e.* hypoxia), or to meet fiber metabolism needs. In contrast, glycolytic fibers were shown here to have a high HIF-1 α content but they express a low level of Mb (Vogt et al., 2001). In this case, we could speculate that the Mb level is not efficient enough to compensate for alterations in O₂ homeostasis (*i.e.* hypoxia), which implies a higher fiber sensitivity to O₂ variations—the higher HIF-1 α content in glycolytic fibers would be necessary to provide satisfactory hypoxia protection for these fibers. Moreover, a balance between HIF-1 α and Mb was recently documented in a study of Mb *-/-* knocked-out mice (Grange et al., 2001). Indeed, these mice were viable and showed significant phenotypic modifications to compensate for the Mb absence, including HIF-1 α upregulation and metabolism transitions from oxidative to glycolytic muscle fibers (Grange et al., 2001). Conversely, a recent study of partially HIF-1 α *-/-* knocked-out mice showed no variation in fiber type composition in soleus and gastrocnemius muscles (Mason et al., 2004). However, a clear shift from glycolytic to oxidative muscle metabolism was detected during exercise of these modified mice in comparison to wild-type mice. Taken together, these points could be

explained by the presence of an O₂ regulation system based mainly on Mb in oxidative fibers and on an HIF-1 complex in glycolytic fibers.

In conclusion, our data confirmed differences in the HIF-1-mediated oxygen regulation system between normoxic oxidative and glycolytic skeletal muscle fibers. The higher HIF-1 α expression observed under the lower O₂ supply conditions in glycolytic fibers underlines the importance of HIF-1 for maintaining muscle homeostasis and HIF-1 involvement in muscle plasticity to tailor the metabolism, and therefore the mechanical properties, to meet the functional demand.

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FIGURE LEGENDS

Figure 1. Comparison of HIF-1 α expression in slow (soleus) versus fast (gastrocnemius, tibialis anterior, quadriceps) skeletal muscles. A) Protein analysis by Western blotting on mouse skeletal muscle nuclear protein extracts. HIF-1 α was detected as a specific 120 kDa band, and a 80 kDa non-specific band stained with amido-black is shown as an even-lane loading control. B) RNA analysis performed by semi-quantitative RT-PCR on skeletal muscle RNAs. Partial HIF-1 α mRNAs were amplified and quantified and 18S rRNAs were used for normalization. The band intensity was measured, in linear PCR amplification range, and the results are expressed in arbitrary units after normalization. Histograms represent mean \pm S.E.M. of three independent experiments from two independent RNA or protein preparations. * : significantly different from soleus muscle (A: P<0.001; B: P<0.03).

Figure 2. HIF-1 α expression in differentiated C₂C₁₂ myotubes treated with 200 μ M CoCl₂. A) Total cell proteins were extracted and analyzed by Western blotting using anti-HIF-1 α antibody. An HIF-1 α -specific 120 kDa band was detected, and a 80 kDa non-specific band stained with amido-black is shown as an even-lane loading control. B) RNAs were prepared and analyzed by semi-quantitative RT-PCR. Primers specific to HIF-1 α mRNA (for analysis) and to 18S rRNA (for normalization) were used. Band intensities were measured in the linear amplification range, and the results are expressed as ratios between the different time points and the T0-band intensities after normalization by 18S rRNA variations. The results represent mean \pm S.E.M. of three independent experiments.

Figure 3. Comparison of HIF-1 α mRNA expression in soleus and gastrocnemius muscles atrophied by unloading versus control muscles. Total RNAs isolated from rat muscle pools were analyzed by semi-quantitative RT-PCR. HIF-1 α mRNAs were amplified for analysis and

18S rRNAs were amplified for normalization. Band intensity was measured in the linear amplification range and the results are expressed in an arbitrary unit after normalization by 18S rRNA variations. Histograms represent mean \pm S.E.M. of three independent RT-PCR experiments from two independent RNA preparations. * : significantly different from control (soleus muscle: $P < 0.007$).

Figure 1.

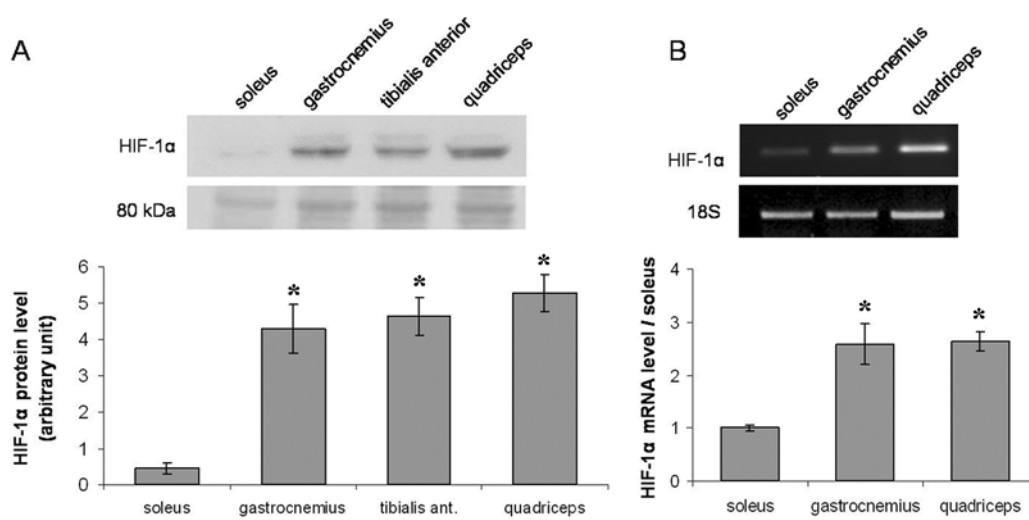


Figure 2.

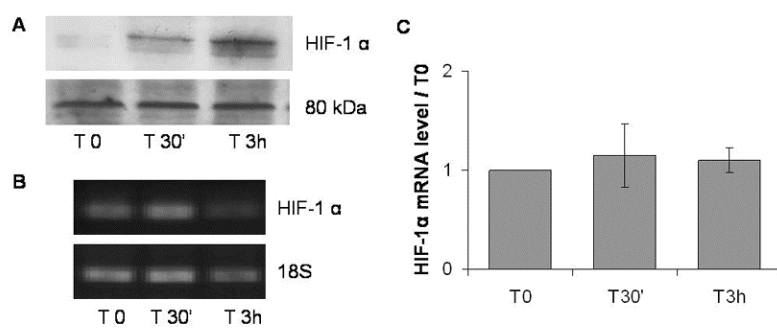


Figure 3.

