Response to normobaric hypoxia in slow and fast muscles of the rat

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Abstract
In this study the HIF-1 signaling pathway was comparatively studied in fast (EDL) and slow (soleus) muscles in basal normoxic conditions and under normobaric hypoxia (10% oxygen). In normoxia HIF-1α transcription was determined by Real Time PCR and found similar in EDL and in soleus, whereas HIF-1α dependent transcription, explored by Real Time PCR of VEGF which is known as a HIF-1α regulated gene and by expression of luciferase under control of a HIF1α responsive promoter, was higher in soleus than in EDL muscle. Under normobaric hypoxia conditions, HIF1α mRNA showed a small but significant increase in both EDL and soleus and this change was accompanied by an increase of both VEGF transcription and luciferase expression. Interestingly, the time course of the response VEGF and luciferase to hypoxia was different in EDL compared to soleus and, in addition, some discrepancy was detected between VEGF and luciferase pattern. Taken together the results point to diversity between fast and slow muscles in the HIF-1α signaling pathway both in normoxic and hypoxic conditions.
Key words: HIF-1, hypoxia, skeletal muscle, rat.

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Hypoxia inducible factor-1 (HIF-1) is a hetero-dimeric transcriptional factor involved in the cellular response to hypoxia in different tissues. It is composed of two subunits, the constitutive subunit β (91-94 kDa) and the subunit α (120 kDa) which, in normoxia conditions, is hydroxylated and degraded by the proteasome system [8, 9, 13]. When oxygen levels suddenly fall, the degradation of the α subunit is inhibited and the heterodimer is stabilized and may translocate in the nucleus where, thanks to transcriptional coactivators, binds to the Hypoxia Responsive Element (HRE) present on target genes involved in the hypoxia response. HIF-1 activity is also regulated by several non hypoxic factors, like citokines, insulin, growth factors, nitric oxide, trombin, angiotensin and mechanical stress [14, 15].

Whereas the scientific community is particularly interested in the HIF-1 involvement in tumor growing processes, neo-angiogenesis and other pathological processes of several tissues [10, 15], no exhaustive data exist on the HIF-1 role in skeletal muscle. The latter tissue is physiologically exposed to large variations of oxygen partial pressure. This fact makes the muscle a good experimental model to study the oxygen sensing mechanisms and the consequent response to hypoxia. The interplay between systemic hypoxia and contractile activity is a crucial aspect in skeletal muscle and Tang et al. [11] has demonstrated the additive character of the response to normobaric systemic hypoxia and the exercise. Moreover, exercise leads to elevated HIF-1α protein levels and a more intense nuclear staining of HIF-1α, together with an activation of the HIF-1 target genes VEGF and EPO [3, 1]. VEGF is considered a HIF-1 target gene actively expressed in response to exercise [see references in 7]; VEGF mRNA levels show an increase immediately after a session of submaximal exercise in normoxia, in resting hypoxic rats and, in particular, in rats exercised in hypoxic conditions [11].

In this study we aimed to test the hypothesis that different mechanisms of oxygen consumption are used in different fiber types in relation to specific oxygen sensing properties and to the mechanisms of response to hypoxia. In particular our attention was directed at HIF-1α activity in two opposite types of fibers, fast, white, glycolytic (mainly expressing MHC of type 2X and 2B) and slow, red oxidative fibers (expressing MHC of type 1 and 2A). To this end we investigated the differences of HIF-1α activity in basal conditions and in normobaric systemic hypoxia in rat EDL (fast and glycolytic) and soleus (slow and oxidative) muscles measuring the HIF-1α transcriptional activity with dual reporter Luciferase

assay and mRNA expression of its target gene VEGF. Moreover, the gene expression of HIF-1α in basal and systemic hypoxia conditions was evaluated by a Real Time PCR approach using the comparative Ct method.

Material and Methods

Animals

All the experiments were performed using male Wistar rats weighting 120-150g born and maintained at the animal facilities of the Department of Anatomy and Physiology, University of Padova. Animals were housed under controlled environmental conditions (20-22°C, 12:12-h light-dark cycle) and were allowed free access to standard rodent chow (ALTROMIN-R, A. Rieper Spa, Vandoies, BZ, Italy) and tap water. The animals were maintained according to the European Union guidelines for the care and use of laboratory animals.

Plasmids and transfections

Under general balanced anesthesia, administered intraperitoneally and composed of Zoletil (tyletamine + zolazepam) 30 mg/Kg and xilazine 8 mg/Kg, the soleus and the EDL of both hind limbs muscles were transfected with a reporter construct carrying the firefly Luciferase gene under control of concatamers of Hypoxia Response Elements (HRE) [12]; in this system the Luciferase gene acts as a target of HIF-1α, allowing a measure of the transcriptional activity of HIF-1. A second plasmid was co-transfected carrying the Renilla Luciferase gene under control of viral promoter, to normalize the luciferase signal. We injected 50 µl of solution containing the two plasmids (20µg of HIF-1 sensor and 5 µg of TK Renilla). After the closure of the surgical wound, to increase the transfection efficiency, the transfected muscles were electroporated with the ECM 830 electroporator (BTX, Harvard Bioscience) using the following parameters: voltage = 220V/cm, number of the impulses = 6, length of the impulse = 20 msec, interval among the impulses = 180 msec. We allowed 7 days from the transfection to the dissection and Luciferase activity analysis to increase the integration and expression of the plasmid carrying the Luciferase reporter genes and to minimize the inflammation related variables.

Normobaric Hypoxia

One week after the transfection the rats were randomly assigned to three groups: two experimental groups exposed to systemic normobaric hypoxia obtained connecting a bottle, containing a certified mixture of 10% Oxygen and 90% Nitrogen, with an airtight chamber in which the animals had free access to the food and water, and one control group. The concentration of oxygen in the chamber was controlled with a commercial oxymeter (MicroPac, Draeger). After 8 hours the first group of animals (n= 8) was sacrificed by cervical dislocation under deep anesthesia induced by an intraperitoneal administration of xylazine (15 mg kg^{-1} body mass) and Soleus and EDL muscles were quickly dissected out avoiding reoxygenation. The specimens of the second group (n= 8) were collected in the same way after 24 hours from the beginning of the experiment, A control group (n =8) was kept for 24 hours in normoxic conditions and muscles sampled in exactly the same way. After dissection, the soleus and EDL muscles were immediately immersed in melting isopentane, cooled in liquid nitrogen and stored at –80°C for subsequent analyses.

RNA extraction and Real Time PCR

Total RNA was extracted from 100 mg of muscle tissue using the Trizol® Reagent from Invitrogen (Paisley, UK). The integrity of RNA was checked on 2% agarose gels and total RNA concentration was estimated by a spectrophotometer (Pharmacia). 2 µg of total RNA was reverse-transcribed to synthesize cDNA by using the SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen) after treatment with DNAse I (Invitrogen) to remove contaminating genomic DNA. Real Time PCR amplification reactions were carried out on 30-µl aliquots (containing 3 µl cDNA in a 1:8 dilution) on an ABI 7500 Real Time PCR System (Applied Biosystems, Italy) by SYBR Green I dye chemistry detection under amplification conditions reported elsewhere (10). HIF-1α and VEGF mRNA levels were analyzed against β-actin, a generally used reference gene. Briefly, Real Time conditions for the SYBR green assay was: 2 min at 50°C, 10 min at 95 °C, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. Fluorescence signal baseline and threshold were set manually for each detector generating a threshold cycle (Ct) for each sample. Standard curves were prepared for the target and the endogenous reference gene (β-actin). The specificity of the PCR amplification was always verified with melting curve analysis, while the mean C_T values of the β-actin were not different among groups. Quantification of the mRNA data was done by using the comparative C_T method as previously described [5, 4].

Primers for each detector were designed using the Primer Express 3.0 (Applied Biosystems) software so that primers spanned an intron junction. In particular, HIF-1α primers were designed on the AY057308 rat sequence at position 2241-2261 for the forward and at position 2361-2382 for the reverse primer. VEGF primers were designed on AY702972 rat sequence at position 313-334 for the forward primer and at position 369-392 for the reverse primer. The β-actin primers were: for, 5' CCG TAA AGA CCT CTA TGC CA, rev, 5' AAG AAA GGG TGT AAA ACG CA 3'.

Data analysis was performed by means of parametric (one way ANOVA) and non-parametric (General Linear Model) tests in order to detect the outcome differences between two groups (one normoxia muscle and one muscle under hypoxia condition, used as calibrator sample, n=6) or to compare multiple groups (normoxia,

8 hours hypoxia, 24 hours hypoxia, n=6) using the SigmaStat version 2.03 (SPSS Inc., CA, USA). The level of statistical significance was set at $P<0.05$ for all analyses.

Luciferase assay

Luciferase activity was measured from frozen homogenate samples. The analysis was performed using a Dual-Luciferase Reporter Assay System (Promega Inc.) following the supplier instructions and the subsequent Firefly/Renilla ratio measurements with a luminometer TD20/20 (Turne Design). Statistical analysis was performed by means of one way ANOVA. The level of statistical significance was set at $P<0.05$ for all analyses.

Results

Transcriptional activity of HIF-1 in slow and in fast muscles in normoxia and under systemic normobaric hypoxia.

In basal conditions, i.e. in normoxia, the Firefly/ Renilla ratio was significantly higher in the soleus muscle compared to the EDL (Fig. 1). Under normobaric hypoxia, in the soleus muscle, the Firefly/ Renilla ratio showed a trend to increase that reached statistical significance compared to basal conditions only after 24 hours (Fig. 1A). In the EDL, the HIF-1 transcriptional activity showed a significant increase after 8 hours of hypoxia while after 24 hours it returned to values not different from those reported in basal conditions (Fig. 1B).

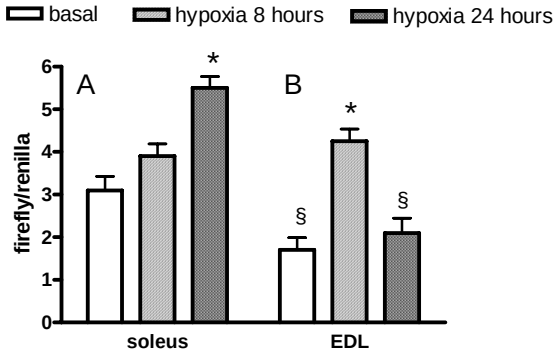

Fig. 1 *HIF-1 transcriptional activity monitored in the rat muscles under normoxia and in normobaric systemic hypoxia (FiO_2 10 %) by means of a dual luciferase assay and measured as firefly/Renilla ratio. A) The HIF-1 activity in the soleus showed an increasing trend, statistically significant, after 24 hours of treatment. B) In the EDL the HIF-1 activity peaked significantly after 8 hours of hypoxia and after 24 hours returned to basal levels. * significant difference compared to basal (normoxia), $ significant difference compared to soleus.*

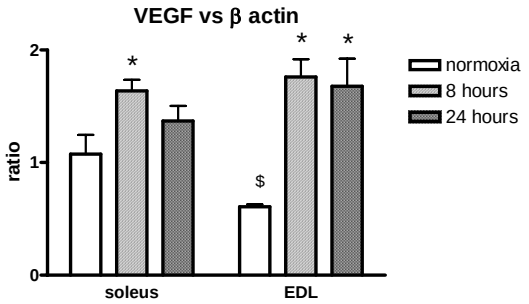

Fig. 2 *VEGF mRNA levels in the soleus and in the EDL muscle in normoxic and normobaric hypoxic conditions measured with a Real Time PCR approach. In normoxic conditions the VEGF expression was statistically different between the two muscles ($, $p<0.005$); after 8 hours of hypoxia mRNA levels increased significantly (*) in both muscles and, after 24 hours, the difference between the two muscles increases compared to the value at 8 hours.*

HIF-1 transcriptional activity was also measured by means of the Real Time PCR for the VEGF target gene (Fig. 2). In basal conditions, the VEGF mRNA levels were significantly higher in the soleus respect the EDL. After 8 hrs of hypoxia a significant increase was observed for both muscles. After 24 hours the mRNA levels slightly decreased in the soleus and showed no further increase in the EDL (Fig. 2).

HIF-1α expression in slow and fast muscles and under systemic normobaric hypoxia

In normoxic conditions, the basal HIF-1α mRNA level was similar in the EDL compared to the soleus muscle and after 8 hours of hypoxia the mRNA levels increased for both muscle with a similar statistically significant pattern. After 24 hours the increase of HIF-1α mRNA was only present in the soleus (Fig. 3).

Discussion

In this study the transcriptional activity of HIF-1 was studied in basal normoxic conditions and in normobaric systemic hypoxia comparing fast and slow muscles. Two different approaches were followed: i) to determine with Real Time PCR the transcription of a gene, VEGF, which is known to be controlled by HIF-1 and ii) to measure the accumulation in muscle fibers of luciferase, whose gene, associated in a plasmidic construct with a HIF-1 responsive promoter, was transfected in muscle fibers.

Experiments carried out using the luciferase assay showed a higher basal HIF-1 transcriptional activity in slow oxidative muscles compared to fast glycolytic muscles. This data was confirmed by the expression analysis carried out on VEGF mRNA, a target of HIF-1.

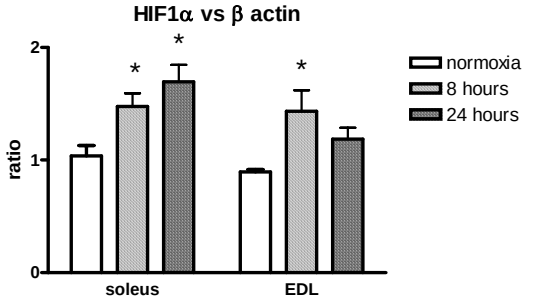

Fig. 3. *HIF-1α mRNA levels in the soleus and in the EDL muscle in normoxic and under normobaric hypoxic conditions measured with a Real Time PCR approach. In basal conditions the HIF-1α expression was different but without statistical significance between the two muscles. After 8 hours of hypoxia mRNA levels increased significantly (*) in both muscles while after 24 hours only the soleus showed an increasing significant trend.*

The higher HIF-1 activity in the oxidative muscles seems consistent with morphological evidence of greater development of blood vessels in this type of muscle compared to the glycolytic muscles.

In contrast, HIF-1α mRNA basal level which was also determined in the two muscles was not significantly different. Only one study [7] has previously compared HIF-1α signaling in different muscle types and has reached conclusions which are not consistent with our work. That study has compared the soleus (taken as a model of slow fiber) and the whole gastrocnemius (which is a mixed muscle composed of slow and fast fibers) and has showed, by a semiquantitative PCR, that the HIF-1α protein and mRNA levels are lower in the oxidative muscle. In basal conditions slow oxidative muscles are more active than fast muscles [2] as slow motor units are characterized by continuous low frequency discharge related with tonic postural activity, whereas fast motor units are characterized by burst of high frequency discharge related with phasic movements with long intervals of complete rest. A direct effect of muscle contractile activity on HIF-1α protein accumulation and on VEGF transcription has been demonstrated by Tang et al [11] and this might suggest a relation between the greater contractile activity and the more pronounced HIF-1 dependent transcription in slow muscles. Oxygen consumption per unit mass is higher in slow oxidative muscles than in fast muscles at rest as well as during exercise [6], but this does not imply a difference in oxygen pressure at cellular level due to the different blood flow.

Under hypoxic conditions, the luciferase assay data showed that the kinetic of the HIF-1α response to hypoxia was different in the two muscles since it had a more transient character in the EDL muscle and a regular increasing trend in the soleus muscle. The results from VEGF Real Time PCR experiments confirmed partially this data showing a less transient response in the EDL muscle; these differences in the results from the two approaches may be explained taking into account the more complex mechanism acting on the promoter of the VEGF gene in comparison to the HRE concatamer present in the luciferase plasmide. To our best knowledge no data are available in the scientific literature on the response of fast compared to slow muscles. Surprisingly, in hypoxic conditions the transcriptional activity of HIF-1α was similar in both muscles, especially after 8 hours of hypoxia.

In conclusion our data point to a difference in the basal HIF-1 transcriptional activity between the two types of muscles, in the pattern of the response to the acute hypoxia and in the time course of the response suggesting a possible contribution of HIF-1 signaling to the phenotypical diversity between muscles.

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