Hypoxia inducible factor 1α links fast-patterned muscle activity and fast muscle phenotype in rats

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Non-technical summary Muscle fibres change when they are used differently, such as by exercise. Genetic studies have shown that a hyperactive form of the gene regulatory protein hypoxia inducible factor 1α (HIF-1α) occurs twice as often among strength-trained athletes as in the normal population. HIF-1α is 'sensing' the oxygen levels in cells, and the oxygen levels change by an order of magnitude in working muscle. We show that an 'endurance' type of activity reduces the level of HIF-1α, while short intense bursts of activity increases it. When HIF-1α was produced in higher quantities by introducing artificial genes in the muscle fibres they became larger and faster, and with a less oxidative metabolism. Thus, oxygen itself could be a trigger for changes in muscle. The composition of muscle fibres is strongly correlated to major lifestyle conditions such as diabetes and chronic obstructive pulmonary disease, and HIF-1α might provide a new molecular link.

Abstract Exercise influences muscle phenotype by the specific pattern of action potentials delivered to the muscle, triggering intracellular signalling pathways. P_{O_2} can be reduced by an order of magnitude in working muscle. In humans, carriers of a hyperactive polymorphism of the transcription factor hypoxia inducible factor 1α (HIF-1α) have 50% more fast fibres, and this polymorphism is prevalent among strength athletes. We have investigated the putative role of HIF-1α in mediating activity changes in muscle. When rat muscles were stimulated with short high frequency bursts of action potentials known to induce a fast muscle phenotype, HIF-1α increased by about 80%. In contrast, a pattern consisting of long low frequency trains known to make fast muscles slow reduced the HIF-1α level of the fast extensor digitorum longus (EDL) muscle by 44%. Nuclear protein extracts from normal EDL contained 2.3-fold more HIF-1α and 4-fold more HIF-1β than the slow soleus muscle, while von-Hippel-Lindau protein was 4.8-fold higher in slow muscles. mRNA displayed a reciprocal pattern; thus FIH-1 mRNA was almost 2-fold higher in fast muscle, while the HIF-1α level was half, and consequently protein/mRNA ratio for HIF-1α was more than 4-fold higher in the fast muscle, suggesting that HIF-1α is strongly suppressed post-transcriptionally in slow muscles. When HIF-1α was overexpressed for 14 days after somatic gene transfer in adult rats, a slow-to-fast transformation was observed, encompassing an increase in fibre cross sectional area, oxidative enzyme activity and myosin heavy chain. The latter was shown to be regulated at the mRNA level in C2C12 myotubes.

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Abbreviations bHLH, basic helix–loop–helix; COPD, chronic obstructive pulmonary disease; CSA, cross sectional area; EDL, extensor digitorum longus; FIH-1, factor inhibiting HIF 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPDH, α-glycerophosphate dehydrogenase; HIF, hypoxia inducible factor; MyHC, myosin heavy chain; PAS, Per Arnt Sim; SDH, succinate dehydrogenase; SOL, soleus; VHL, von-Hippel-Lindau.

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Introduction

Muscle cells are in a very different state during rest and in activity, and it is well established that the state of activity creates signals responsible for triggering the phenotypic changes caused by exercise (for review see Schiaffino *et al.* 2007; Gundersen, 2011). Since muscle is largely a post-mitotic tissue, changes are caused by alterations in the subset of genes expressed in pre-existing fibres (Gorza *et al.* 1988).

Most research on establishing the signalling pathways connecting contractile activity to transcription of the genes determining muscle phenotype has been focused on signalling systems detecting the dramatic increase in the free intracellular Ca^{2+} caused by activity. Muscle contractile activity has, however, other dramatic effects on muscle cells such as changes in mechanical stress, metabolites and oxygen tension (reviewed in Cacciani *et al.* 2008; Gundersen, 2011).

Changes in intracellular P_{O_2} during muscle activity have been investigated by ^{1}H magnetic resonance spectroscopy of myoglobin. P_{O_2} values are reduced from >20 mmHg at rest, to values <4 mmHg during exercise (Richardson *et al.* 1995, 2001). The alteration of P_{O_2} could serve as a reliable activity-dependent trigger for plastic changes and there are well characterised signalling mechanisms sensing oxygen levels, such as those related to the basic helix–loop–helix (bHLH) Per Arnt Sim (PAS) domain transcription factor hypoxia inducible factor (HIF)-1α. HIF-1α in muscle has previously been shown to be increased both by reducing P_{O_2} in inspired air, and by eliciting action potentials in the muscle (Tang *et al.* 2004).

In this signalling pathway prolyl hydroxylases use molecular oxygen as a substrate and hence act as oxygen sensors. Specifically, HIF-1α prolyl hydroxylases (PHD1–3) undermine the stability of the HIF-1α protein by hydroxylation, which takes place in normoxia, enabling the interaction of HIF-1α and the von-Hippel-Lindau (VHL) protein, leading to subsequent ubiquitination and proteasomal degradation. Asparaginyl hydroxylation by factor inhibiting HIF (FIH-1) can block the interaction of HIF-1α with cofactors and thereby negatively regulate HIF-1α activity. When activating gene transcription, HIF-1α forms a heterodimer with another helix–loop–helix PAS protein, ARNT, also called HIF-1β (Bracken *et al.* 2003; Semenza, 2009). We suggest here that this signalling system plays an active role in muscle plasticity.

Aspects of muscle function such as contraction speed, endurance, metabolism and strength are all influenced by exercise. Although various properties can be regulated independently, under normal conditions they are usually coupled to more or less distinct fibre types. Rodent limb muscle fibres are usually classified into four such types according to the myosin heavy chain (MyHC) isoenzymes they express: type I, IIa, IIx and IIb.

The MyHC type is linked to metabolic properties, which are related to endurance and fibre cross sectional area (CSA), which is generally proportional to strength. Thus, type I fibres are generally slow, oxidative, fatigue resistant and thin/weak, while type IIb fibres are fast, glycolytic, fatiguable and large/strong. The IIa and IIx types display intermediate values for these parameters. When fibre type changes occur, they usually happen in the sequential order I $\rightleftharpoons$ IIa $\rightleftharpoons$ IIx $\rightleftharpoons$ IIb (Schiaffino & Reggiani, 1996; Pette & Staron, 2000).

It is well established that the direction of the plastic changes is dependent upon the pattern of action potentials evoked in the muscle fibres by the motor neurons. Thus, when various fibre types are electrically stimulated by a pattern mimicking the activity in fast motor units (brief high frequency bursts of action potentials) the phenotype shifts in the fast direction, while slow activity (high amounts of action potentials delivered in long low frequency trains of impulses) induces changes in the opposite direction (Gundersen & Eken, 1992; Gundersen, 1998; for review see Gundersen, 2011).

In this study we show that HIF-1α levels are higher in normal fast muscles and increased in muscles subjected to fast-patterned electrical activity, while reduced in fast muscles subjected to slow-patterned activity. When HIF-1α was overexpressed in myotubes or in adult rat muscles after somatic gene transfer, we observed a shift from slow to fast fibre type.

Methods

Animal experiments

All surgical procedures were performed on male Wistar rats weighing 200–300 g under deep anaesthesia induced either by isoflurane gas (Forene, Abbott) or by intraperitoneal injections of 5 μl g^{-1} Equithesin (Rikshospitalet, Norway). Depth of anaesthesia was monitored during the experiments by pinching the metatarsus region. Animals were killed by neck dislocation while anaesthetised. The animal procedures were performed under University of Oslo guidelines and governance. The experiments were reviewed and approved by the Norwegian Animal Research Authority and were conducted in accordance with Norwegian legislation and the policies of *The Journal of Physiology* (Drummond, 2009).

Electrical stimulation

Stimulation of the sciatic nerve was performed essentially as described previously (Windisch *et al.* 1998; Ekmark *et al.* 2007). A silicon cuff connected to Teflon-coated

steel electrodes (AS632, Cooner Sales, Chatsworth, CA, USA) was placed around the right leg sciatic nerve, which innervates the lower leg muscles, including the extensor digitorum longus (EDL) and soleus muscle. The nerve was cut proximally to the electrodes in order to avoid reflexes, and electrical stimulation lasted for 6 h while the animal was anaesthetised. Contralateral left leg muscles served as controls. Contraction was monitored visually and by palpation and the contractions appeared to be as vigorous at the end of the 6 h period as in the beginning. Chronic stimulation with similar activity patterns has not revealed muscle damage as judged histologically (Windisch *et al.* 1998; Ekmark *et al.* 2007), or immunohistochemically by labelling for embryonic MyHC (Gorza *et al.* 1988). Stimulated and contralateral control soleus and EDL muscles were harvested at the end of stimulation, snap frozen in liquid nitrogen and stored at −80°C.

Plasmids

For overexpression of HIF-1α, pCEP4HIF-1α (ATCC number MBA-2 (JHU-38), LGC Promochem) was used (Jiang *et al.* 1997). To identify transfected muscle fibres *in vivo*, pAP-lacZ was used to overexpress β-galactosidase (Kisselev *et al.* 1995). In C2C12 cells, the pGL3-basic plasmid (Promega) expressing firefly luciferase was co-transfected for normalization purposes. Sham controls were transfected with the empty pCEP4.

Expression of recombinant HIF-1α *in vivo* and *in vitro*

In vivo electroporation of muscle fibres was performed essentially as described previously (Rana *et al.* 2004). Muscles were co-transfected with 50 μg of pAP-lacZ and 50 μg pCEP4HIF-1α DNA in 0.9% NaCl, with pAP-lacZ transfection alone (50 μg) serving as sham control in the contralateral soleus or EDL. In some muscles, minor areas appeared damaged by the electroporation, and such areas were excluded from the analysis. All muscles were labelled for embryonic MyHC by the BF-G6 antibody indicative of regenerating fibres (Schiaffino *et al.* 1986); only a few positive fibres were observed in a small number of muscles.

Since only a minority of the fibres are transfected (<5%), recombinant protein cannot be detected in homogenates. Thus, in order to confirm the expression of HIF-1α into a full-length protein from the pCEP4HIF-1α plasmid, human embryonic kidney cells (HEK-293) were transfected using Lipofectamine 2000 (Invitrogen), and protein harvested after 48 h. Subsequent immunoblotting demonstrated that the nuclear fraction of the transfected cells displayed more of a protein of the expected 120 kDa size than sham transfected cells displaying only the endogenous HIF-1α of identical size (Fig. 1).

Cultured C2C12 myoblasts were co-transfected (FUGENE, Roche) with either pCEP4HIF-1α + pGL3-basic or the empty pCEP4 + pGL3-basic (control) 24 h before differentiation into myotubes, and harvested for subsequent RNA extraction 72 h after initiation of differentiation.

mRNA analysis

mRNA was extracted from snap-frozen muscles using Trizol reagent (Invitrogen) and from C2C12 cells using the TRI reagent (Ambion), and was subsequently stored in RNA Storage Solution (Ambion) at −80°C. RNA quantity and quality were validated using Nanodrop (Thermo Scientific) and the 2100 Bioanalyser (Agilent), respectively. RNA integrity number above 8 was considered acceptable. RNA was treated with Turbo DNase (Ambion) and cDNA synthesis was performed using Superscript III Reverse Transcriptase (Invitrogen), as described previously (Ellefsen *et al.* 2008). Real-time RT PCR was run on the Lightcycler 480 using the Master SYBR Green I kit (Roche Applied Science), as described previously (Ellefsen *et al.* 2008). Cycling consisted of an initial denaturation step at 94°C for 10 min before 39 repeats of 94°C for 10 s, 60°C for 12 s and 72°C for 8 s. Primer sequences are given in the online Supplemental Material, Table S1. β2-Microglobulin was used for normalisation. To analyse MyHC composition in C2C12 cells, gene-family profiling was performed as described previously (Ellefsen & Stensløkken, 2010).

Protein analysis

Protein fractions were isolated from snap-frozen muscles or HEK-293 cells using the Compartmental Protein Extraction Kit (Chemicon International). Protein

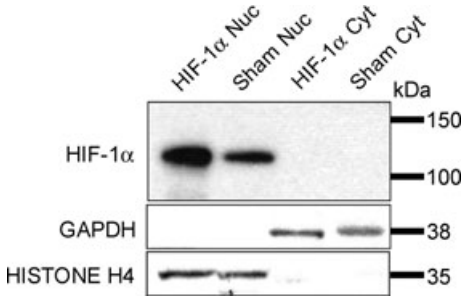

Figure 1. Transfection of HEK-293 cells with pCEP4HIF-1α led to expression of the HIF-1α protein
Nuclear protein extracts (Nuc) from HEK-293 cells transfected with pCEP4HIF-1α (HIF-1α) yielded a stronger 120 kDa band, the expected size of the HIF-1α protein, on a Western blot probed with a HIF-1α antibody, than nuclear extracts from pAP-lacZ transfected cells (sham). Cytoplasmic protein extracts (Cyt) displayed no band. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and histone H4 were used as loading controls for the cytoplasmic and nuclear protein fractions, respectively.

concentration was measured using the Micro BCA Kit (Pierce). Twenty to thirty-five micrograms of cytoplasmic and nuclear protein was run on NuPAGE Novex Bis-Tris Gels (Invitrogen), followed by immunoblotting (NuPAGE Western Transfer Protocol, Invitrogen). HIF-1α protein was detected by the monoclonal antibody NB100-105 (Novus Biologicals), HIF-1β by the monoclonal antibody NB100-124 (Novus Biologicals), and the VHL protein by the polyclonal antibody Sc-1535 (Santa Cruz Biotechnology), all at a 1:500 dilution. Purity of protein fractions was validated using antibodies against the cytoplasmically located glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme (Sc-20357, Santa Cruz Biotechnology), the nuclear histone H4 (Sc-8658, Santa Cruz Biotechnology) or OCT-4 (3576-100, BioVision, Mountain View, CA, USA), diluted 1:500. Secondary horseradish peroxidase (HRP)-conjugated antibodies used were anti-mouse, anti-goat or anti-rabbit (catalogue numbers 1031, 6160 and 4030) from Southern Biotechnology (Birmingham, AL, USA), diluted 1:1750. Blots were developed using the ECL Western blotting detection kit (GE Healthcare).

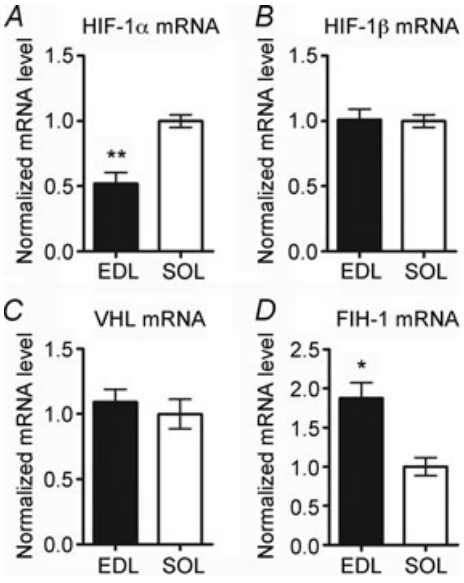

Figure 2. mRNA level of the HIF-1a signalling system in the slow soleus and the fast EDL
mRNA expression levels of components of the HIF-1 transcription factor system: HIF-1α (*A*), HIF-1β (*B*), von-Hippel Lindau (VHL) protein (*C*) and factor inhibiting HIF-1 (FIH-1) (*D*) relative to β2-microglobulin in soleus (SOL) and EDL muscle homogenates from six rats presented as means ± S.E.M. relative to the mean SOL levels. Differences in mRNA expression levels were tested using Wilcoxon's matched pairs test: $^{*}P < 0.05$; $^{**}P < 0.01$.

Histochemistry

Muscles were frozen by submersion in melting isopentane in a slightly stretched condition. Transverse, serial cryo-sections (10 μm) were prepared, and histological analyses of MyHC fibre type, β-galactosidase and CSA were determined 14 days after electroporation essentially as described previously (Ekmark *et al.* 2003; Lunde *et al.* 2007). MyHC type was determined using the monoclonal antibodies BA-D5 (I), SC-71 (IIa), and BF-35 (non-IIx) (Schiaffino *et al.* 1989) according to Table S2. Histochemical staining reflecting enzyme activity for succinate dehydrogenase (SDH) (Bancroft, 1975) and GPDH (Dubowitz, 1985) was performed and quantified as described previously (Lunde *et al.* 2007).

Statistics

Differences in mRNA expression or HIF-1α protein after stimulation were tested using a Wilcoxon's matched pairs test. Differences in MyHC-expression in C2C12 cells were tested using a Student's *t* test, and differences in fibre type distributions in muscle were tested using Fisher's exact test. Differences in protein levels were tested using a repeated measures one-way ANOVA with Bonferroni *post hoc* testing. For CSA, SDH and GPDH, average values of neighbouring control fibres were set to 100, and differences tested by a Mann–Whitney test. The level of significance was set to 5%.

Results

Fast and slow muscles display differences in the HIF-1 signalling system

We first measured the mRNA level for HIF-1α, HIF-1β, VHL protein and FIH-1 in the typical slow soleus and the typical fast EDL muscle. The HIF-1α mRNA level was almost 2-fold higher in the soleus compared to the EDL (Fig. 2*A*), while there were no significant differences in HIF-1β (Fig. 2*B*) or VHL protein (Fig. 2*C*). FIH-1 mRNA showed the opposite pattern of HIF-1α, being higher in EDL than in soleus (Fig. 2*D*). These observations suggest that HIF-1α signalling may be boosted in slow muscles at the transcriptional level.

The protein levels, however, did not correlate well with the mRNA levels. The HIF-1α protein level in nuclear extracts was 2.3-fold higher (Fig. 3*A* and *B*) and HIF-1β 4-fold higher (Fig. 3*A* and *C*) in the EDL compared to soleus. VHL, which contributes to HIF-1α breakdown, was 4.8-fold and 1.9-fold higher in the soleus nuclear and cytoplasmic fractions respectively (Fig. 3*A* and *D*). Thus, at the protein level the fast EDL showed multiple signs of stronger HIF-1α signalling than the slow soleus. As mRNA and protein levels of HIF-1α displayed reciprocal patterns,

the HIF-1α protein/mRNA ratio was actually more than 4-fold higher in the EDL than in soleus. These results suggest that there are major differences between the two muscles with respect to post-transcriptional regulation of HIF-1α signalling, i.e. more degradation through VHL in soleus.

HIF-1α levels are altered by activity in a pattern-dependent fashion

In order to investigate if HIF-1α is dependent on the pattern of electrical activity elicited in the muscle, we stimulated the sciatic nerve on one side of anaesthetised animals, with the contralateral leg serving as a resting control. Two different stimulation patterns were used: a 'slow' pattern mimicking slow type I motor units in the soleus (Hennig & Lømo, 1985), consisting of 20 Hz pulse trains each lasting 10 s and repeated every 30 s, and a 'fast' pattern consisting 150 Hz brief trains of 0.17 s (25 pulses) every 15 s. The fast pattern was designed based on the motorneuronal discharges observed in assumed type IIa or IIx motor units in the EDL (Hennig & Lømo, 1985). We have previously shown that these patterns over time will transform muscles in the slow and fast direction, respectively (Eken & Gundersen, 1988; Windisch *et al.* 1998).

In the fast EDL muscle the fast pattern increased the HIF-1α protein level by 80% compared to contralateral inactive muscles while the slow pattern reduced the levels by 44% after 6 h (Fig. 4). In the slow soleus muscle stimulation with the slow (native) pattern had no significant effect compared to inactive muscles, while a fast pattern on average raised the HIF-1α protein level by 82% (Fig. 4). These results show that HIF-1α protein levels are activity dependent in a highly pattern-specific fashion, such that HIF-1α signalling is increased during fast-patterned muscle activity and decreased during slow patterned activity.

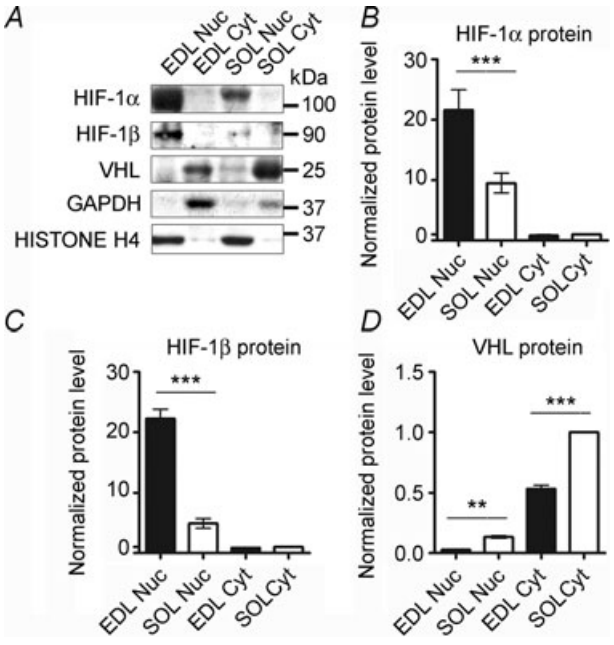

Figure 3. Protein level of the HIF-1 signalling system in the slow soleus and the fast EDL
Protein expression levels of components of the HIF-1 transcription factor system: HIF-1α (*A* and *B*), HIF-1β (*A* and *C*), and von-Hippel-Lindau (VHL) protein (*A* and *D*), in soleus (SOL) and EDL of six rats presented as representative immunoblots (*A*) and as means ± S.E.M. relative to the mean SOL cytoplasmic (Cyt) level (*B–D*). GAPDH and histone H4 protein levels were used to control the purity of Cyt and nuclear (Nuc) protein fractions (*A*). Differences in protein expression levels were tested using repeated measures one-way ANOVA and Bonferroni's *post hoc* test: $^{**}P < 0.01$; $^{***}P < 0.001$.

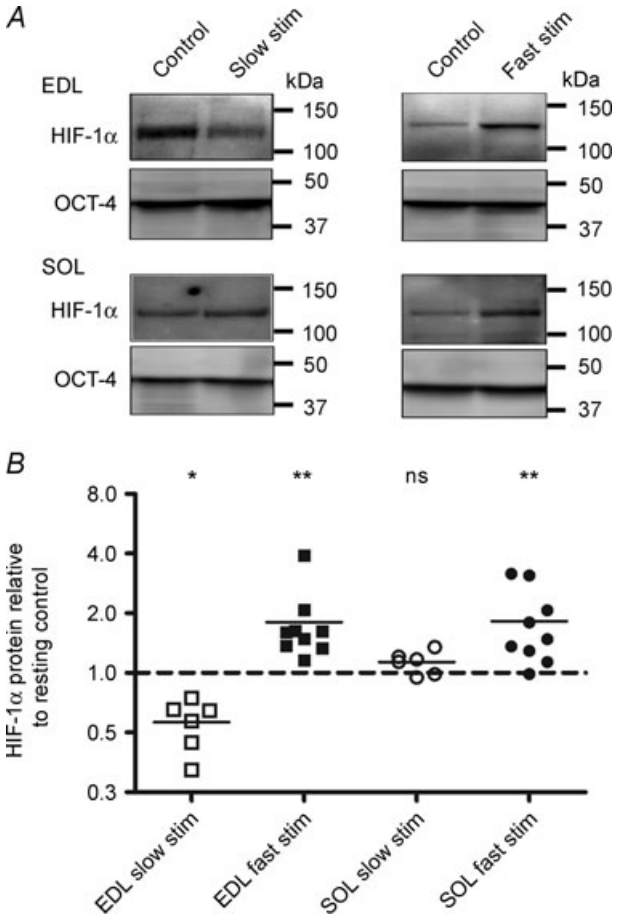

Figure 4. HIF-1α level is altered by activity in a pattern-dependent fashion
Nuclear protein level of HIF-1α in EDL and soleus (SOL) muscles of rats after slow-patterned and fast-patterned electrical stimulation for 6 h via the nerve, and in contralateral resting control muscles; presented as representative immunoblots (*A*) and as single data points relative to control muscles from the same rat plotted on a logarithmic scale (*B*). OCT-4 was used as loading control. $^{*}P < 0.05$; $^{**}P < 0.01$; statistically different from controls using the Wilcoxon's matched pairs test; ns: non-significant.

Overexpression of HIF-1α induces MyHC isoform changes in the fast direction

Since HIF-1α responded to activity patterns known to induce phenotypic changes in muscle, we investigated the effects of overexpressing HIF-1α in single muscle fibres 14 days after electroporation of an expression vector. First we investigated if the transcription factor could influence MyHC expression, i.e. the fibre type composition, by analysing neighbouring sections for the β-galactosidase reporter and with a battery of antibodies raised against various MyHCs (Fig. 5*A*).

The EDL is composed of almost exclusively type II fibres, and among the type II fibres, a shift in the direction of the fastest MyHC isoform was observed. Thus, the proportion of IIb fibres increased from 54% to 79% of the total population when comparing HIF-1α expressing fibres to normal controls. IIa and IIx fibres both diminished in frequency (Fig. 5*B*).

In the soleus, HIF-1α overexpression resulted in an increase in type II fibres at the cost of type I, as type IIa and I/IIa hybrids increased from 11% to 27% of the total fibre type population. The frequency of pure IIa fibres was tripled from 3% to 9% and hybrids more than doubled (from 8% to 18%) (Fig. 5*C*).

To investigate if HIF-1α could act in a cell autonomous fashion and at the transcriptional level, we transfected C2C12 myotubes with the same HIF-1α construct that we used *in vivo*. Differentiated myotubes were analysed with respect to mRNA for MyHC isoforms. Both developmental and adult limb MyHC isoforms were investigated. The developmental isoforms dominated, with embryonic and neonatal MyHC constituting 88% and 3% of the total MyHC expression, respectively. The remaining 9% was adult isoforms. HIF-1α did not influence the ratio between developmental and adult isoforms, but when the relative distribution of adult isoforms was analysed, an increase in IIb expression and a reduction in type I was observed (Fig. 6). This is in agreement with the immunohistochemical observations *in vivo*.

Collectively, these *in vitro* and *in vivo* experiments indicate that HIF-1α induces MyHC isoform transitions in the fast direction at the transcriptional level.

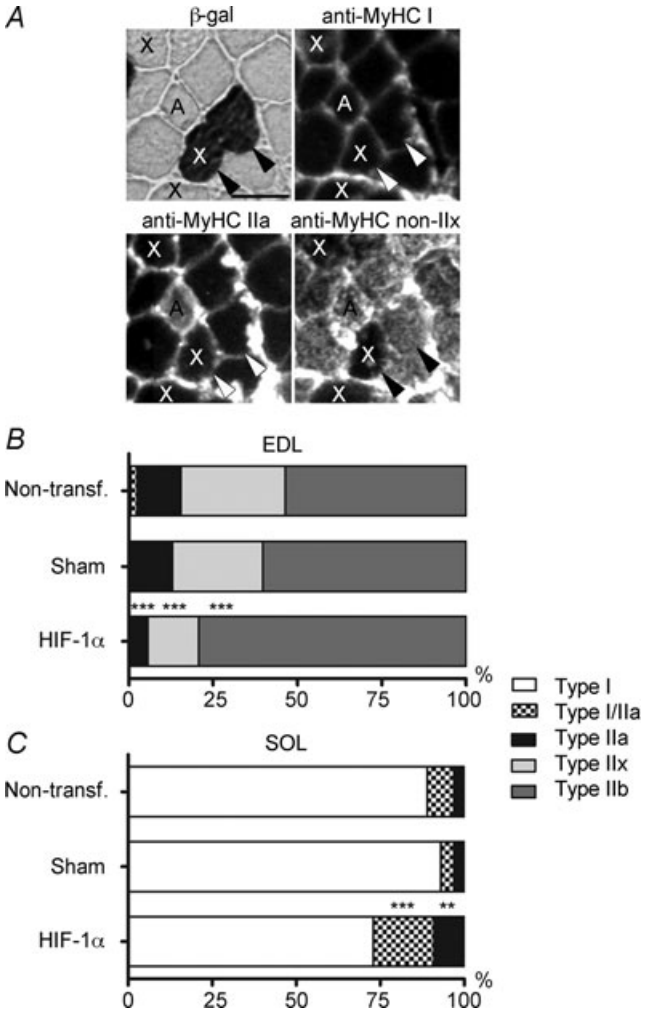

Figure 5. HIF-1α induces MyHC fibre type transformation in the fast direction
The identification of transfected fibres and fibre type is illustrated on transverse serial sections of the EDL muscle (*A*). Transfected fibres (arrows) were detected 14 days after transfection by histochemical β-galactosidase staining (β-gal) yielding a dark reaction product (*A*, upper left panel). MyHC fibre type was determined by immunofluorescence using monoclonal antibodies against MyHC I, IIa and non-IIx (*A*, upper right and lower panels). Type IIa and IIx fibres are marked A and X, respectively, while IIb fibres are unmarked. Scale bar is 50 μm. Fibre counts are given as distributions (%) of 1090 fibres from 10 different EDL muscles (*B*), and 706 fibres from eight different soleus (SOL) muscles (*C*). Data are given for HIF-1α -transfected, sham-transfected and neighbouring non-transfected fibres. Statistical differences from non-transfected fibres were tested using Fisher's exact test: $^{**}P = 0.0013$, $^{***}P \leq 0.0008$. There were no significant differences between the sham and non-transfected distributions.

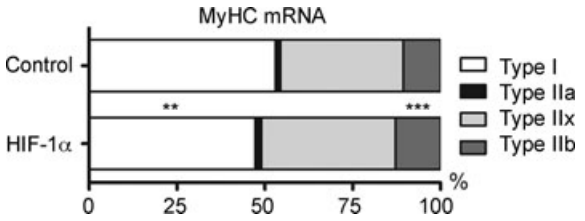

Figure 6. HIF-1α induces fast MyHC expression in C2C12 cells
C2C12 myotubes were either co-transfected with HIF-1α expression vector or the empty vector as a control, and firefly luciferase. The relative mRNA expression (see Methods) for the four adult limb MyHC isoforms are presented as a result of six parallel experiments 72 h after initiation of differentiation. Luciferase mRNA level was used to normalise for transfection. Differences in distribution of the MyHC isoforms were tested using Student's *t* test: $^{**}P < 0.01$, $^{***}P < 0.001$.

Overexpression of HIF-1α leads to reduced oxidative capacity

We next investigated if the change in MyHC fibre type in the fast direction was accompanied by the lower oxidative capacity normally characterising such fibres. The level of succinate dehydrogenase (SDH), a key enzyme in oxidative metabolism, was indeed shown to be markedly lower in IIb fibres of the EDL than type I, IIa and IIx (Fig. 7*A*). In HIF-1α transfected fibres, SDH activity was on average reduced by 25% compared to non-transfected fibres (Fig. 7*B*). This could, however, reflect the increased proportion of IIb fibres among the HIF-1α transfected fibres. Hence we investigated the SDH level of the different fibre types separately. Limiting the analysis to HIF-1α-transfected IIb fibres, we found a 17% decrease in SDH activity (Fig. 7*A*, *C*) compared to non-transfected, neighbouring fibres of the same type, while there were no significant differences for the other fibre types. Thus, HIF-1α seemed to induce a reduction in SDH activity in muscles not only by changing fibre type, but also by attenuating SDH activity of IIb fibres.

In the soleus, no significant reduction in SDH activity was found when all HIF-1α-transfected fibres were considered together (Fig. 7*D*), nor when considering type I or IIa separately. However, the I/IIa hybrids that constituted 18% of the HIF-1α expressing population, and only 8% of the control population, displayed a 15% reduction in SDH activity (Fig. 7*E*).

In summary, although the effects of HIF-1α on oxidative capacity varied among different fibre types, HIF-1α appeared to be able to reduce muscle SDH activity not only by changing fibre type but also by changing the enzyme levels within specific fibre types.

Glycolytic fibres have been shown to have not only lower levels of oxidative enzymes, but also higher levels of enzymes related to non-oxidative metabolic pathways. These tend to be reduced during fast-to-slow transformations (Hintz *et al.* 1980; Henriksson *et al.* 1986; Hughes *et al.* 1999). We therefore investigated if α-glycerophosphate dehydrogenase (GPDH) would be increased during our slow-to-fast transformations. For normal fibres, we confirmed that the enzyme activity is higher in glycolytic fibres (Fig. 8*A*). HIF-1α over-expression had, however, no significant effect overall (Fig. 8*B* and *C*), nor when each fibre type was analysed separately.

Overexpression of HIF-1α leads to hypertrophy in the EDL muscle

In EDL the HIF-1α transfected fibres were on average 28% larger than internal control fibres (Fig. 9*A*). This was partly due to transformation into IIb fibres, which is normally a larger fibre type (Ekmark *et al.* 2003). When analysing CSA of the different fibre types separately, IIb fibres were on average 16% larger than non-transfected control fibres of the same type (Fig. 9*B*). There were no significant effects on size for the other fibre types in EDL.

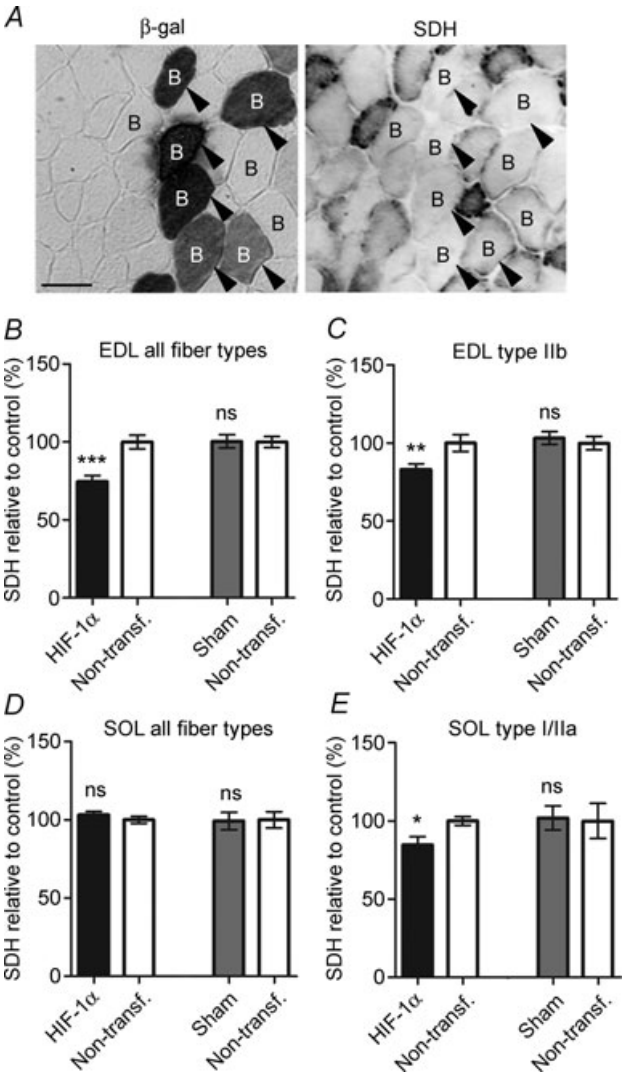

Figure 7. HIF-1α reduces SDH activity of IIb fibres in the EDL and I/IIa hybrids in the soleus after *in vivo* transfection
Succinate dehydrogenase (SDH) activity level was visualised by histochemical staining of muscle fibres on transverse serial sections after 14 days of HIF-1α overexpression (*A*–*E*). In *A*, transfected IIb fibres in EDL, identified by positive β-galactosidase (β-gal) staining (arrows), were observed to have weaker staining than neighbouring control type IIb fibres (marked *B*). Scale bar is 50 μm. For quantification, SDH activity was normalised to non-transfected neighbouring fibres for each muscle section and MyHC fibre type. Data are given as means ± S.E.M. of 791 and 522 fibres from ten and eight EDL and soleus (SOL) muscles, respectively, and are shown for all fibre types pooled (*B* and *D*), and for the specific fibre types (*C* and *E*). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$: statistical difference from non-transfected neighbouring fibres in a Mann–Whitney test; ns: non-significant.

In the soleus, there were no significant effects on size when comparing all the HIF-1α transfected fibres to controls (Fig. 9*C*), nor when type I and IIa fibres were analysed separately. HIF-1α transfected I/IIa hybrid fibres, however, were found to be on average 17% smaller than I/IIa control fibres (Fig. 9*D*). The reduction in these fibres might seem paradoxical, but in the rat soleus the IIa fibres are actually smaller and more oxidative than type I fibres in violation of the general rule that fast fibres are larger (Delp & Duan, 1996; Nakatani *et al.* 1999). Thus, both in the soleus and in the EDL, the changes in size seem to follow the change in MyHC leading to changes in opposite directions in the two muscles.

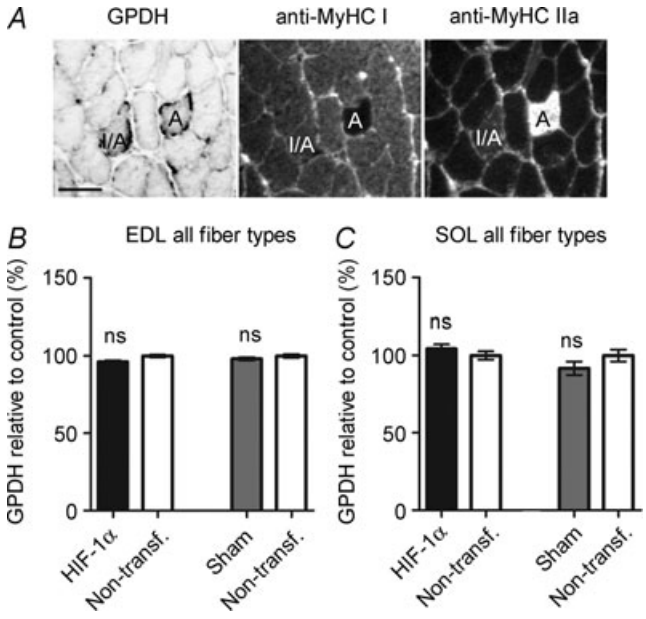

Figure 8. HIF-1α does not regulate GPDH activity after *in vivo* transfection
After 14 days of HIF-1α overexpression α-glycerophosphate dehydrogenase (GPDH) activity level was visualised by histochemical staining of muscle fibres on transverse serial sections. IIa positive fibres are marked A; one of these was a I/IIa hybrid also positive for type I MyHC, and thus marked I/A. The remaining unmarked fibres were positive for MyHC type I only. Scale bar is 50 μm. The GPDH staining was quantified in HIF-1α-transfected, sham-transfected and neighbouring non-transfected fibres, and given as means ± S.E.M. of 796 and 582 fibres from ten and eight different EDL (*B*) and SOL (*C*) muscles, respectively, and is shown for all fibre types pooled (*B* and *D*). GPDH activity was normalised to non-transfected neighbouring fibres for each muscle section and MyHC fibre type. ns: not significantly different from neighbouring fibres in a Mann–Whitney test.

Discussion

HIF-1α is a link between fast-patterned activity and a fast muscle phenotype

While there is a wealth of information on signalling related to endurance and endurance training, signalling coupled to strength training and shifts in the fast direction has long remained more obscure (Gundersen, 2011). The present data show that the hypoxia-regulated transcription factor HIF-1α is dependent on the pattern of muscle activity. Thus, when a pattern suited to trigger a phenotype change in the fast direction (Eken & Gundersen, 1988) was delivered to the muscles, HIF-1α protein levels increased, while a pattern triggering a slow phenotype (Eken & Gundersen, 1988) reduced the levels. Our data also connect HIF-1α causally to the changes in phenotype since elevating the level of HIF-1α in adult rats *in vivo*, without altering activity, triggered a fast phenotypic programme encompassing MyHC fibre type, metabolic enzymes and size.

Interestingly, the microvascular P_{O_2} is lower in fast muscles compared to the slow soleus (McDonough *et al.* 2005). This is consistent with the higher HIF-1α levels we observe in the EDL, and thus HIF-1α may play a role in maintaining normal properties in fast muscles. Genetic evidence supports such ideas in humans, since in our species a Pro582Ser polymorphism with increased HIF-1α transactivating ability (Tanimoto *et al.* 2003) is linked to a higher percentage of fast fibres (Ahmetov *et al.* 2008).

One of the striking features of activity-regulated changes in muscle properties is the strict dependence of pattern (Westgaard & Lømo, 1988; Gundersen & Eken, 1992; Gundersen, 1998). If hypoxia is a major mediator of activity effects in muscle the signalling system would be required to decode such patterns, and the HIF-1α pathway has been shown to be dependent on the pattern of hypoxia. For cells in culture, intermittent hypoxia (IH) (for example 1.5% oxygen for 30 s followed by 20% normoxia for 4–5 min) is much more potent in activating HIF-1α than continuous hypoxia of the same duration and average severity (Yuan *et al.* 2005, 2008; Nanduri & Nanduri, 2007; Nanduri *et al.* 2009). Moreover, while the elevated level of HIF-1α induced by continuous hypoxia is reduced after just a few minutes of recovery in normoxia, after IH HIF-1α levels remain virtually undiminished for at least 90 min after recovery (Yuan *et al.* 2008). This persistence is related to IH not only stabilizing HIF-1α (as described in the introduction), but to IH, as opposed to continuous hypoxia, also stimulating the synthesis of HIF-1α (Yuan *et al.* 2008).

In agreement with our findings, a HIF-1α knockout mouse displays a shift towards a more oxidative metabolism (Mason *et al.* 2004, 2007), while PHD1 deficient mice (expected to have higher HIF-1α levels) display a less oxidative metabolism (Aragones *et al.* 2008).

These animals failed to show any major shift in MyHC type although a minor shift in the slow direction, from IIa to I, was observed in the soleus of HIF-1α knockouts. Furthermore, these mice did not display any differences in single muscle fibre force suggesting that there was no change in size. Transgenic animals like these, however, are not perfectly suited to illuminate adult muscle plasticity since the gene is knocked out also during development, something that can trigger compensatory mechanisms or cause developmental perturbations. Our transgene product appeared *de novo* in a subset of normal muscle fibres in adult animals, which is a model more relevant for exercise effects.

HIF-1α signalling is related to other signals involved in muscle plasticity

There has been little information about pathways coupled to strength training and shifts in the fast direction (Gundersen, 2011), and it has even been suggested that fast properties are a default state, occurring in the absence of slow signalling activity (Butler-Browne *et al.* 1982; Esser *et al.* 1993; Jerkovic *et al.* 1997). Indeed, the transcription factor nuclear factor of activated T-cells c1 (NFATc1) that is activated by slow stimulation has been established as an inhibitor binding directly to a fast gene promoter (Rana *et al.* 2008), but specific effects of fast activity have been described as well (Rana *et al.* 2005), specifically the MyoD (Ekmark *et al.* 2007) and Six1/Eya1 (Grifone *et al.* 2004) transcription factors have been shown to activate a fast phenotypic programme in adult animals. Both HIF-1α and MyoD are bHLH transcription factors and may share co-factors, such as for instance the E- and Id-proteins that are positive and inhibitory binding partners, respectively, for bHLH molecules. These partners are regulated by activity in muscle (Gundersen & Merlie, 1994; Carlsen & Gundersen, 2000), and interestingly, Id-1 has been shown to induce atrophy (Gundersen & Merlie, 1994). However, in cancer cells Id-1 seems to stabilize HIF-1α (Lee *et al.* 2006; Kim *et al.* 2007).

Although HIF-1α is regulated by the pattern and severity of hypoxia, and it seems likely that both these factors are activity dependent, the present data provide no direct evidence that anoxia *per se* is regulating muscle phenotype. HIF-1α is regulated also by oxygen-independent mechanisms, and it is conceivable that activity-regulated factors other than hypoxia might be important. Thus, HIF-1α synthesis is regulated by the PI(3)K/Akt/mTOR pathway (Semenza, 2009), a pathway crucial for use-related hypertrophy (Bodine *et al.* 2001). Our findings suggest that HIF-1α could act downstream of this pathway and contribute to hypertrophy. Furthermore, muscle activity elicits calcium transients, and calcium could activate calcineurin, which has been suggested to increase HIF-1α signalling (Semenza, 2009). However, it seems improbable that this mechanism should dominate in the context of muscle activity since calcineurin is thought to be stimulated by slow-patterned activity, and to activate a slow muscle programme (Gundersen, 2011).

Is muscle hypoxia and HIF-1α signalling important during exercise and in disease?

Genetic data suggests that HIF-1α signalling is important for athletic performance in humans. Thus, the hyperactive Pro582Ser HIF-1α polymorphism occurred twice as often in a group of weight lifting athletes compared to the control group (Ahmetov *et al.* 2008).

In agreement with our findings in the soleus it has been shown that HIF-1α levels are increased in this muscle

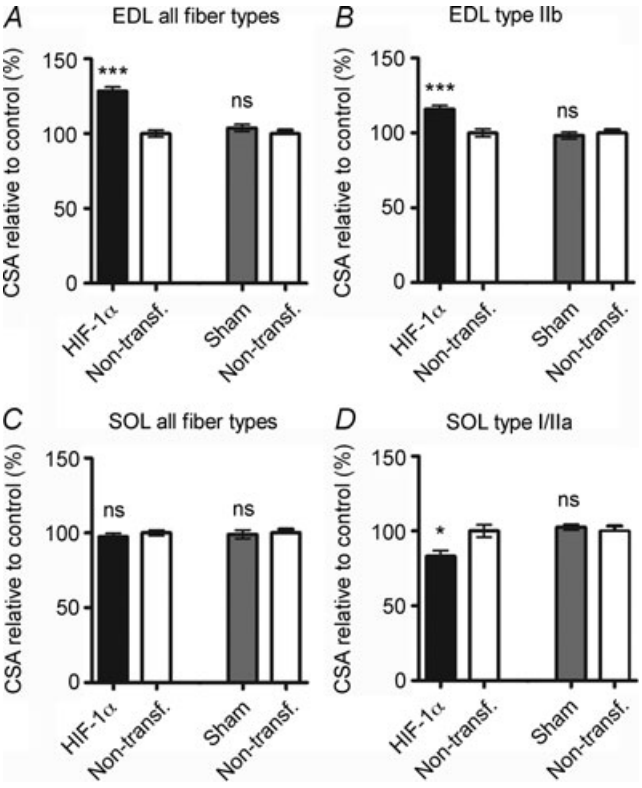

Figure 9. HIF-1α induces hypertrophy of IIb fibres in the EDL and atrophy of I/IIa hybrids in the soleus after in vivo transfection
Cross sectional area (CSA) of HIF-1α-transfected, sham-transfected, and non-transfected neighbouring fibres is presented as means ± SEM. Data is given for a total of 964 and 660 fibres from ten and eight different EDL and soleus (SOL) muscles, respectively, and is shown for all fibre types pooled together (*A* and *C*) and for the specific fibre types being significantly different 14 days after HIF-1α transfection (*B* and *D*). CSA was normalised to non-transfected neighbouring fibres of the same MyHC type for each muscle. $^{*}P < 0.05$; $^{***}P < 0.0001$ indicates statistically difference from neighbouring fibres in a Mann–Whitney test; ns: non-significant.

during hindlimb suspension. Under these conditions the fibres become faster, more glycolytic, and atrophic (Pisani & Dechesne, 2005), similar to what we observe when overexpressing HIF-1α. On the other hand, a bout of endurance-type exercise seems to increase HIF-1α protein levels in human muscles (Ameln *et al.* 2005), but to our knowledge the effect of different types of training on fast and slow muscles has not been compared with respect to HIF-1α signalling.

Several studies suggest that hypoxia *per se* is involved in muscle plasticity. The effects of ischaemic strength training were reviewed recently (Wernbom *et al.* 2008). In general it has been shown that the effects of low intensity strength training are boosted by applying a tourniquet. It has even been shown that intermittent ischaemia without concurrent activity can counteract disuse atrophy in patients (Takarada *et al.* 2000; Clark *et al.* 2006; Kubota *et al.* 2008). Thus, a relationship between ischaemia and prevention of muscle wasting seems to be established in a clinical context, and our data suggest that interfering directly with the HIF-1α pathway should be explored as an avenue for clinical interventions.

Patients with metabolic syndrome are characterized by physical inactivity, obesity, cardiovascular disease and type 2 diabetes (Grimaldi, 2005). Such individuals have muscles with reduced oxidative capacity, increased glycolytic capacity and a high percentage of fast muscle fibres (Tanner *et al.* 2002). Similarly, it is well established that patients with chronic obstructive pulmonary disease (COPD) display a slow-to-fast shift in muscle fibre type (Satta *et al.* 1997; Jobin *et al.* 1998; Whittom *et al.* 1998; Maltais *et al.* 1999; Gosker *et al.* 2002; Green *et al.* 2009). Such patients are exercise intolerant, and the muscle changes could simply be related to inactivity caused by the breathing problems, but it has also been suggested that hypoxia *per se* might influence the changes in muscle phenotype (reviewed in Wust & Degens, 2007). Our findings suggest that hypoxia might be a causative factor for the muscle changes in the fast direction. Thus, activation of the HIF-1α signalling system in muscle might be a common factor of COPD and metabolic syndrome. Both diseases are becoming increasingly common, and are projected to be among the most prevalent causes of morbidity and death in the near future (Murray & Lopez, 1997). HIF-1α might provide a link between these morbidities.

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Author contributions

I.G.L., J.C.B., S.E. and K.G. contributed to conception and design, analysis and interpretation of data. S.L.A. and Z.A.R. contributed to analysis and interpretation of data. K.G. drafted the paper with the help of I.G.L. and all the authors contributed by revising it critically for important intellectual content. All authors approved the final version to be published. The experiments were performed in K.G.'s laboratory at the University of Oslo.

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