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Transitions Towards Either Slow-Oxidative or Fast-Glycolytic Phenotype Can Be Induced in the Murine WTt Myogenic Cell Line

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ABSTRACT

Contraction and energy metabolism are functions of skeletal muscles co-regulated by still largely unknown signals. To help elucidating these interconnecting pathways, we are developing new cellular models that will allow to control the switch from a neonatal to an adult slow-oxidative or fast-glycolytic phenotype of myofibers, during in vitro differentiation. Thus, our purpose was to direct the differentiation of the newly characterized WTt clone, from a mixed towards either fast or slow phenotype, by modifying amounts of two transcription factors respectively involved in control of glycolytic and oxidative energy metabolism, namely HIF-1α and PPARδ. Our data support the idea that HIF-1α protein stabilization would favor expression of fast phenotypic markers, accompanied or not by a decreased expression of slow markers, depending on treatment conditions. Conversely, PPARδ over-expression appears to enhance the slow-oxidative phenotype of WTt myotubes. Furthermore, we have observed that expression of PGC-1α, a coregulator of PPAR, is also modified in this cell line upon conditions that stabilize HIF-1α protein. This observation points to the existence of a regulatory link between pathways controlled by the two transcription factors HIF-1α and PPARδ. Therefore, these cells should be useful to analyze the balance between oxidative and glycolytic energy production as a function of phenotypic transitions occurring during myogenic maturation. The newly characterized murine WTt clone will be a good tool to investigate molecular mechanisms implicating HIF-1α and PPARδ in the coordinated metabolic and contractile regulations involved in myogenesis. J. Cell. Biochem. 111: 82–93, 2010.

KEY WORDS: MYOBLASTS; MUSCLE DIFFERENTIATION; METABOLIC PHENOTYPE; PPARδ; HIF-1α

During skeletal muscle development or repair, muscle fibers of slow-oxidative or fast-glycolytic types are formed. The mechanisms responsible for these phenotypic determinations are not completely understood. Environmental conditions as diverse as oxygen supply, extra cellular matrix components, signaling molecules and innervation, influence these differentiation processes [for review, see Hawke and Garry, 2001; Wozniak et al., 2003]. Satellite cells are quiescent myoblasts, located between the sarcolemma and basal lamina of skeletal muscle fibers and play a major role in striated muscle growth and repair [Campion, 1984]. In order to obtain insights into the mechanisms of coordinated regulations involved in normal or pathological skeletal muscle differentiation processes, we have chosen to develop new murine cell lines able to differentiate into myofibers expressing adult contractile markers in culture conditions. Such a cellular model is lacking as rodent muscle cell lines or primary cultures of satellite cells do not reach differentiation stages with expression of adult contractile markers [see references in Peltzer et al., 2008]. Therefore, muscle clonal cell lines were derived from satellite cells of the transgenic H-2kb-tsA58 mouse also called immortomouse [Morgan et al., 1994]. These cells will develop a sarcomeric apparatus expressing fast and/or slow adult myosin heavy chain (MHC) isoforms in culture, contrary to commonly used rodent cell lines [Miller et al., 2003; Peltzer et al., 2008]. We have shown that clones derived from slow-oxidative muscles will differentiate preferentially into myotubes of slow contractile phenotype when submitted to optimal culture conditions [Peltzer et al., 2008]. One cell line derived from newborn satellite cells, named WTt clone, has been analyzed in more details, using markers previously developed for rodent in vivo studies dealing with coordinated modifications that take place in striated muscles [Hourdé et al., 2005; Noirez et al., 2006]. Culture conditions favoring in vitro WTt cell maturation have been defined [Peltzer et al., 2008]. We have demonstrated that in vitro differentiation of WTt cells yields myotubes expressing adult MHC isoforms, including the slow

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MHC1 and the fast MHC2b. Therefore, the WTt myogenic clone differentiates into a mixed phenotype. As it is derived from neonatal satellite cell, it appears as an adequate model for our attempts to mimic in vitro the ontogenetic choices leading to maturation towards either fast-glycolytic or slow-oxidative phenotype.

In order to direct the differentiation of the WTt clone towards a more homogeneous fast-glycolytic or slow-oxidative phenotype, we have modified the amounts of two transcription factors involved in control of the glycolytic and oxidative energy metabolism respectively. Our working hypothesis was that modifying pathways involved in energy metabolism would induce modifications of contractile protein isoform expressions, in a coordinated manner, similar to what occurs in the animal [Hourdé et al., 2005]. The two transcription factors chosen for this study were: HIF-1α controlling expressions of many glycolytic enzymes, and PPARδ known to control the balance of oxidative energy metabolism. Results presented here are in keeping with our working hypothesis.

THE HYPOXIA-INDUCIBLE FACTOR 1 ALPHA (HIF-1α)

HIF-1α is a heterodimeric transcription factor, discovered for its role in adaptation of cells to hypoxic stress [Semenza, 1999]. During hypoxia, cytoplasmic HIF-1α is imported into the nuclei, where it participates in a DNA binding complex, interacting with the hypoxia response element (HRE) of responsive genes. Responsive genes include those encoding erythropoietin, VEGF and several glycolytic enzymes, among them enolase [Semenza et al., 1996; Discher et al., 1998; Rouet et al., 2005]. Indeed, adaptation to hypoxia involves a transition from energy production by mitochondrial oxidative pathway to that produced by anaerobic glycolysis [Hoppeler et al., 2003]. In normal conditions, the HIF-1α protein is very unstable. Hypoxic signals induce protein stabilization, which will then allow the HIF-1α protein to bind HRE on target genes. In accordance with this mechanism, we have attempted to stabilize the HIF-1α protein in the WTt myogenic clone, with the idea that the resulting enhanced glycolysis might induce coordinated regulations, resulting in a transition of MHC contractile proteins towards the fast isoforms. We have treated the WTt cells with cobalt chloride ($CoCl_2$), which is known to stabilize HIF-1α protein and is commonly used to mimic hypoxic stress in cultivated cells [Itoh et al., 2001; Pisani and Dechesne, 2005]. Our data indicate that $CoCl_2$ treatment does induce changes in the expression of the contractile MHC isoforms, which differ depending on $CoCl_2$ duration of treatment. Our results strongly suggest that HIF-1α might regulate fast-glycolytic and slow-oxidative myofiber phenotypes through independent pathways.

THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR DELTA (PPARδ)

Strong evidence indicates that the peroxisome proliferator-activated receptors (PPARs) function to mediate adaptive metabolic responses to changes in systemic fuel availability [Muoio et al., 2002]. We have focused our studies on one member of this family, PPARδ which is detected in all tested tissues, including skeletal muscles. PPARδ is likely involved in lipid metabolism, since fatty acids activate this receptor [Kliewer et al., 2001]. Furthermore, muscle-specific over-expression of PPARδ in transgenic mice results in an increased expression of genes implicated in oxidative metabolism and of the corresponding enzymatic activities [Luquet et al., 2003; Wang et al., 2004]. Analyses revealed a profound change in muscle fiber composition of these animals, due to hyperplasia and /or shift to more oxidative myofibers. As fibers had become more fatigue-resistant, these mice were also named ''marathon-mice'' [Luquet et al., 2003; Wang et al., 2004]. These results suggested that PPARδ might play an important role during muscle development, maturation and specification of myofibers towards the slow-oxidative phenotype. In addition, the PPAR gamma co-activator 1 alpha (PGC1-α) is known to interact with all PPARs and is expressed in several tissues including skeletal muscle where it is responsible for activation of oxidative metabolism [Lin et al., 2002, 2005]. In the present study, we have over expressed PPARδ by retroviral infection of the WTt clone, in order to investigate whether this treatment would induce a transition towards a slow-oxidative phenotype of muscle cells in vitro, similar to what had been observed in striated muscles of transgenic animals [Luquet et al., 2003; Wang et al., 2004]. Data presented here support this hypothesis.

Taken together, results obtained in this study support the idea that both PPARδ and HIF-1α transcription factors are major players in controlling coordinated regulations of metabolic and contractile pathways of striated muscle cells, and that the original myogenic cell line, WTt, represents a convenient tool for deciphering the underlying molecular mechanisms.

MATERIALS AND METHODS

CULTURE CONDITIONS FOR THE WTt MYOGENIC CLONE

We have previously described the optimal culture conditions for WTt cells [Peltzer et al., 2008]. Briefly, cells were grown on plastic coated with Matrigel (BD Biosciences) diluted in DMEM at 2 mg/ml. They were grown at 33°C in proliferation medium containing 20 U/ml of γ interferon (Euromedex) until reaching 90% confluence. Cells were then incubated at 37°C for 24 h, without medium change. Medium was then replaced by differentiation medium (DMEM containing 1 g/L glucose, 1% penicillin/streptomycin and 4% horse serum (HS) from PAA). Cells were then kept differentiating up to 10 days with medium changes every 2 days. All sets of kinetic experiments, corresponding to various treatments, were performed at least in duplicate, including controls. For each kinetic experiment, assays were performed at least in duplicate.

$CoCl_2$ TREATMENT

$CoCl_2$ at 100 and 250 μM concentrations are commonly used in publications [Itoh et al., 2001]. In our hands, 100 μM of $CoCl_2$ allowed optimal differentiation of WTt cells. $CoCl_2$ was applied at the end of the proliferation stage for either 24 or 120 h. Cells were then collected at different times thereafter. Medium was complemented with $CoCl_2$ up to D4 in the case of 120-h treatment.

Cell viability was not affected following 4 days of treatment according to Trypan blue test. Briefly, WTt cells were cultivated for

4 days in 24-well plates with 100 or 250 μM $CoCl_2$. Assays were performed at least in duplicates, with 1,000 and 2,000 cells per well, using Trypan blue (Sigma) 0,2% in culture medium.

OVER-EXPRESSION OF PPARδ

PPARδ coding sequence was transduced into WTt cells using replication defective recombinant retrovirus. Virus was produced in the ecotropic packaging cell line BOSC23 [Pear et al., 1993]. BOSC23 cells at 50–70% of confluence were transfected using Fugene 6 (Roche Molecular Biochemicals) with 8 μg of either pBizeoneo empty vector or pBizeoneo-PPARδ retroviral vectors [Bastie et al., 1999]. After 48 h, supernatants were collected and used to infect WTt cells at 50% confluence in growth medium in the presence of 1 μg of Polybrene (Sigma). Six hours after infection, the medium was changed for fresh growth medium and cells were further incubated for 48 h. Infected WTt cells were selected in growth medium supplemented with 0.5 mg/ml geneticin (Invitrogen). WTt cells were always maintained at 33°C. Thirty PPARδ overexpressing and thirty control (empty vector) clones were isolated. Detailed results are shown for one clone of each group.

PROTEIN EXTRACTION AND ANALYSIS

Cell pellets were recovered from dishes by scraping and kept frozen until protein extraction. Total protein extracts were treated for analysis by Western blot as previously described [Peltzer et al., 2008]. In order to enable direct comparison, extracts from treated and control cells were deposited on the same polyacrylamide gel. For the sake of simplicity, only parts of the blots are shown in the final figures. Original results are shown in supplementary material. Polyclonal and monoclonal antibodies directed against enolase and MHC isoforms were as described [Peltzer et al., 2008]. Rabbit polyclonal antibody directed against HIF-1α was a gift from J. Pouyssegur. Similar results were obtained with a commercial antibody (sc 10790 from Santa Cruz biotechnology). Membranes were then incubated with relevant secondary antibodies conjugated with peroxidase (Bio-Rad). Immunoreactivity was detected by chemiluminescence using ECL kit (Roche); for semi-quantitative analysis, signal intensity of bands was quantified using the gel analyzer function of the ImageJ software [Rasband W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA, http: //rsb.info.nih.gov/ij/, 1997–2009]. Data are expressed relative to an internal standard sample, as indicated in legend to figures.

IMMUNOCYTOCHEMISTRY

Cells were fixed in 2% formaldehyde in PBS for 20 min and then permeabilized using 0.1% Triton X-100 for 4 min. All-MHC (MY32), MHC1 (Novocastra) and PGC-1α H300 (Tebu-bio) antibodies were used at 1/1,000, 1/20 and 1/50 dilution, respectively. Fluorescent secondary antibodies were Alexa 488 conjugated anti-mouse IgG and Alexa 546 anti-rabbit IgG raised in donkey. Fluorescence images were acquired from a BH-2 Olympus microscope coupled to a Scion CFW1310 monochrome camera. Image processing and analysis were performed using the 3FluoLableling Exploring Tools for ImageJ available on line from the NIH web site [see Keller et al., 2007].

EXPRESSION OF TRANSCRIPTS: RT-qPCR METHODOLOGY

Total RNA was extracted from cells with RNA Insta-Pure System (Eurogentec) and treated with DNase as recommended by the provider (Ambion), and further treated as previously described [Peltzer et al., 2008]. Briefly, cDNA was synthesized from 2 μg total RNA by using Random Hexamer Primer and SuperScript II RNase H^- Reverse Transcriptase (both from Invitrogen). Gene expression was quantified by real-time PCR using the LightCycler Fast Start DNA Master (Roche) with 0.2 μl cDNA corresponding to 100 ng total RNA in a 20 μl final volume, 3 mM magnesium chloride and 0.5 μM of each primer (final concentration). PCR was performed for 45 cycles at 95°C for 15 s, at the specific annealing temperature for 25 s and at 72°C for 30 s. Amplification specificity was checked using a melting curve following the manufacturer's instructions. Specific gene primers for each enzyme or factor of interest were designed for real-time PCR analysis using Primer3, PrimerBank and Ensembl softwares, as previously described [Peltzer et al., 2008]. Only primers made in addition to those used in Peltzer et al. [2008] are shown in Table I. Results were analyzed with LightCycler software v.3.5 (Roche) using the second derivative maximum method to set the threshold cycle (CT). In order to obtain robust data, quantitative analysis was carried out using standard curves and normalization with the GeNorm software and methodology [Vandesompele et al., 2002], with four reference genes: TFIID, GAPDH, β actin and 18S ribosomal RNA.

RESULTS

HIF-1α STABILIZATION IN WTt CLONE INDUCES MODIFICATIONS TOWARDS A FAST-GLYCOLYTIC PHENOTYPE

It has been largely demonstrated that changes induced by hypoxia are mostly the consequences of HIF-1α protein stabilization [see Pouyssegur and Mechta-Grigoriou, 2006]. However, transcriptional regulation has also been reported [Hoppeler and Vogt, 2001; Pisani and Dechesne, 2005]. This prompted us to analyze HIF-1α changes at both protein and transcript levels, following the pseudo-hypoxic shock produced by $CoCl_2$ addition to medium. As indicated by Trypan blue tests, satisfactory WTt cell viability was maintained during the protocol chosen for $CoCl_2$ treatments (data not shown).

Effects of 100 μM $CoCl_2$ treatment for 24 h on WTt cell phenotypic markers. *Short $CoCl_2$ treatment induces a transient increase in HIF-1α expression.* It is difficult to visualize the HIF-1α protein in control cells, as it is normally unstable [Semenza, 1999]. However, in our control culture conditions, a transient accumulation at D3 and D4 of WTt cell differentiation allowed us to detect the protein at these stages (Fig. 1A, left). Following the short $CoCl_2$ treatment (performed for 24 h before differentiation), an increase in HIF-1α protein, was again visible, and again only in a transient fashion, at D3 and D4 in differentiation medium (Fig. 1A, right and Supplementary Fig.). Semi-quantitative analysis indicates that the levels of protein were increased by a factor 2.3 ± 0.3 in treated as compared to control cells. This increase of protein level was correlated with an earlier transient increase of HIF-1α transcript level, visible following the 24-h $CoCl_2$ treatment, at the time of placing cells in differentiation medium (D0) (Fig. 1B, left), but at no other time (not shown).

TABLE I. PCR Primers Used to Investigate Muscle Cell Differentiation

Gene	Reference or accession #	Sense	Anti-sense	Fragments	Size
MHC2a	NM144961	GGACCCACTGAACGAGACC	TGGCAGCTCCACCACTACTT	1803–1902	119
MHC2x	XM354615	TCACGCCAGCTAGACGAA	CGGGAGGACTGAAGAGCA	3979–4127	149
CS	BC029754	GAGAGGCATGAAGGGACTTG	CCTTAGGCAGCATTTTCTGG	15–121	107
HAD	NM178878	AGCAACACGAATATCACAGGAAG	CCACCGGCATCCAGAAGTT	345–566	241
Myokinase	NM_021515	ATGGCTTCCTGATCGACGG	GTCGTTGGGTCATGGTCTCG	311–414	123
PPARδ	NM011145[a]	TCCAGAAGAAGAACCGCAACA	GGATAGCGTTGTGCGACATG	622–702	80
pPPARδ	NM011145 and sequence plasmid Bizeoneo	TGCAGTGGCTAAAGAAGACG	CCGGTCGGTCCAGAACTC	33–306	274
PGC-1α	NM_008904	AGACGGATTGCCCTCATTTGA	TGTAGCTGAGCTGAGTGTTGG	303–449	167
HIF-1α	AF003695	TTGCCACTTCCCCACAATG	CCACCGGCATCCAGAAGTT	387–487	101

PCR primers corresponding to newly designed markers, in addition to those given in Peltzer et al. [2008]: adult MHC, citrate synthase (CS) and HAD correspond to oxidative metabolic enzymes; PGC-1α and PPARδ are transcription factors; pPPARδ: primers specifically recognizing PPARδ linked to the plasmid vector.
[a][Dressel et al., 2003] four reference genes always were used: TFIID, GAPDH, β-actin, and 18S [Peltzer et al., 2008].

Short $CoCl_2$ treatment induces transient changes in expression of metabolic enzymes. An increase in transcript expression of a direct target of HIF-1α, the ubiquitous isoform of a glycolytic enzyme, α enolase [Semenza et al., 1996], was observed at the same time (Fig. 1B, right). The corresponding increase in α enolase protein was visible only starting at day 5 of differentiation (Fig. 1C). Semi-quantitative analysis indicates that control values are multiplied by 1.8 ± 0.1 ($n = 4$) in the treated cells.

Quantitative RT-PCR analyses demonstrated a decreased expression of the oxidative enzymes, citrate synthase and HAD (Hydroxy AcylCoA Dehydrogenase), only at D0 of differentiation (Fig. 1D, left), together with the observed transient increase in HIF-1α transcript level reported above (Fig. 1B, left). At later times, no difference between control and treated cells was visible for these markers. At D3, we observed an increased expression of two muscle specific isoenzymes usually associated with glycolytic myofibers, the glycolytic enzyme β enolase (muscle-specific isoform) and the myokinase (Fig. 1D, right). Such increases were not detected at other times. Thus, we observed transient modifications consisting in a negative regulation of oxidative enzymes accompanied by a later activation of muscle-specific glycolytic enzymes.

Short $CoCl_2$ treatment induces an increased expression of fast MHC isoforms. We first examined whether we could observe expression of MHC2b protein, the marker of fast-glycolytic fibers, in WTt myotube extracts. Signals obtained with the specific antibody, following Western blotting, are weak, but they were clearly increased in the $CoCl_2$ treated myotubes, as compared to controls, especially at D3 and D11 (Fig. 2A and Supplementary Fig.). Semi-quantative analysis of the data indicates that control values are multiplied by 2.4 ± 0.4 ($n = 7$). Therefore, MHC2b protein level appears more than doubled following the 24-h $CoCl_2$ treatment.

Analyses of transcript accumulation for each MHC isoforms pointed to increased expression of the fast isoforms, as shown here at D10 of differentiation, and only for the fast MHC2x and 2b isoforms (Fig. 2B). No significant differences were observed either with the developmental (embryonic and neonatal) MHC isoforms (data not shown) or with the two isoforms associated to the slow oxidative myofibers, MHC1 and MHC2a.

In an effort to visualize whether fast MHCs accumulated at significantly higher levels in treated WTt myotubes, as compared to controls, we conducted immunocytochemical labeling of D10 differentiated cells. Best immunocytochemical data were obtained using MY32 antibody that recognizes all adult fast and the neonatal MHC isoforms. Following the 24-h $CoCl_2$ treatment, we observed a strikingly stronger labeling of myotubes at D10 of differentiation (Fig. 2C, bottom left panel). MHC labeling of control WTt (Fig. 2C, above left panel) was weak, but clearly above that obtained when omitting primary or secondary antibodies (data not shown).

These data demonstrate that, a short 24-h treatment of WTt cells with 100 μM $CoCl_2$, immediately prior to a switch to differentiation medium, induces transient changes in expression of HIF-1α and metabolic enzymes. This treatment appears to induce an accumulation of fast MHCs in treated WTt myotubes. It most probably corresponds to adult isoforms, as no change in transcript levels of developmental isoforms was observed. It was therefore interesting to determine the effects of applying a stronger pseudo hypoxic stress to the WTt cells.

Effects of 100 μM $CoCl_2$ treatment during 120 h on WTt cell phenotypic markers. *Long $CoCl_2$ treatment induces transient decrease in expression of oxidative enzymes.* As shown in Figure 3A, long $CoCl_2$ treatment had no effect on HIF-1α expression at later times, D3 and D10 of differentiation. However, at D1, corresponding to a 48-h treatment, $CoCl_2$ treatment decreased HIF-1α transcript amounts (Fig. 3A), by contrast to the short 24-h treatment that increased it at D0 (see Fig. 1B, left). The decrease in citrate synthase and HAD expression was less transient than in the short treatment, being now still visible at D3, although by D10 expression had returned to control levels (Fig. 3B).

Decreased expression of PGC-1α transcripts. The observed diminution of oxidative enzyme expression (Figs. 1D and 3B) prompted us to investigate the behavior of the transcription factor known to control oxidative metabolism, PGC-1α [Lin et al., 2002]. Our data show that the 120-h $CoCl_2$ treatment induced a rapid continuous decrease in PGC-1α transcript levels, as early as D1 (Fig. 3C), in contrast to the shorter 24-h treatment, which had no effect (data not shown). The decrease in PGC-1α was still visible at later times, up to D10 (Fig. 3C).

Long $CoCl_2$ treatment induces an increased expression of fast MHC isoforms associated to a decrease in slow MHC expression. Quantitative RT-PCR analysis revealed that the longer 120-h treatment induced an increased accumulation of transcripts, visible at D10 of differentiation, corresponding to the fast isoforms MHC2x and 2b isoforms, but no significant change in MHC2a expression (Fig. 4). It is worth noting that the accumulation of MHC2x is double that of

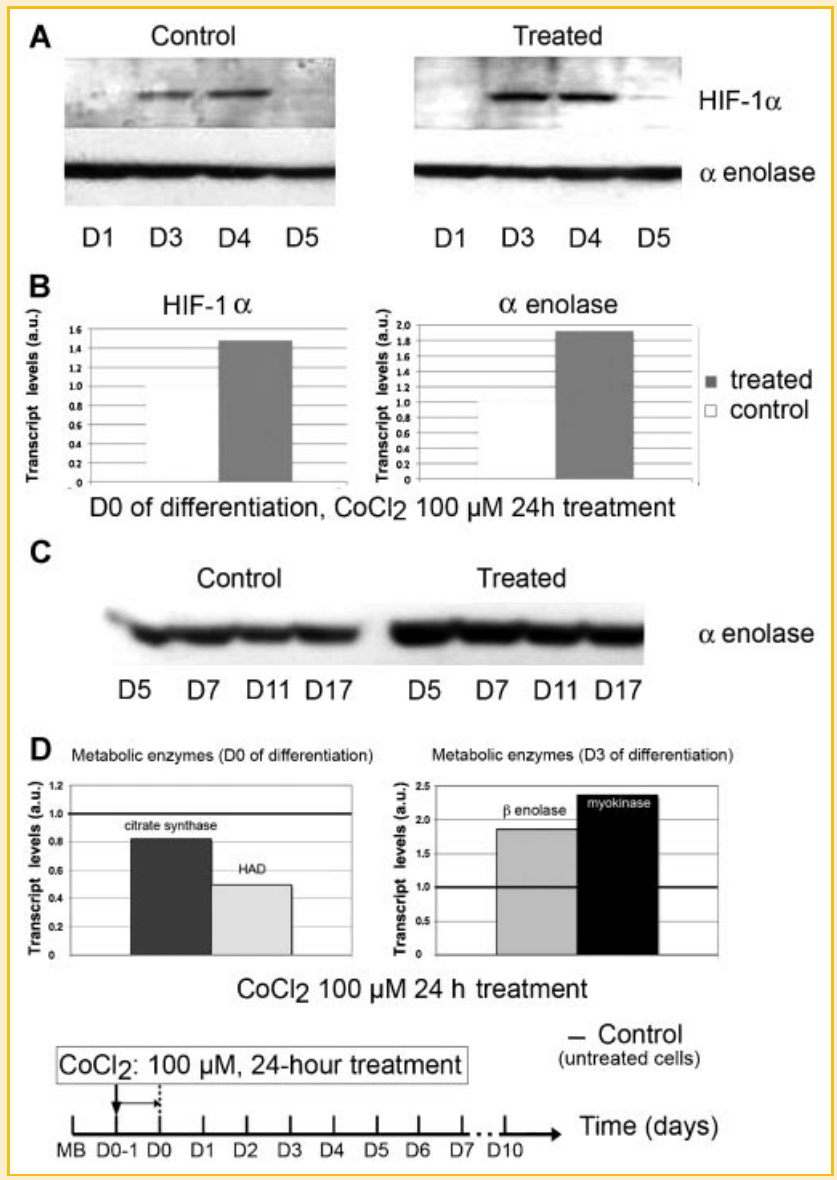

Fig. 1. Kinetic studies of HIF-1α and metabolic markers during WTt cell differentiation. A: HIF-1α protein expression following 24 h treatment with 100 µM $CoCl_2$. Western blots show HIF-1α expression (120 kDa) in WTt control (left) and in $CoCl_2$ treated cells (right). Fifteen micrograms protein were deposited per well; α enolase was used for comparison. Extracts were obtained from cells in differentiation medium for 1–5 days (D1–D5). B: HIF-1α (left) and α enolase (right) transcript levels at D0 of differentiation, in $CoCl_2$ treated and control (untreated) cells. Control level was set at 1 (white column). Transcript expressions were quantified by real time quantitative RT-PCR and expressed in arbitrary units (a.u.). C: Western blot showing α enolase protein expression. Control and treated cells were analyzed from 5 to 17 days of differentiation. Thirty micrograms protein were deposited per well. D: metabolic enzyme expressions following 24 h treatment with 100 µM $CoCl_2$. Left: results are shown at D0 of differentiation for the oxidative enzyme transcripts, CS and HAD. Right: results are shown at D3 of differentiation for enzyme transcripts specific of the muscle glycolytic phenotype, β enolase and myokinase. Control level was set at 1 and indicated by a black line. Timing of $CoCl_2$ treatment is indicated on the diagram at bottom of the figure.

MHC2b transcripts. By contrast, this stronger treatment induced an associated decrease in expression of the slow MHC1 isoform, which was not observed in the milder treatment (Fig. 2B). Similar MHC1 changes were observed at both transcript (Fig. 5A) and protein levels (Fig. 5B,C). This is shown here with cells treated with 100 µM, but was observed as well with 250 µM $CoCl_2$ treatment (data not shown). Decreased MHC1 expression had already occurred at D1 (the first examined time) with MHC1 transcript levels at least sevenfold lower than in controls. Thereafter, we could observe some increase as a function of time, without ever reaching control levels. Western blot analyses revealed parallel changes in the MHC1 protein. The strikingly decreased amounts of this slow MHC isoform were barely detectable at early times of treatment. Interestingly, although increased expression of MHC2b transcripts had been observed (Fig. 4A), no modification of the fast MHC2b protein was visible (Fig. 4B). Thus, the data obtained on MHC protein isoform

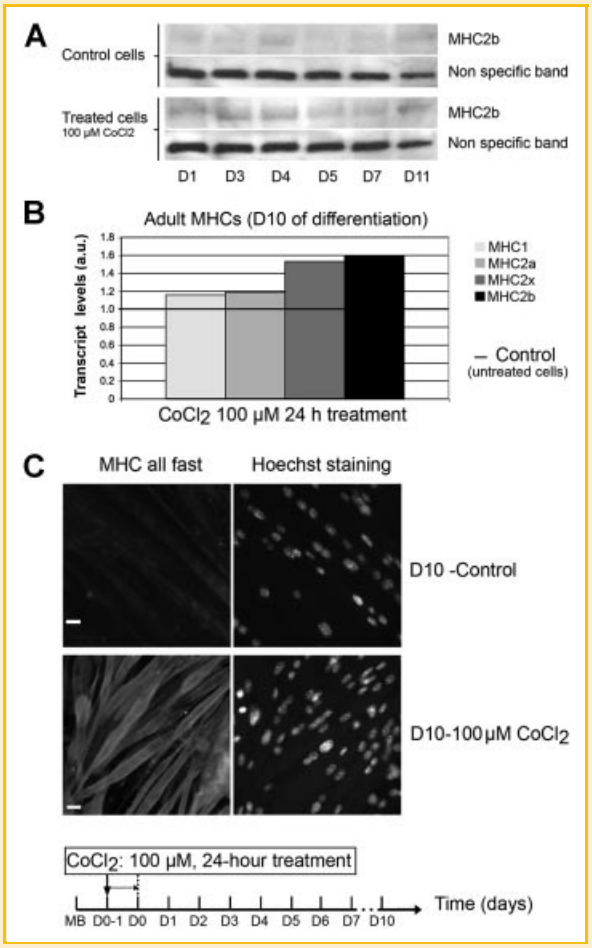

Fig. 2. Changes in expression of MHC isoforms following 24 h treatment with 100 µM $CoCl_2$. A: MHC2b expression as a function of time, respectively in control and treated cells; 30 µg of protein per well. The MHC2b isoform migrates as a 200 kDa protein; the antibody also reveals a non-specific band of 180 kDa, used for comparison [see Peltzer et al., 2008]. B: MHC transcript expressions (see legend to figure 1B) are shown at D10 of differentiation. Control (untreated WTt cells) is set at 1 (black line). C: Immunocytochemical analysis of untreated (Control, top line) and treated (100 µM $CoCl_2$, bottom line) WTt cells, at D10 of differentiation. Labeling was obtained using MY32 monoclonal antibody directed against all adult fast and neonatal MHC isoforms in green (left column), and Hoechst reagent for nuclei in blue (right column).

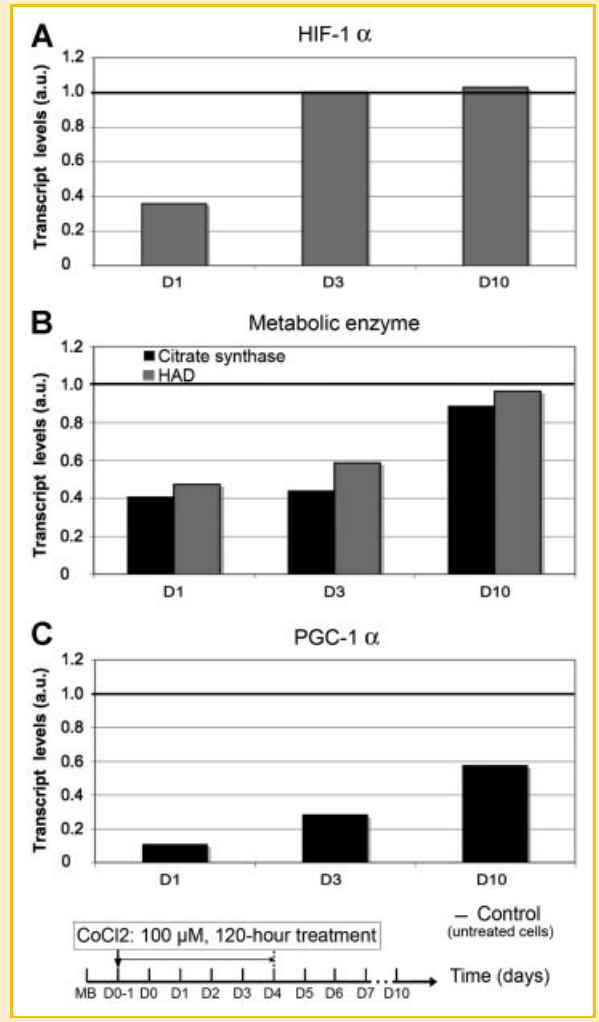

Fig. 3. Transcript expression of various markers in WTt cells treated with 100 µM $CoCl_2$ for 120 h. Expression of HIF-1 α (A), metabolic enzymes (B) and PGC-1 α (C), analyzed by real time quantitative RT-PCR. Legend as in Figure 2B.

expression show that the 120-h treatment induced a decrease in the slow contractile phenotype along with an increase in MHC2x and 2b transcript expression, whereas the 24-h treatment resulted only in an enhanced fast contractile phenotype.

In order to verify whether changes in transcript expressions were accompanied by similar changes of the corresponding proteins, immunocytochemical analyses were conducted. Immunolabeling data are shown using a fire color chart. They reveal that WTt cells submitted to the long treatment (Fig. 6, middle line) exhibited decreases in both PGC-1α and MHC1 protein accumulations as compared to the short $CoCl_2$ treatment (Fig. 6, top line).

Taken together, these results demonstrate that 120-h treatment of the WTt myogenic cells, with 100 µM $CoCl_2$, induces a number of coordinated changes, characterized by the reduced expression of oxidative enzymes, accompanied by decreased expressions of PGC-1α transcription factor and of slow MHC1 isoform, together with increased expressions of the fast isoforms MHC2x and MHC2b.

PPARδ OVER-EXPRESSION IN WTt CLONE INDUCES MODIFICATIONS TOWARDS A SLOW-OXIDATIVE PHENOTYPE

In the present experiment, WTt cells were infected by either a retrovirus transducing the PPARδ transcription factor or by the corresponding control retrovirus. Based on PPARδ protein expression, we have selected 30 clonal colonies for each construct (data not shown).

Changes in expression of PPARδ transcription factor and of metabolic markers as a function of differentiation time. Transcript expression was analyzed by quantitative RT-PCR in all experiments. Similar PPARδ expression data were obtained with any of the 30 clones of each group (containing empty vector or overexpressing PPARδ). Among the best differentiating clones, we have chosen one clone of each group for

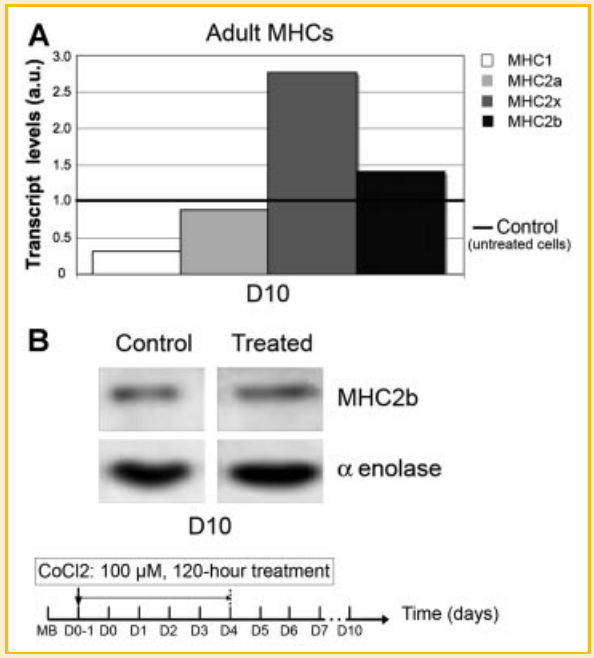

Fig. 4. MHC transcript levels in WTt cells treated with 100 µM $CoCl_2$ for 120 h. A: Transcript expression shown at D10 differentiation. Legend as in Figure 3. B: Expression of the fast MHC2b protein isoform, at D10 of differentiation, was analyzed by Western blots, α enolase was used for comparison. Protein amount per well was always 30 µg.

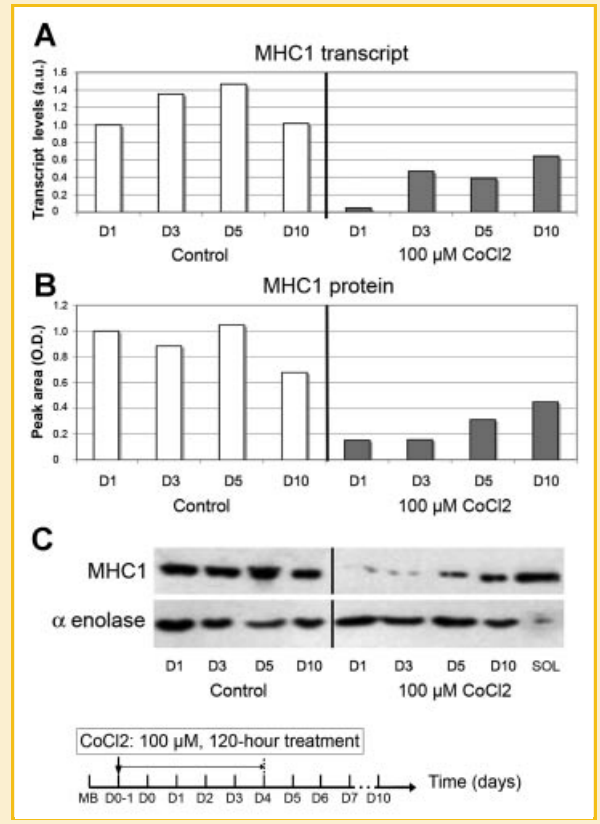

Fig. 5. MHC1 expression in WTt cells treated with 100 µM $CoCl_2$ for 120 h. A: MHC1 transcripts in control and treated cells. B: MHC1 protein levels in the same cells, were obtained from scanning of Western Blot. C: Western blot images for control and 100 µM $CoCl_2$ treated cells. SOL: 200 ng soleus muscle extract. Time course data are from a representative experiment. Cell extracts: 30 µg per well. α enolase was used for comparison. OD: optical density.

further studies. Here, we show results obtained comparing parallel experiments conducted during in vitro differentiation of an over expressing PPARδ clone named PPARd, and of a control clone infected with the empty vector.

PPARδ expression in control cells expressing the empty vector, was similar to that of the WTt original clone (data not shown) and transcripts were expressed at low level, with no significant change occurring during differentiation (see Fig. 7A, inset). In contrast, a large 40-fold increase in PPARδ expression was already visible at the myoblast stage, in the PPARd clone, over-expressing the PPARδ transcription factor, as compared to values obtained with the control clone (Fig. 7A). By 10 days of differentiation, PPARδ transcript level was about 150-fold higher in the PPARd clone than in the control cells.

As already described for the original WTt cell line [Peltzer et al., 2008], in vitro differentiation is accompanied by a decreased expression of the ubiquitous and embryonic alpha enolase (Fig. 7B), associated with an increase of the muscle specific beta enolase isoform (Fig. 7C). Thus, even after infection, these WTt cells can mimic in vitro, the isozymic transition that naturally occurs with this glycolytic enzyme, during in vivo ontogenesis. No major difference in transcript levels is observed between control and PPARd cells.

However, expression of citrate synthase (CS), the enzyme marker of the Krebs cycle, was strikingly different in PPARd cells as compared to control cells (Fig. 7D). In control cells, after a slight increase in expression at the beginning of differentiation (D3), CS transcript levels did not change significantly later in differentiation. By contrast, this level more than doubled between D3 and D5 of differentiation, in PPARδ over expressing cells. These high levels were maintained at D10 (Fig. 7D).

Changes in expression of PGC1-α transcription factor during differentiation. In transgenic mice, the PGC1-α co-activator will drive the formation of slow-twitch muscle fibers in muscles normally rich in fast fibers [Lin et al., 2002; Grimaldi, 2005]. It was therefore of interest to analyze whether over-expression of PPARδ would have an effect on PGC-1α expression.

We observed that the behavior of PGC-1α, was quite different from that of PPARδ. An increased expression occurred during differentiation of all examined clones, including control cells (Fig. 7E). Interestingly, at the beginning of differentiation (D3), the increase appeared more pronounced in the control than in the other clone. At later times (D10), however, transcript levels were equivalent in control and PPARd clones (Fig. 7E).

Changes in expression of MHC isoforms during differentiation. We expected to reproduce in our in vitro model cell line the transition towards the slow-oxidative phenotype induced in vivo by PPARδ over expression in striated muscles [Luquet et al., 2003; Wang et al., 2004]. Indeed, using quantitative

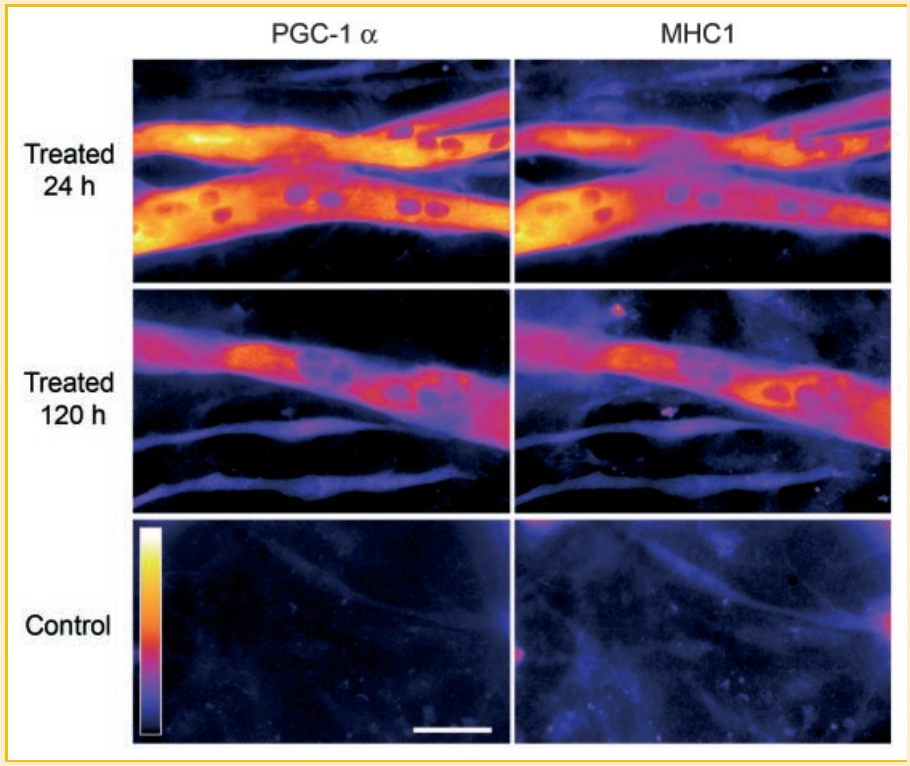

Fig. 6. Immunocytochemical analysis of PGC-1α and MHC1 parallel decreases following 120 h 100 μM of $CoCl_2$. Double labeling was obtained using rabbit polyclonal antibody directed against PGC-1α (left column), and monoclonal anti MHC1 (right column). Images are shown using a fire color chart indicative of labeling intensity: blue is lowest, yellow is highest. All analyzes were performed at D10 of WTt cell differentiation, following 100 μM $CoCl_2$ treatments for either 24 h (top line) or 120 h (middle line); negative control (bottom line) was obtained by primary antibody omission (120-h treatment). Bar: 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RT-PCR experiments, we did observe an important increase of MHC1 transcript levels, by 10 fold at D10, as compared to levels in the control clone (Fig. 8A). This increased expression of the slow MHC isoform was accompanied by a decreased expression of all fast MHC isoforms (MHC2a, MHC2b and MHC2x) during differentiation of the PPARd cells (Fig. 8B–D).

Western blot scanning data demonstrate that PPARδ over expression resulted in a moderate 40% increase of MHC1 protein expression (data not shown). Although less important, this is in keeping with the observed increase of MHC1 transcripts (Fig. 8A), whereas no change is observed with the developmental and fast MHC isoforms (data not shown).

DISCUSSION

In this study, we were able to induce transitions of a myogenic clone towards either a fast-glycolytic or a slow-oxidative phenotype. These results were obtained by modifying expressions of two transcription factors in the WTt myogenic clone, respectively HIF-1α and PPARδ. Taken together, our data demonstrate that it is possible to induce coordinated modulations of metabolic and contractile phenotypic markers in WTt cells, during in vitro differentiation. Furthermore, changes in PGC-1α expression, observed following a long hypoxic-like treatment, underlines a link between the molecular control cycles of oxidative and glycolytic pathways, in these cells.

ROLE OF HIF-1α STABILIZATION IN THE TRANSITION OF WTt MYOGENIC CLONE TOWARDS A FAST-GLYCOLYTIC PHENOTYPE

The sparse reports concerned with HIF-1α in cultured myogenic cells are often in contradiction with otherwise reported data from in vivo observations. Thus, Pisani and Dechesne [2005] reported an increase in HIF-1α protein, but no variation in mRNA contents in differentiated C2C12 myotubes stressed for 3 h with 200 μM $CoCl_2$. By contrast, increases of skeletal muscle HIF-1α mRNA have been reported in chronic conditions, such as adaptation to altitude [Hoppeler and Vogt, 2001] and to muscle disuse [Pisani and Dechesne, 2005], conditions known to induce changes in muscle fibers towards a fast phenotype. It was therefore of interest to investigate the effects of HIF-1α expression changes occurring in a new myogenic cell line exhibiting relatively advanced maturation in vitro.

In order to stabilize the HIF-1α protein, we made use of the common $CoCl_2$ treatment of cells provoking a pseudo hypoxic stress [Itoh et al., 2001]. Our data show that, indeed, modifications of metabolic pathways induced by cobalt treatment are followed by changes in expression of contractile MHC isoforms. Two tested protocols differed in the duration of treatment with 100 μM $CoCl_2$

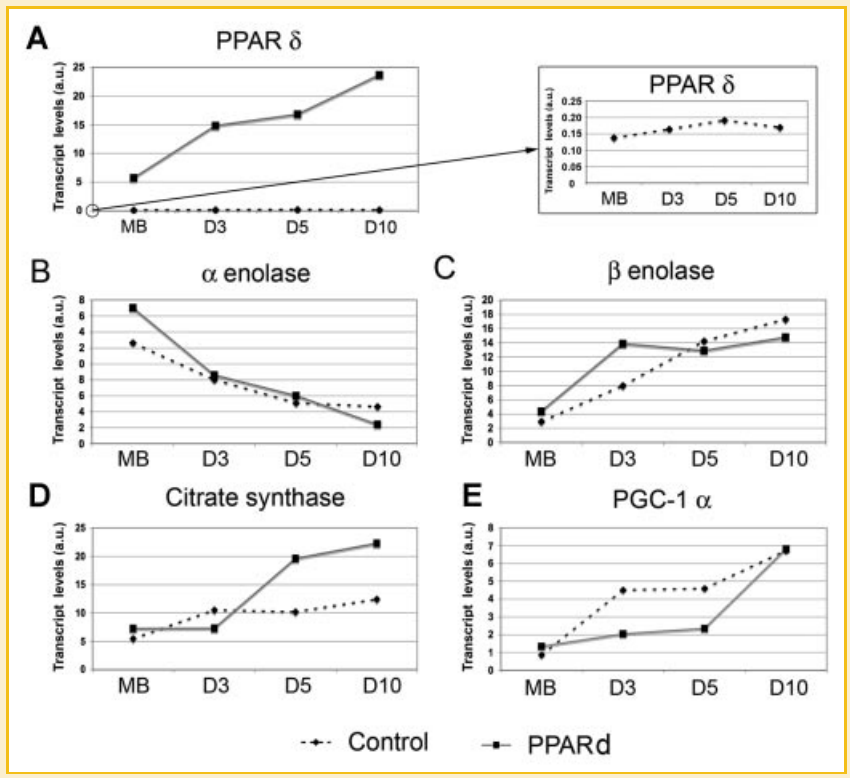

Fig. 7. Expression of transcription factors and metabolic enzymes during differentiation of PPARδ infected WTt cells. A: Time course evolution of PPARδ transcript expression is shown in one WTt infected clone (clone PPARd). Levels were obtained from real time quantitative RT-PCR analysis, at various differentiation times. The inset shows the data obtained for the control clone, but with a different scale, as the low level of PPARδ expression makes it apparently not detectable in the main diagram. B–D: transcript levels of α enolase, β enolase, and citrate synthase, respectively. E: PGC-1α transcript levels. Time course data are from a representative experiment. Control: WTt clone infected by empty bizeoneo vector. PPARd: WTt clone over-expressing PPARδ. MB: myoblast stage; D3, D5, D10: 3, 5, and 10 days in differentiation medium. a.u.: arbitrary units.

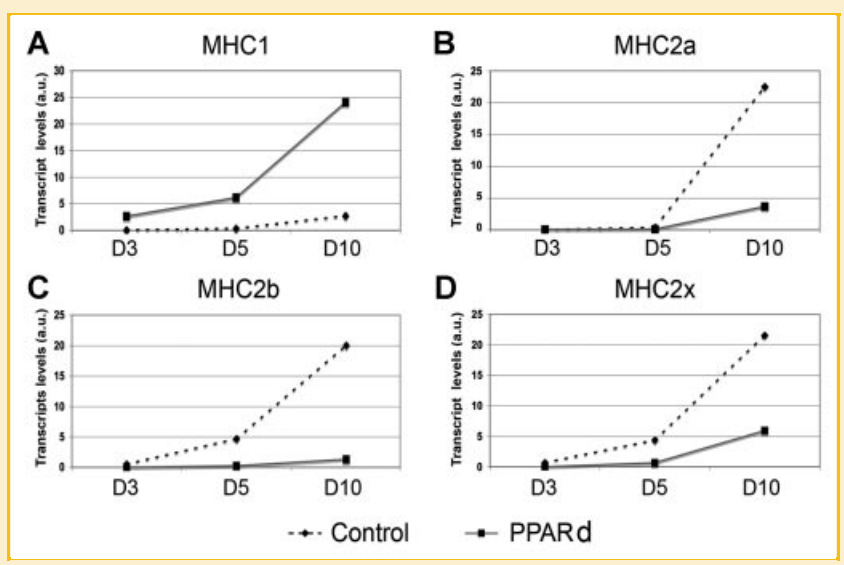

Fig. 8. Expression of MHC isoforms during differentiation of infected WTt cells. Transcript levels of adult MHC isoforms. A: MHC1. B: MHC2a. C: MHC2b. D: MHC2x. Legend as in Figure 7.

and revealed the complexity of mechanisms involved in the control of metabolic phenotypes.

SHORT TIME $CoCL_2$ TREATMENT INDUCES THE SEQUENTIAL EXPRESSION OF GLYCOLYTIC AND CONTRACTILE FAST MHC IN WTt MYOTUBES

We have observed a sequence of events, remindful of the hypothesis derived from older studies, suggesting that phenotypic transition of striated muscle cells would be induced by metabolic changes and then followed by modifications in expression of contractile proteins [Nemeth et al., 1989]. Thus, when WTt cells were treated with $CoCl_2$ for a short time (24 h), HIF-1α transcript level was transiently increased at the very beginning of differentiation, that is, exactly at the end of the 24-h $CoCl_2$ treatment. This transient increase in transcript accumulation was followed by a transient increase of the corresponding protein, at D3 and D4 of differentiation. Indeed, this protein increase might also be the consequence of protein stabilization by $CoCl_2$ treatment. A decrease in expression of the oxidative enzymes, HAD and citrate synthase, occurs at D0, together with the observed HIF-1α transcript increase. By contrast, the increased expressions of enzymes associated to the glycolytic phenotype (β enolase and myokinase) occur later, at D3. This is at the time of increased HIF-1α protein, in support of a control mechanism of these enzymes by HIF-1α protein. The increase in transcripts of fast contractile MHC isoforms occurs even later.

Interestingly, it has been reported from in vivo experiments, that the specific deletion of HIF-1α gene induced a contractile phenotype in mouse skeletal muscles similar to that observed in the human diseases caused by defects in glycogenolysis and glycolysis [Mason et al., 2004]. This is in keeping with our data, supporting a role for HIF-1α in coordinated contractile and metabolic regulations of muscle cells.

LONG TIME $CoCL_2$ TREATMENT IS NECESSARY TO INDUCE A DECREASE IN SLOW MHC EXPRESSION IN WTt MYOTUBES

When this same WTt clone was treated with $CoCl_2$ for a longer time period (120 h), the effect on HIF-1α expression was strikingly different. Instead of an increase, a transient decrease in HIF-1α transcript level was observed after 48 h of treatment (D1). In contrast to what occurs during the short treatment, we were not able to visualize the HIF-1α protein in WTt cells, at any differentiation time. This suggests that a more drastic $CoCl_2$ treatment induces some control mechanism preventing HIF-1α protein accumulation in muscle cell. This kind of treatment had also a very different effect on phenotypic differentiation. By contrast to results obtained with the short treatment, the early decreased expression in oxidative enzymes (HAD and citrate synthase) lasted longer and was now accompanied by a decreased expression of the slow contractile MHC1 isoform, transcripts and protein, and of PGC-1α expression as well. Only at later times, an increase in expression of the fast isoforms was observed, more important for MHC2x than for MHC2b.

Thus, comparison of data obtained with short and long $CoCl_2$ treatments suggests that HIF-1α might regulate fast-glycolytic and slow-oxidative myofiber phenotypes through independent pathways. The role of HIF-1α in control of oxidative enzyme expression might be indirect as changes in the latter cannot be correlated with any visible change in HIF-1α protein expression.

An impressive amount of publications has documented a key-role for HIF-1α in many functions, so that it has been evaluated that at least 2% of all genes in humans are under HIF-1α control [see Ke and Costa, 2006]. This probably explains the many regulatory pathways involved in controlling its expression. Therefore, our $CoCl_2$ treatments of WTt cells most probably act on multiple pathways, together controlling HIF-1α protein stability. However, it cannot be excluded that the bivalent ion Co2+ may act on WTt muscle cell differentiation through a HIF-1α-independent mechanism. Therefore, it would be of interest to test if other treatments such as hypoxia or application of a specific prolyl hydroxylase inhibitor such as dimethyloxalylglycine [Jaakkola et al., 2001] would yield results similar to those reported here. In the future, it would be important to examine whether similar results would be obtained by over expressing a stable mutant of the HIF-1α protein in the WTt myogenic clone [Pajusola et al., 2005].

OVER-EXPRESSION OF PPARδ IN WTt MYOGENIC CLONE INDUCES COORDINATED IN VITRO MODIFICATIONS, SIMILAR TO WHAT HAS BEEN OBSERVED BY OTHERS IN TRANSGENIC MICE

PPARs are ligand-activated transcription factors that have emerged as key metabolic regulators. Indeed, they enhance insulin-stimulated glucose disposal, lipid catabolism, energy expenditure, cholesterol efflux, and decrease triglycerides in skeletal muscle [Smith and Muscat, 2006]. Several PPAR subtypes (α, β/δ, and γ) have been uncovered. Relatively little is known about the most widely expressed subtype, PPARδ. Two groups have investigated the roles of PPARδ in skeletal muscles, by generating mouse transgenic lines, in which over-expression of PPARδ was promoted in a tissue-specific manner [Luquet et al., 2003; Wang et al., 2004]. Over-expression of PPARδ greatly influenced energy metabolism of mouse muscles by promoting an important increase of fibers with an oxidative metabolic capability [Luquet et al., 2003], and enabled the generation of a strain of mice with a ''long-distance running'' phenotype [Wang et al., 2004]. These changes in muscle phenotype are, in some aspects, reminiscent of muscle remodeling that occurs during adaptation to endurance exercise in rodents [Ishihara et al., 1991; Kelley, 1996] and humans [McCall et al., 1996].

In our experiments, analyses of the PPARd clone, obtained by over-expression of the PPARδ transcription factor in the WTt clone, demonstrated the increased expression of PPARδ but not of its co-activator PGC-1α, suggesting that other regulatory pathways might be involved. The PPARδ increased expression was associated with an increased expression of oxidative markers, in keeping with observations reported by others in another cell type, C2C12 [Holst et al., 2003]. Furthermore, our transcript data demonstrate the increased expression of the slow contractile MHC1 isoform, accompanied by the decreased expression of the three fast adult MHC. A mild increase in the slow MHC1 protein was also visible. Thus, we were able to induce in vitro, the phenotypic transition of the mixed (fast and slow) WTt myogenic clone, towards a slow-oxidative phenotype, in the WTt-derived PPARd clone. Recently, in vivo data were obtained in adult rats and are in keeping with our own results obtained in a cellular model [Lunde et al., 2007]. These

authors over expressed PPARδ in adult muscle fibers, which induced a transition towards a slow-oxidative phenotype with an increase in type I myofibers and oxidative capacity [Lunde et al., 2007].

We demonstrate here, for the first time in cell culture, that over-expression of PPARδ a factor known to be involved in control of metabolic pathways, induces coordinated regulations resulting in a switch in contractile MHC isoform expression, in a myogenic clone. The morphology of these myotubes is remarkable as is resembles that of clones derived from slow-oxidative muscle [Peltzer et al., 2008].

CONCLUSIONS

The modifications observed following $CoCl_2$ treatments and PPARδ overexpression, support the hypothesis put forward in older studies, suggesting that phenotypic transition of striated muscle cells would be induced by metabolic changes and then followed by modifications in expression of contractile proteins [Nemeth et al., 1989]. HIF-1α activates transcription by binding to specific response elements (HRE) of numerous target genes. Similarly, PPARδ activates transcription by binding to specific response elements (PPRE) of oxidative enzyme genes. In a preliminary in silico study, we searched for both HRE and PPRE domains in enhancer regions of MHC genes. Using the Genomatix software, we found corresponding domains in all MHC genes. It remains to elucidate whether such sequences might be functionally active. Our results do not contradict the idea that a direct action of HIF-1α and PPARδ on metabolic and contractile genes could explain the observed coordinated regulations. However, we cannot exclude the participation of indirect mechanisms involving these factors in the control of coordinated modifications occurring in skeletal muscle.

Our data obtained following a long hypoxic-like treatment, demonstrate expression changes in the PPAR cofactor, PGC-1α. This is indicative of indirect mechanisms creating a link between the molecular control cycles of oxidative and glycolytic pathways. In the future, it will be important to find out whether PPARδ and HIF-1α act directly by their respective interaction with the PPRE and HRE sequences that were found by in silico searches on MHC genes, and to clarify other possible indirect mechanisms.

Indeed, ongoing research has been very active in the past few years, aimed at understanding mechanisms involved in control of myogenic cell differentiation and maturation. For example, AMP-activated protein kinase (AMPK) has arisen as an important regulating partner in the complex dynamic control of striated muscle phenotype. It has been proposed to be a regulator of energy balance in response to nutritional environmental variations [Viollet et al., 2009]. Interestingly it appears to control myoblast differentiation through a PGC-1α -dependent mechanism [Williamson et al., 2009]. Our own results, presented here, do point to a central role of PGC-1. Such converging conclusions shed light on putative interconnections between differentiation pathways investigated by different authors.

Skeletal muscle is a metabolically flexible tissue and has a paramount role in whole body energy balance. This is the reason why this newly developed WTt model of in vitro myogenic maturation might represent a very useful tool for the elucidation of PPAR and HIF molecular mechanisms and functions in skeletal muscle. This is supported by the fact that our in vitro data parallel those obtained in vivo, by others, for both PPAR [Lunde et al., 2007] and HIF [personal communication and Lunde et al., 2009]. It is important to increase knowledge on these factors and their selective modulators, as they are emerging as critical targets in the battle against obesity, metabolic syndrome, type II diabetes, and dyslipidemia.

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