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Interactions between bioenergetics and mitochondrial biogenesis

Scot C. Leary [a], Brendan J. Battersby [1,a], Richard G. Hansford [b], Christopher D. Moyes [a,*]

[a] *Department of Biology, Queen's University, Kingston, Ont. K7L 3N6, Canada*

[b] *Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA*

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Abstract

We studied the interaction between energy metabolism and mitochondrial biogenesis during myogenesis in C2C12 myoblasts. Metabolic rate was nearly constant throughout differentiation, although there was a shift in the relative importance of glycolytic and oxidative metabolism, accompanied by increases in pyruvate dehydrogenase activation state and total activity. These changes in mitochondrial bioenergetic parameters observed during differentiation occurred in the absence of a hypermetabolic stress. A chronic (3 day) energetic stress was imposed on differentiated myotubes using sodium azide to inhibit oxidative metabolism. When used at low concentrations, azide inhibited more than 70% of cytochrome oxidase (COX) activity without changes in bioenergetics (either lactate production or creatine phosphorylation) or mRNA for mitochondrial enzymes. Higher azide concentrations resulted in changes in bioenergetic parameters and increases in steady state COX II mRNA levels. Azide did not affect mtDNA copy number or mRNA levels for other mitochondrial transcripts, suggesting azide affects stability, rather than synthesis, of COX II mRNA. These results indicate that changes in bioenergetics can alter mitochondrial genetic regulation, but that mitochondrial biogenesis accompanying differentiation occurs in the absence of hypermetabolic challenge.

Keywords: Myogenesis; Muscle; C2C12; Cytochrome oxydase; Energy metabolism

1. Introduction

Genetic control of mitochondrial biogenesis requires the coordinated expression of genes in both the nucleus and mitochondria. Mitochondrial DNA (mtDNA) copy number is a primary regulator of mitochondrial gene expression [1] whereas expression of nuclear DNA is increased through both transcriptional [2] and post-transcriptional mechanisms [3]. Coordination of nuclear gene transcription is thought to be achieved by shared activators including the nuclear respiratory factor (NRF) family [4], OXBOX [5], REBOX [6], YY1 [7] and MT1, -3, and -4 [8,9]. Apart from coordinating nDNA expression, there is evidence that several of these factors also influence mtDNA replication, transcription and mRNA processing [9–12]. While an increasing number of respiratory genes are found to possess these regulatory elements, the regulatory links between physiological stressors (i.e. hypermetabolic stress) and control of respiratory gene expression remain elusive [13].

* Corresponding author. Fax: +1 (613) 545-6617; E-mail: MoyesC@Biology.QueensU.ca

[1] Present address: Montreal Neurological Institute, 3801 University Street, McGill University, Montreal, Que. H3A 2B4, Canada.

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Mitochondrial proliferation occurs in muscle in response to chronic hypermetabolic conditions, such as endurance training, electrical stimulation and hyperthyroidism [13,14]. A number of studies have demonstrated that factors which decrease metabolic rate or mitochondrial efficacy alter the expression of genes for respiratory proteins, supporting the hypothesis that prolonged elevations in metabolic rate directly or indirectly induce mitochondrial enzyme synthesis. Tetrodotoxin treatment of neurons leads to a decrease in mRNA for COX proteins [15]. Hypoxia exerts a reciprocal control on glycolytic (increase) and mitochondrial (decrease) enzymes in myotubes [16]. Paralysis of myoblasts, however, does not lead to changes in the activities of oxidative enzymes [17]. There is little evidence for direct links between regulators of metabolic rate (e.g. phosphorylation potential [18]) and induction of mitochondrial biogenesis under hypermetabolic conditions. Cytoplasmic translation is regulated by GTP:GDP ratios [19], although it is not clear if the changes which occur in vivo are great enough to have physiological relevance [20]. High levels of ATP have been shown to both inhibit mitochondrial RNA polymerase activity [21] and stimulate mitochondrial translation [22].

Differentiation of myoblasts into myocytes is also accompanied by mitochondrial biogenesis, as indicated by increases in mtDNA, marker enzyme activities and mRNA levels [16,23–25]. Mitochondria-deficient myoblasts fail to form into myotubes [26], but the connections between mitochondrial biogenesis, myogenesis and bioenergetics have not been established. At present, it is not known if the regulatory control of mitochondrial biogenesis in hypermetabolic adaptation is fundamentally similar to that in cellular differentiation. In contrast to the apparent coordinated expression during hypermetabolic challenges [14], the expression of respiratory genes during cellular differentiation can be asynchronous [25]. It is also unknown if the differentiation-induced mitochondrial biogenesis is accompanied by changes in either metabolic rate or bioenergetic regulation.

Although cultured myoblasts are widely used in studies of mitochondrial biogenesis and myogenesis, relatively little is known about their metabolic properties. In the present study, we use an immortalized mouse skeletal muscle cell line (C2C12) to investigate the relationship between energy metabolism and mitochondrial biogenesis. We initially consider how bioenergetics change in response to differentiation. We then examine the influence of azide on respiratory gene transcript levels in differentiated myocytes, since the responsiveness of myocytes to energetic stress is superimposed upon the intrinsic relationships between respiratory genes and myogenesis.

2. Materials and methods

2.1. Cell culture

C2C12 cells were grown in Dulbecco's minimum essential medium (DMEM), containing high glucose, glutamine and pyruvate, supplemented with 20% fetal bovine serum. At 70% confluency, the medium was changed to DMEM supplemented with 2% horse serum. Penicillin, streptomycin and neomycin were included in all media. All media, sera and antibiotics were supplied by Gibco-BRL.

2.2. Enzyme assays

Cultured cells were extracted in 1 ml of enzyme solubilization medium (20 mM HEPES, pH 7.2, 0.1% Triton X-100, 1 mM EDTA). Citrate synthase (CS), cytochrome oxidase (COX) and complex I activities were assayed spectrophotometrically from whole cell extracts using previously described protocols [25]. Dichlorophenol-indophenol was used as the acceptor for complex I assays.

A radiometric assay for pyruvate dehydrogenase (PDH) was employed to assay both total PDH and the proportion present in the active, dephospho form. Cells were extracted in a medium that prevents changes in phosphorylation (2 mM EDTA, 5 mM dichloroacetate, 2 mM dithiothreitol, 0.2% Triton X-100 in 50 mM HEPES, pH 7.7). Conversion of PDH into its dephospho form was accomplished by incubation of the cells with uncoupler (10 μM FCCP) for 10 min prior to extraction. This concentration (in the presence of DMEM+2% horse serum) gave maximal rates of oxygen consumption in trypsinized suspensions of myoblasts (data not shown). Higher FCCP concentrations or longer incubations did not increase measurable PDH activity. Incuba-

tions were conducted in flasks sealed with rubber caps which contained a center well holding a glass fiber circle (934 AH). Enzyme was added to the assay medium (2 mM NAD^+, 0.6 mM coenzyme A, 0.5 mM cocarboxylase, 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.1% Triton X-100, 20 mM Tris-HCl pH 8.0) for a 4 min pre-incubation in a 37°C water bath. The assay was started by addition of pyruvate (100 μM final concentration) with approximately 200 000 DPM [^{14}C]1-pyruvate. Incubations were stopped with 0.1 volume of 70% perchloric acid. Hyamine hydroxide (150 μl) was injected into the center well and $^{14}CO_2$ collected for 90 min. Filters were removed and counted in scintillant containing 0.1% acetic acid to reduce chemiluminescence. These reaction conditions gave a linear rate of $^{14}CO_2$ production for 20 min, although assays were routinely carried out for only 10 min.

2.3. Metabolic rate determinations

Oxygen consumption was measured in C2C12 cells at 0, 1, 3, 6, 9 and 12 days of serum starvation. Culture flasks were filled with a solution of DMEM/2% horse serum buffered with 6 mM HEPES and equilibrated to 10% CO_2, and oxygen consumption was monitored polarographically at 37°C using a Clark-type electrode interfaced with Vernier Instruments Data Logger software. The rate of oxidative ATP production per mg protein was calculated by multiplying the rate of oxygen consumption by a conversion factor that accounted for the volume of solution in the flask, the maximal saturation of oxygen in water at 37°C, and the production of 5 mol of ATP per mol of consumed O_2.

The rate of glycolytic ATP production was also calculated at 0, 1, 3, 6, 9 and 12 days of serum starvation by measuring lactate concentrations in the supernatant. Culture media was replaced with 10 ml fresh DMEM/2% HS on each sampling day, and an aliquot of the supernatant removed immediately. Plates were incubated for 4 h, and a second aliquot of the supernatant was taken. Plates were washed twice with phosphate buffered saline, and cells extracted in 1 ml of solubilization medium (20 mM HEPES, pH 7.2, 0.1% Triton X-100, 1 mM EDTA).

2.4. Metabolite assays

Lactate, phosphocreatine (PCr) and creatine (Cr) were analyzed using conventional spectrophotometric techniques modified for use on microtiter plates. Lactate was assayed using lactate dehydrogenase (LDH) and the 'hydrazine sink' method. Lactate oxidation by LDH leads to equimolar NADH production, detectable at 340 nm. Inclusion of hydrazine and use of high pH ensures the reaction goes to completion. PCr was assayed using the enzymes creatine phosphokinase (CPK), hexokinase and glucose-6-phosphate dehydrogenase. Cr was assayed using the enzymes CPK, pyruvate kinase and LDH. The ratio of PCr/(PCr+Cr) is an index of energy status, through interactions of the adenylate pool with creatine pool mediated by endogenous CPK, which is assumed to be near-equilibrium (by day 6, the onset of azide treatments, CPK activity has risen to near maximum activities [25]).

2.5. Mitochondrial mRNA and mtDNA

Total RNA was purified from guanidinium thiocyanate extracts using the acid phenol method. RNA was glyoxylated and electrophoresed on 1.2% agar-

Table 1
Primer sequence designed for amplifying rat mitochondrial and nuclear DNA gene products

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing T (°C)	Size (bp)
COX I	actattcggagcctgagcgg	gtgatatggtggaggcagc	58.0	1451
COX III	gtccatgaccattaactggagc	tgctgcggcttcaaatcc	56.0	678
ADNT1	ggcatcattgattgtgtcgt	cctgcacagagacactgaaa	57.5	390
PK	gggactgccttcattcagacc	cggcatccttacacagcacagg	60.0	1406
COX IV	agcctaattggcaag(a/c)gagc	gtcgtagtcccacttggc	58.0	468

COX I, III, IV: subunits 1, 3 and 4 of cytochrome *c* oxidase; PK, pyruvate kinase; ADNT1, adenine nucleotide translocase isoform.

ose gels. Probes for mtDNA-encoded COX II and ATPase VI were obtained as previously described [25]. Probes for COX I, COX III, COX IV, PK and ADNT1 were constructed as outlined in Table 1. COX III, COX IV, PK and ADNT1 were amplified from first strand cDNA prepared from total RNA of rat gastrocnemius, and the nucleotide sequence confirmed by MOBIX (McMaster University, Canada). COX I cDNA was amplified from a plasmid containing the entire mouse mitochondrial genome and a pure PK fragment respectively (both generously supplied by R.G. Hansford, National Institute of Health). Blots were corrected for loading differences using a probe for α-tubulin mRNA (generously supplied by W. Bendena, Queen's University). The probes for mtDNA and mRNA were radiolabelled using random primers and ^{32}P-dCTP. Blots were exposed to a phosphoimager screen, and quantified using a phosphoimager (Molecular Dynamics) driven by Imagequant software.

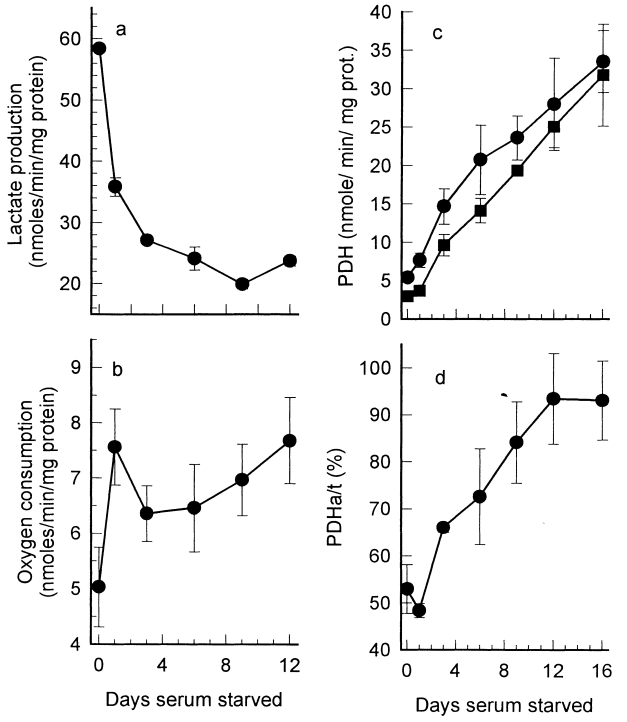

Fig. 1. Changes in energy metabolism during differentiating C2C12 cells. See Section 2 for complete description of techniques. (a) Glycolytic rate. (b) Respiration. (c) Changes in pyruvate dehydrogenase activity, expressed as total activity (PDHt, ●) and that present in the active, dephospho form (PDHa, ■). (d) PDHa activity expressed as % of total activity. All data are presented as mean (±S.E.M.).

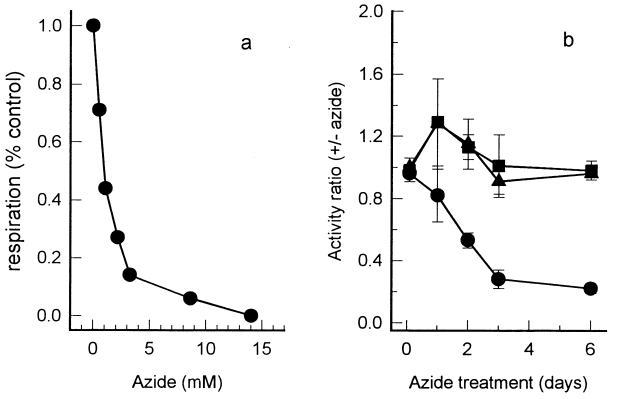

Fig. 2. Effects of acute and chronic azide treatments on C2C12 myoblasts and myotubes. (a) Respiration was measured on cells collected 1 day after serum starvation. Rates are expressed relative to the untreated suspension. (b) Chronic effects of low azide concentrations. Myotubes (day 6) were treated with azide (10 μM) and sampled over 6 subsequent days for cytochrome oxidase (●), citrate synthase (■) and complex I (▲) activities.

2.6. Statistical analysis

Significant differences in steady state mRNA levels between treatment groups were detected using one-way ANOVAs, and identified using Student-Newman-Keuls test. A repeated measures one-way ANOVA was used to test for significant differences in the rate of ATP production as a function of time following serum starvation.

3. Results

3.1. Changes in metabolic rate with differentiation

Metabolic rate was highest in proliferating cells with approximately 30% of the ATP being contributed by OXPHOS (Fig. 1b). Once proliferation ceased, the metabolic rate decreased slightly and then remained constant throughout the differentiation period. Although the total metabolic rate was constant, there was a steady shift toward a greater reliance on mitochondrial pathways (Fig. 1a,b). By day 12, mitochondrial pathways contributed 61% of the total ATP used by the cells. This shift was due to an increase in mitochondrial respiration coupled with a decrease in glycolytic rate.

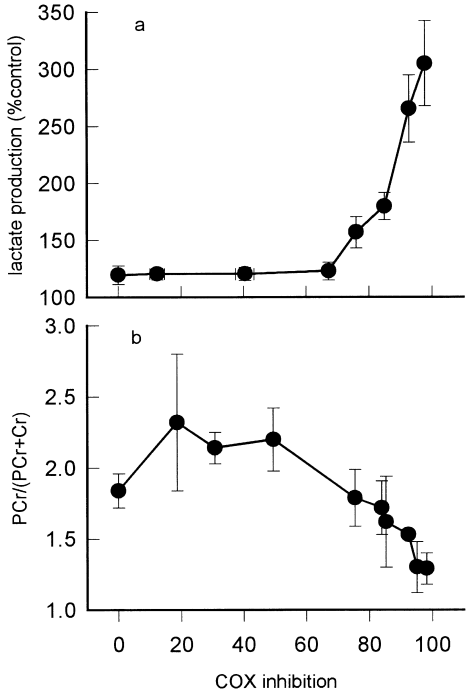

Fig. 3. Effects of azide concentration on C2C12 enzymes and bioenergetic parameters. Myotubes (day 6) were treated with various azide concentrations (0–500 μM) for 3 days. (a) Lactate production over 24 h was measured in the culture medium. Plates were washed and extracted for cytochrome oxidase activity and protein determinations. Data are expressed relative to the activity in untreated control. Paired data were ranked by degree of inhibition of COX. Each data point represents 10 separate determinations. (b) Creatine (Cr) and phosphocreatine (PCr) were measured after 3 days azide treatment. The degree of COX inhibition was determined on parallel plates. Cells were given fresh medium 4 h before sampling. Plates were quickly rinsed in ice-cold phosphate buffered saline then extracted immediately in 7% perchloric acid.

3.2. Changes in pyruvate dehydrogenase

PDHt increased approximately six-fold (Fig. 2c) during differentiation. As well as an increase in total enzyme, there was an increase in the proportion present in its active, dephospho form (PDHa). In myoblasts the ratio of PDHa/t was approximately 50%, but as myotubes formed, PDH became increasingly active to the point of being almost completely activated.

3.3. Azide effects on mitochondrial parameters and bioenergetics

Acute azide treatment inhibited respiration by myoblast suspensions with an I_{50} of approximately 1 mM (Fig. 2a). Chronic low level azide treatment led to pronounced changes in the activity of COX. The degree of inhibition increased with time until reaching a maximum effect by 3 days (Fig. 2b). The activities of CS and complex I were not affected by azide.

As much as 70% of COX activity could be inhibited without affecting lactate production (Fig. 3a), creatine phosphorylation (Fig. 3b) or steady state levels of COX II mRNA (Fig. 4a). Greater degrees of inhibition stimulated glycolysis (Fig. 3a), decreased creatine phosphorylation (Fig. 3b) and increased COX II mRNA approximately four-fold ($n=3$) (Fig. 4b, Fig. 5). While mitochondrial DNA copy number was unaffected by the degree of COX inhibition (Fig. 4a), COX II mRNA levels were highest following 3 days of azide treatment (Fig. 6a). Steady state levels of other mitochondrial and nuclear mRNAs were unchanged using identical azide treatment protocols (Fig. 6b).

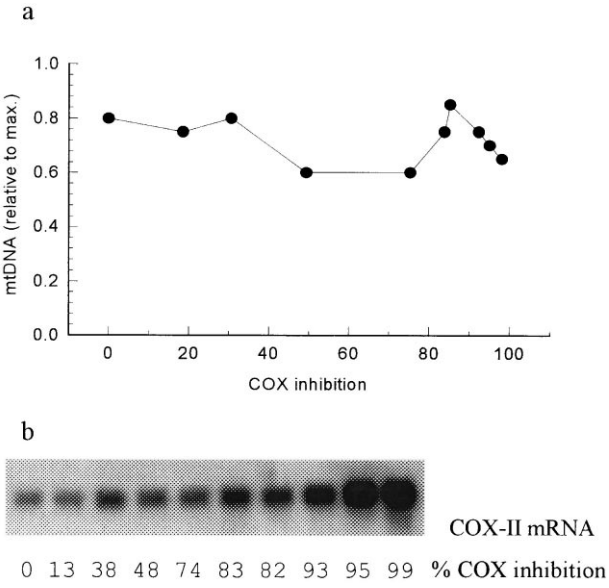

Fig. 4. Changes in mtDNA and mRNA in relation to azide treatment. Cells were treated with various azide concentrations for 3 days beginning at 6 days of serum starvation. Two series of plates were harvested for enzyme analysis, then subsequently extracted for DNA. A third series was extracted for total RNA. ^{32}P-dCTP labelled COX II was used as a probe for both the mtDNA dot blot and the Northern blot (10 μg RNA/lane). Data for mtDNA copy number are presented as mean of two separate azide titrations (each point in triplicate). Degree of COX inhibition is expressed relative to untreated control.

4. Discussion

4.1. Mitochondrial energetics in proliferating cells

Undifferentiated C2C12 myoblasts appear similar to tumor cells in their dependence upon glycolysis [27]. Despite the availability of oxidizable fuels (glucose, pyruvate, amino acids) much of the energy required for proliferation is derived from glycolysis. In the earliest stages of differentiation, approximately 60% of the energy demands of the cell are met by lactate production from glucose (Fig. 1a). However, several lines of evidence suggest this reliance upon anaerobic glycolysis is not due to mitochondrial inadequacy. Addition of an uncoupler of OXPHOS (FCCP) more than doubles the respiration rate of suspended myoblasts (data not shown), suggesting mitochondria are capable of greater flux under the appropriate regulatory influences. The activity of PDHa (3.6 nmol/min/mg protein, Fig. 1c), the first step in oxidation of mitochondrial pyruvate, suggests a capacity to yield 54 nmol ATP/min/mg protein (3.6×15 ATP/pyruvate oxidized), compared to an observed rate of oxidative ATP production of 25 nmol/min/mg protein (Fig. 1b). Furthermore, only 50% of PDH in myoblasts is present in its active, dephospho form. In many oxidative tissues, changes in PDHa/t accompany changes in energetic demands [28]. These observations suggest that oxidative metabolism could meet the bioenergetic demands of proliferating cells under the appropriate regulatory conditions.

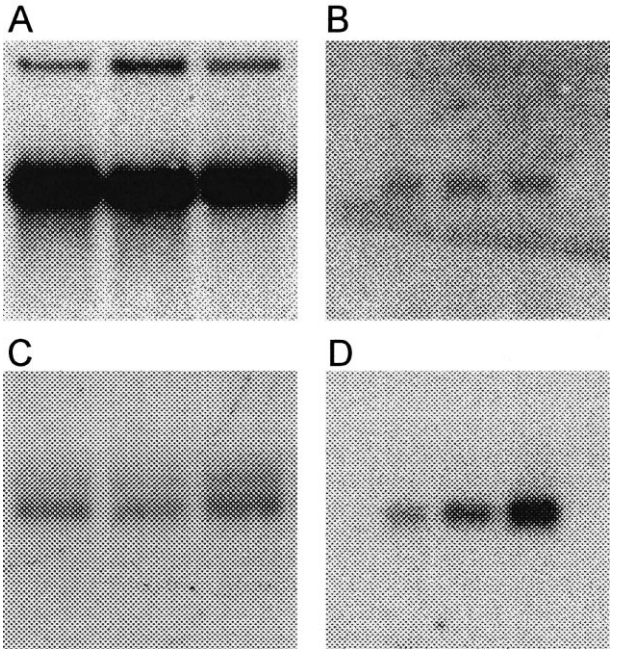

Fig. 5. Representative autoradiographs of mRNA levels in C2C12 cells treated 3 days with azide. Each panel is a autoradiograph of blot of a 1.2% agarose gel using 5 μg (B and D) or 20 μg (A and C) glyoxylated total RNA per lane. (A) ATPase VI; (B) ADNT1; (C) α-tubulin; (D) COX II. In each panel, the left lane is 0 azide, center 50 μM, right 500 μM. Azide treatment increased lactate production two-fold at 50 μM and five-fold at 500 μM.

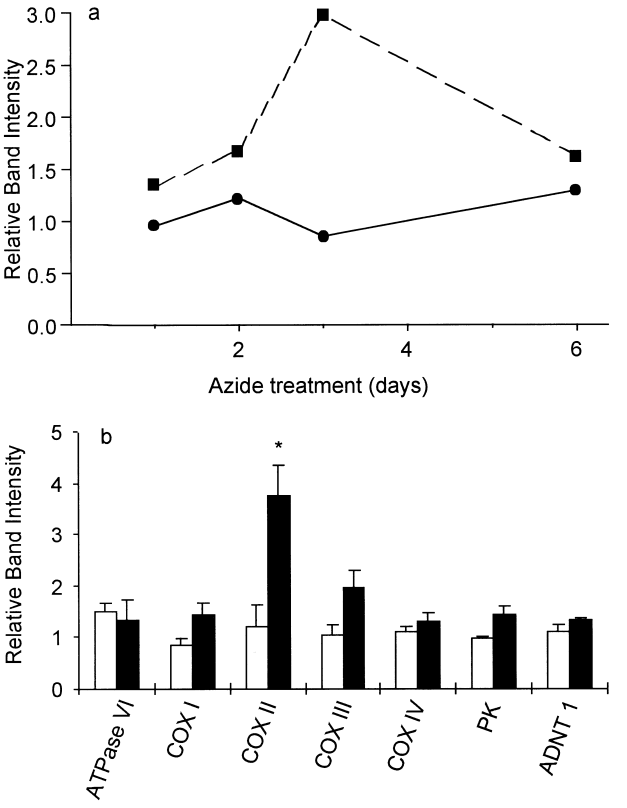

Fig. 6. Levels of mRNA in C2C12 cells treated with azide. All data were obtained by probing total RNA blots (5 μg for mitochondrial signals, 25 μg for nuclear signals) with ^{32}P-dCTP labelled cDNA probes, normalized with α-tubulin, and expressed relative to control. (a) Changes in COX II mRNA levels in control (●) and azide-treated (■) (500 μM) C2C12 cells over 6 days relative to day 0 control. (b) mRNA levels relative to control for mitochondrial and nuclear transcripts in C2C12 cells treated with 50 μM (open bar) and 500 μM (closed bar) azide for 3 days (* denotes significant difference; $P < 0.05$).

4.2. Mitochondrial energetics during myogenesis

In mammalian skeletal muscle, hypermetabolic

treatments such as exercise training and chronic electrical stimulation lead to mitochondrial proliferation [13,14]. It is not clear if mitochondrial biogenesis is induced directly by neural/hormonal factors or indirectly from persistent disturbances in energy metabolism arising through the recruitment pattern. In mitochondrial myopathies, the resulting bioenergetic disturbance may induce expression of the nuclear-encoded respiratory genes ADNT1 and ATPβ [29]. It is unlikely that, during myogenesis, metabolic demand per se is the ultimate cause of mitochondrial biogenesis, given that the metabolic rate during differentiation (Fig. 1a,b, sum of oxidative and glycolytic rates) is relatively constant at 55–85 nmol ATP/min/mg protein.

Manifold changes in the activity of PDH (Fig. 1c,d) and other mitochondrial enzymes [25] coincided with a shift in energy metabolism from primarily glycolytic (66%) to predominantly oxidative (60%). A fundamental change in the relationship between glycolysis and respiration is also predicted in previous studies which showed a shift in adenine nucleotide translocase (ADNT) isoform expression. ADNT2 is an isoform expressed in glycolytic tissues whereas ADNT1 predominates in oxidative tissues such as skeletal and cardiac muscles. In C2C12 myoblasts, levels of mRNA decline for ADNT2 but increase for ADNT1 with the onset of differentiation and myotube formation [30]. Although there is no direct evidence for functional differences in the isoforms, the tissue distribution suggests ADNT2 may have a role in transferring glycolytic ATP into mitochondria (see [30]).

The elevated oxidative capacity accompanying C2C12 differentiation is perhaps best illustrated by the changes in the capacity for flux through PDH (Fig. 1c). The calculated maximal flux through myoblast PDHa is 54 nmol ATP/min/mg (PDHa = 3.6 nmol/min/mg × 15 nmol ATP/nmol pyruvate). This capacity approaches that predicted from respiration studies (25 nmol/min/mg) (Fig. 1b). During myogenesis there was a pronounced increase in both PDHt (Fig. 1c) and PDH activation state (Fig. 1d), leading to an increase in capacity to 480 nmol ATP/min/mg (15 nmol ATP/nmol pyruvate × 32 nmol/min/mg PDHa, Fig. 1c), 12.5-fold greater than that which would account for oxidative ATP requirements (37.5 nmol/min/mg, Fig. 1b). The increased capacity and high PDH activation state do not reflect a cellular response to an increased metabolic demand, as metabolic rate did not increase during differentiation (Fig. 1a). It can further be argued that the differentiating cell was not in an energetic deficit as glycolytic flux actually declined during myogenesis (Fig. 1a). This disparity suggests that regulatory conditions within the mitochondria (acetyl CoA/CoA, $NADH/NAD^+$, Ca^{2+}) were highly favorable for PDH interconversion towards PDHa, but were not necessarily indicative of energetic demand. There is some potential for these same regulatory factors to influence myogenesis and mitochondrial biogenesis. REBOX factor binds to the element in an NADH-dependent manner [6], although the conditions required (0–15 mM NADH) are not physiologically realistic. Ca^{2+} is an important regulator of both myogenesis and bioenergetics. Myogenesis is strictly dependent on the extracellular Ca^{2+} concentration [31]. The Ca^{2+} ionophore A23187 leads to an increase in expression of mitochondrial enzymes in primary cultures of rat muscle cells [17]. Thus, it has not been established if any of these bioenergetic regulators directly or indirectly influence mitochondrial gene expression. More information exists, at present, on the presence of *cis*-elements of nuclear-encoded mitochondrial genes than control of expression of *trans*-factors binding to these elements.

4.3. Azide effects on mitochondrial energetics and gene expression

Azide is a well known non-competitive inhibitor of COX, in this study demonstrating an I_{50} of approximately 1 mM (Fig. 2a). However, chronic treatment of C2C12 cells with azide in the μM range also reversibly decreases COX activity (Fig. 2b). Bennett et al. [32] treated rats with low levels of azide via osmotic pumps and created Alzheimer-like symptoms, including a depression of measurable COX activity. Chandel et al. [33] found that chronic hypoxia reduced the COX catalytic activity, although the mechanism by which this occurs is unclear. Similarly, the molecular mechanism by which low levels of azide inhibits COX activity is unknown. It did allow us to introduce small, measurable degrees of metabolic inhibition to examine the relationship between metabolic stress, mitochondrial energetics and gene ex-

pression. The azide effects were specific to COX, as neither CS nor complex I activity was affected (Fig. 2b). Azide did not cause changes in lactate production (Fig. 3a) or creatine phosphorylation (Fig. 3b) until more than 70% of the COX activity was inhibited.

Effects on COX II mRNA levels coincided with the point in the azide titration where effects on bioenergetic parameters were apparent (70% inhibition of COX). However, the changes in COX II mRNA with azide treatment are likely due to post-transcriptional regulation (e.g. mRNA stability) rather than an increased rate of transcription. Mitochondrial transcription is generally thought to be regulated by gene amplification and azide did not affect mtDNA copy number (Fig. 4a). Also, because of the polycistronic transcript, mRNA for COX II is synthesized stoichiometrically with COX I, COX III, and ATPase VI mRNA, the levels of which did not change with azide treatment (Fig. 5).

Although these results argue for an azide-induced increase in COX II mRNA stability, the mechanism by which this occurs is not clear. There is evidence for the presence of cytoplasmic proteins which bind the 3′ untranslated region of mRNA of liver isoforms of COX VIIa, VIII [34] and VIa [35]. However, there is no evidence for COX mRNA binding proteins within mitochondria. Changes in mitochondrial mRNA stability have been reported in developing rat liver [36] but the effect is not selective as all mRNA species are more stable. If the increase in COX II mRNA is due to stability, the nature of the azide sensitivity of the process is unknown. Yeast possess an NTP dependent exonuclease which controls mtRNA turnover [37]. A number of pathways have been shown to be sensitive to either hypoxia or azide. Hypoxia exerts a reciprocal control on transcription of glycolytic (increase) and mitochondrial (decrease) enzymes [16]. It has since been shown that the effects on glycolytic enzymes may be mediated by the oxygen sensitive transcription factor HIF (hypoxia-inducible factor). Some genes have been shown to be similarly sensitive to both hypoxia and azide, suggesting an oxygen independent regulatory pathway (see [38]). To our knowledge there is no evidence for a similar pathway regulating activity of mRNA binding proteins, particularly for mRNA species located within mitochondria.

In summary, pronounced changes in mitochondria and bioenergetics occurred during myogenesis in the absence of a hypermetabolic challenge. When metabolic conditions were perturbed directly by azide, at least one species of mitochondrial mRNA increases, likely due to an increase in mRNA stability.

Acknowledgements

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