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Regulation of skeletal muscle oxidative phenotype by classical NF-κB signalling

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ABSTRACT

Background: Impairments in skeletal muscle oxidative phenotype (OXPHEN) have been linked to the development of insulin resistance, metabolic inflexibility and progression of the metabolic syndrome and have been associated with progressive disability in diseases associated with chronic systemic inflammation. We previously showed that the inflammatory cytokine tumour necrosis factor-α (TNF-α) directly impairs muscle OXPHEN but underlying molecular mechanisms remained unknown. Interestingly, the inflammatory signalling pathway classical nuclear factor-κB (NF-κB) is activated in muscle in abovementioned disorders. Therefore, we hypothesised that muscle activation of classical NF-κB signalling is sufficient and required for inflammation-induced impairment of muscle OXPHEN.

Methods: Myotubes from mouse and human muscle cell lines were subjected to activation or blockade of the classical NF-κB pathway. In addition, wild-type and MISR (muscle-specific inhibition of classical NF-κB) mice were injected intra-muscularly with TNF-α. Markers and key regulators of muscle OXPHEN were investigated.

Results: Classical NF-κB activation diminished expression of oxidative phosphorylation (OXPHOS) sub-units, slow myosin heavy chain expression, activity of mitochondrial enzymes and potently reduced intra-cellular ATP levels. Accordingly, PGC-1/PPAR/NRF-1/Tfam signalling, the main pathway controlling muscle OXPHEN, was impaired upon classical NF-κB activation which required intact p65 trans-activation domains and depended on *de novo* gene transcription. Unlike wild-type myotubes, IκBα-SR myotubes (blocked classical NF-κB signalling) were refractory to TNF-α-induced impairments in OXPHEN and its regulation by the PGC-1/PPAR/NRF-1/Tfam cascade. In line with *in vitro* data, NF-κB blockade *in vivo* abrogated TNF-α-induced reductions in PGC-1α expression.

Conclusion: Classical NF-κB activation impairs skeletal muscle OXPHEN.

1. Introduction

Loss of skeletal muscle oxidative phenotype (OXPHEN) is observed in many diseases associated with chronic low-grade systemic inflammation including chronic obstructive pulmonary disease (COPD), chronic heart failure (CHF) and type II diabetes mellitus (T2DM) [1–8]. OXPHEN is defined as the collective of intrinsic cell-specific features determining fatigue resistance and capacity for mitochondrial substrate oxidation. Observed impairments in muscle OXPHEN in abovementioned disorders include a fibre-type shift from type I fibres towards more glycolytic type II fibres, a reduction in mitochondrial content, reduced activity levels of mitochondrial metabolic enzymes and a diminished muscle ATP content [1,3,9–11]. Illustrative of its significance in chronic disease prevention and progression, a disturbed muscle OXPHEN has been linked to the development of insulin resistance, metabolic inflexibility and progression of the metabolic syndrome and has been associated with progressive disability and a reduced quality of life in chronic disorders [12–15]. Importantly, impairments in muscle OXPHEN in chronic disease reflect pathological mechanisms beyond physical inactivity of which inflammation has been explored as a putative trigger [16].

Abbreviations: ATP, adenosine triphosphate; AV, adenoviral; CHF, chronic heart failure; COPD, chronic obstructive pulmonary disease; CS, citrate synthase; DBD, DNA binding domain; DMEM, Dulbecco's modified Eagle medium; ERR-α, estrogen-related receptor alpha; EM, electron microscopy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAD, β-hydroxy-acylCoA dehydrogenase; HBSS, Hank's balanced salt solution; HCBP, human carnitine-palmitoyl transferase B; HPLC, high-performance liquid chromatography; IKK-β, IκB kinase beta; IL-1β, interleukin 1 beta; IM, intra-muscular; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; KD, kinase-dead; LBD, ligand binding domain; Mfn, mitochondrial fusion gene; MISR, muscle-specific inhibitor of NF-κB super-repressor; MyHC, myosin heavy chain; NRF, nuclear respiratory factor; NF-κB, nuclear factor kappa B; OXPHEN, oxidative phenotype; OXPHOS, oxidative phosphorylation; PBS, phosphate-buffered saline; PGC-1, peroxisome proliferator-activated receptor gamma co-activator; PPAR, peroxisome proliferator-activated receptor; SD, standard deviation; SIRT-1, sirtuin 1; SR, super repressor; T2DM, type II diabetes mellitus; Tfam, mitochondrial transcription factor A; TNF-α, tumour necrosis factor alpha; WT, wild-type

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In the last decade, the PGC-1 signalling axis has emerged as the master intra-cellular signalling pathway controlling skeletal muscle OXPHEN [17]. The 2 main isoforms, PGC-1α and PGC-1β, serve as key co-activator molecules orchestrating a transcriptional programme that directs muscle fibre-type composition as well as mitochondrial capacity towards an improved OXPHEN [18,19]. This programme includes co-activation of nuclear respiratory factor 1 (NRF-1)-driven transcription of mitochondrial transcription factor A (Tfam) gene which is essential for mitochondrial biogenesis. In addition, PGC-1 isoforms facilitate peroxisome proliferator-activated receptor (PPAR) transcriptional activity of which the PPAR-α and PPAR-δ isoforms regulate mitochondrial substrate oxidation processes and muscle fibre-type composition [19]. Reduced expression levels of constituents of the PGC-1/PPAR/NRF-1/Tfam signalling axis have been shown in skeletal muscle of both COPD and T2DM patients and may well underlie impairments in muscle OXPHEN [16,20–22].

Low-grade systemic inflammation is a common denominator of many chronic diseases [4,23,24]. We previously showed that, in COPD, circulating and muscle levels of the pro-inflammatory cytokine tumour necrosis factor α (TNF-α) and its receptors are inversely associated with PGC-1/PPAR and oxidative gene mRNA expression levels in muscle [16,20]. Furthermore, *in vitro*, we showed that TNF-α directly impairs the OXPHEN of cultured muscle cells. Collectively this suggests a role for TNF-α in the (de)regulation of muscle OXPHEN [16].

The ubiquitously expressed classical nuclear factor kappa B (NF-κB) pathway is the main intra-cellular signalling cascade activated by inflammatory cytokines such as TNF-α. Under normal physiological conditions, the NF-κB sub-unit RelA/p65 is maintained primarily in the cytoplasm bound to an IκB repressor molecule. Active classical NF-κB signalling is triggered when inflammatory stimuli, such as TNF-α or interleukin 1 β (IL-1β), initiate the rapid phosphorylation and degradation of IκBα through IKK-β kinase activity. This liberates p65 to move into the nucleus and initiate transcription of NF-κB target genes [25]. Increased activation of the classical NF-κB pathway in muscle has been shown in COPD, CHF, and T2DM [26–30]. However, whether or not classical NF-κB signalling is causally involved in the (de)regulation of skeletal muscle OXPHEN in these disorders is unknown. We hypothesised that activation of classical NF-κB signalling impairs skeletal muscle OXPHEN and its regulation by the PGC-1/PPAR/NRF-1/Tfam signalling cascade. To address this, we modulated classical NF-κB signalling, in absence or presence of inflammatory cytokines, in cultured muscle cells and in mouse muscle tissue *in vivo* and investigated the impact on muscle OXPHEN and its molecular regulation.

2. Materials and methods

2.1. Cell culture

The murine C2C12 skeletal muscle cell line was obtained from the American Type Culture Collection (ATCC CRL1772; Manassas, VA, USA). The stable C2C12 NF-κB transcriptional activity reporter cell line was constructed as described previously [31] and C2C12-IκBα-SR cells (displaying blocked classical NF-κB signalling) were kindly provided by Dr. Guttridge (Ohio State University, OH, USA). C2C12 myoblasts were cultured and differentiated into mature multi-nucleated myotubes as described previously [31]. Mouse primary muscle cells were derived from hind and forelimbs of neonatal C57/BL6 mice [32] and were cultured according to the same protocol as the C2C12 cell line. Immortalised human muscle cells were provided by Dr. De la Garza-Rodea (Leiden University, The Netherlands). Additional detail regarding culture conditions is provided in the online resource.

2.2. Animals

Wild-type male C57/BL6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). MISR (muscle-specific IκBα Super-Repressor) animals were kindly provided by Dr. S. Shoelson (Joslin Diabetes Center, MA, USA). All animals were housed in a temperature-controlled room on a 12:12 h light-dark cycle with food pellets and water provided ad libitum. All procedures were performed with approval of the University's Institutional Animal Care and Use Committee and were in coherence with the EU Directive 2010/63/EU regarding animal experiments. Animals received either intra-muscular (IM) injections of phosphate buffered saline (PBS) (50 μL) or murine TNF-α (40 μg/kg in 50 μL PBS) delivered to the gastrocnemius of both hind limbs with a 30 gauge needle (Ultra-Fine II, Becton Dickinson, NJ, USA) while lightly anesthetised. Seven hours after IM injection, mice were sacrificed by halothane overdose. Gastrocnemius muscles were collected using standardised dissection methods, cleaned of excess fat and connective tissue, snap frozen in liquid nitrogen and stored at −80 °C.

2.3. Chemicals and reagents

TNF-α (Calbiochem, Nottingham, United Kingdom) and interleukin 1β (IL-1β) (Calbiochem) were dissolved in 0.1% bovine serum albumin (BSA) which also served as a vehicle control (0.005% final concentration) to a stock of 200 ng/ml. Actinomycin D (Sigma Aldrich, Saint Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mg/ml. Rosiglitazone-maleate (Alexis Biochemicals, Lausen, Switzerland), GW501516 (Alexis Biochemicals) and WY-14643 (Biomol, Plymouth Meeting, PA, USA) were all dissolved in DMSO to a stock concentration of 75 mM, 1 mM and 50 mM respectively. Pre-made adenoviral (AV) constructs (AV-CMV-IKK-β and AV-CMV-GFP) were purchased from Vector Biolabs (Philadelphia, USA). Viral expression constructs were dissolved in Dulbecco's modified Eagle medium (DMEM) with 2% BSA and 2.5% Glycerol (1×10^{10} PFU/ml) and further diluted in sterile Hank's balanced salt solution (HBSS) (Invitrogen, Leusden, the Netherlands) to a stock concentration of 2×10^8 PFU/ml. A final concentration of 2×10^7 PFU/ml was added to fully differentiated myotubes. SiRNA constructs targeting PGC-1α and scrambled control siRNA constructs were purchased from Invitrogen.

2.4. Western blot and quantitative PCR (Q-PCR)

Details regarding preparation of whole-cell lysates and western blotting procedures as well as information regarding RNA isolation, cDNA synthesis and Q-PCR can be found in the online resource.

2.5. ATP assay

Myotubes were washed in ice-cold PBS and harvested in 200 μl $HCLO_4$ by scraping with rubber policemen. Lysates were incubated on ice for 10 min and subsequently centrifuged for 10 min, 14000 rpm at 4 °C. Supernatant was removed and 8 μl 5 M K_2CO_3 was added. Pellets were used to determine total protein content according to manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Nucleotide profiles were determined by ion exchange high-performance liquid chromatography (HPLC) using a Whatman Partisphere SAX 4.6 × 125 mm column (5-μm particles) and a Whatman 10 × 2.5 mm AX guard column (Whatman Inc, ME, USA). The buffers used were: 9 mM NH4H2PO4, pH 3.5 (buffer A) and 325 mM NH4H2PO4,500 mM KCl, pH 4.4 (buffer B). Nucleotides were eluted with a gradient from 100% buffer A to 90% buffer B in 60 min at a flow rate of 1 ml/min.

2.6. Enzyme activity assays

Activity of β-hydroxy-acylCoA dehydrogenase (HAD) (EC 1.1.1.35) and citrate synthase (CS) (EC2.3.3.1) was measured as described previously [33]. Enzyme activity levels were measured at 37 °C and at PH 7.3 (HADH) or PH 8.0 (CS) and were corrected for total protein content

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