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

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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.
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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-hydroxyacyl CoA 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

which was determined using a detergent compatible protein determination assay (Bio-Rad).

2.7. Electron microscopy

For determination of ultrastructural mitochondrial morphology, C2C12 myotubes cultured in 35 mm dishes were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Postfixation was performed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) supplemented with 1.5% potassium ferrocyanide. The samples were then dehydrated and embedded in epon. Ultrathin sections were examined using a Philips CM100 electron microscope.

2.8. Transfections and plasmids

For the assessment of NF-κB transcriptional activity, C2C12 cells were stably transfected with a 6κB-TK luciferase plasmid (NF-κB reporter) as previously described [31]. For transient transfections, C2C12 cells were grown to be 50% confluent and transfected by nanofectin according to manufacturer's instructions (PAA, Pasching, Austria). For assessment of general PPAR-dependent transcriptional activity, C2C12 myoblasts were transfected with a human CPT-1B promoter (HCBP) luciferase reporter construct which was a kind gift from Dr. M. van Bilsen (Maastricht University, The Netherlands). For assessment of specific PPAR transcriptional activity, the pM1-hPPAR-α (LBD)/Gal4(DBD), pM1-hPPAR-δ(LBD)/Gal4(DBD), pM1-PPAR-γ(LBD)/Gal4(DBD) and UASx4luc reporter constructs were kindly provided by Dr. Kristiansen (University of Southern Denmark, Odense, Denmark) and are previously described [34]. To investigate the involvement of classical NF-κB signalling, the following expression plasmids were transiently transfected into C2C12 myoblasts according to the same protocol: IκBα-SR (pSFFV-NEO-IκBα-SR) (constitutively expressed under control of the SFFV-LTR); kind gift from Dr. Rosa Ten (Mayo Clinic, Rochester, MN, USA), p65 wild-type (p65 WT), IKK-β kinase-dead (IKK-β KD) and constitutive active IKK-β (c.a. IKK-β); all kind gifts from Dr. Michael Karin (University of California, San Diego, La Jolla, CA, USA). p65 mutants with 1 (p65-534), 2 (p65-521), or 3 (p65-313) deleted trans-activation domains were kindly provided by Dr. D. Guttridge (Ohio State University, OH, USA). For assessment of Tfam promoter activity and NRF-1 transcriptional activity, cells were transfected with mTFA-RC4wt/PGL3 or p4xNRF-1LUC reporter construct [35,36]. The β-MyHC (I, slow) promoter reporter construct was kindly provided by Dr. M. Sandri (University of Padova, Italy). The PcDNA3.1 empty vector was used as an empty vector control when appropriate. All transient transfections were corrected for β-galactosidase (β-Gal) activity. Details regarding reporter assays are provided in the online resource.

2.9. Electrophoretic mobility shift assay (EMSA)

Details regarding preparation of nuclear extracts are provided in the online resource. NF-κB DNA binding activity was assessed in nuclear extracts by analysis of complexes binding to an oligonucleotide containing a κB consensus sequence (Santa Cruz, Santa Cruz, CA, USA). Two micrograms of nuclear protein was used per binding reaction and protein–DNA complexes were resolved on a 5% polyacrylamide gel in 0.25× Tris–borate–EDTA buffer at 120 V for 2 h. Gels were dried and exposed to film (X-Omat Blue XB-1, Kodak, Rochester, NY, USA). Supershift reactions were performed by pre-incubation of the nuclear extracts with an antibody specific to the p65 subunit of NF-κB (Santa Cruz).

2.10. Statistics

Data were analysed according to the guidelines of Altman et al. using SPSS (SPSS Inc., Chicago, IL, USA). Non-parametric tests and unpaired *t*-tests were used when appropriate. Data are represented as the mean ± SD. A *p*-value of less than 0.05 was considered to be significant.

3. Results

3.1. Classical NF-κB activation is sufficient to reduce expression and function of mitochondrial proteins

To investigate whether a reduction in protein content of mitochondrial oxidative phosphorylation (OXPHOS) complexes, as observed in response to TNF-α [16], is common to classical NF-κB activation and is associated with correspondingly decreased mRNA expression levels, C2C12 myotubes were stimulated with IL-1β or subjected to IKK-β over-expression. OXPHOS protein as well as mRNA levels were investigated.

IL-1β-induced NF-κB activation was confirmed (Fig. S1A) and over-time not only decreased OXPHOS sub-unit protein levels but also significantly reduced mRNA levels of the respective OXPHOS complexes (Fig. 1a, b). Similarly, genetic NF-κB activation by adenoviral (AV) delivery of IKK-β (Fig. S1B, C) decreased both OXPHOS protein and mRNA levels (Fig. 1c, d). Furthermore, besides reducing OXPHOS expression levels, genetic NF-κB activation in C2C12 myotubes was sufficient to reduce activity levels of mitochondrial enzymes involved in substrate oxidation (citrate synthase: CS; Krebs cycle and β-hydroxyacyl CoA dehydrogenase: HAD; fatty acid β-oxidation) (Fig. 1e, f).

3.2. Classical NF-κB activation is required for TNF-α-induced reductions in expression of mitochondrial proteins

Similar to IL-1β and AV-IKK-β, TNF-α-induced NF-κB activation (Fig. S1A) decreased OXPHOS sub-unit mRNA levels, not only in C2C12 myotubes but also in mouse- and human primary myotubes (Fig. 2a–f). In contrast to its effects on wild-type C2C12 myotubes, TNF-α failed to reduce OXPHOS mRNA and protein expression in C2C12 myotubes (Fig. 2d–g) in which classical NF-κB activation was effectively blocked (IκBα-SR C2C12) (Fig. S1D–F). Moreover, TNF-α stimulation of wild-type C2C12 myotubes reduced cellular ATP content to 50% of control which was completely abrogated in IκBα-SR myotubes (Fig. 2h). In contrast, TNF-α-induced reductions in HAD enzyme activity were not attenuated in IκBα-SR C2C12 myotubes (Fig. 2i). Although basal HAD activity was increased in IκBα-SR myotubes compared to WT myotubes (Fig. 2i), basal OXPHOS sub-unit protein or mRNA expression levels were similar in both cell lines (Fig. S2A, B). In addition, in contrast to IKK-β over-expression, TNF-α stimulation of wild-type C2C12 myotubes failed to affect CS activity levels (data not shown).

3.3. Classical NF-κB activation perturbs mitochondrial morphology

Considering the aberrant expression and activity of essential mitochondrial proteins in conditions of classical NF-κB activation, mitochondrial morphology was assessed in C2C12 myotubes. In response to TNF-α-induced NF-κB activation, mitochondria appeared swollen and less elongated compared to mitochondria from vehicle-treated myotubes (Fig. 3a). As mitochondrial fission and fusion genes are key regulators of mitochondrial dynamics, expression levels of these markers were investigated in response to classical NF-κB activation. TNF-α significantly reduced mRNA expression levels of mitochondrial fusion (mfn-1, mfn-2 and Opa1) and fission genes (MTP18 and FIS1) in C2C12 myotubes (Fig. 3b, c). Similar findings (with exception of decreased Opa1 mRNA levels) were found in mouse primary myotubes in response to TNF-α (Fig. 3d). In addition, IL-1β stimulation as well as AV-IKK-β delivery to wild-type C2C12 myotubes similarly decreased selected fusion and fission gene mRNA expression levels (Fig. 3e, f). In

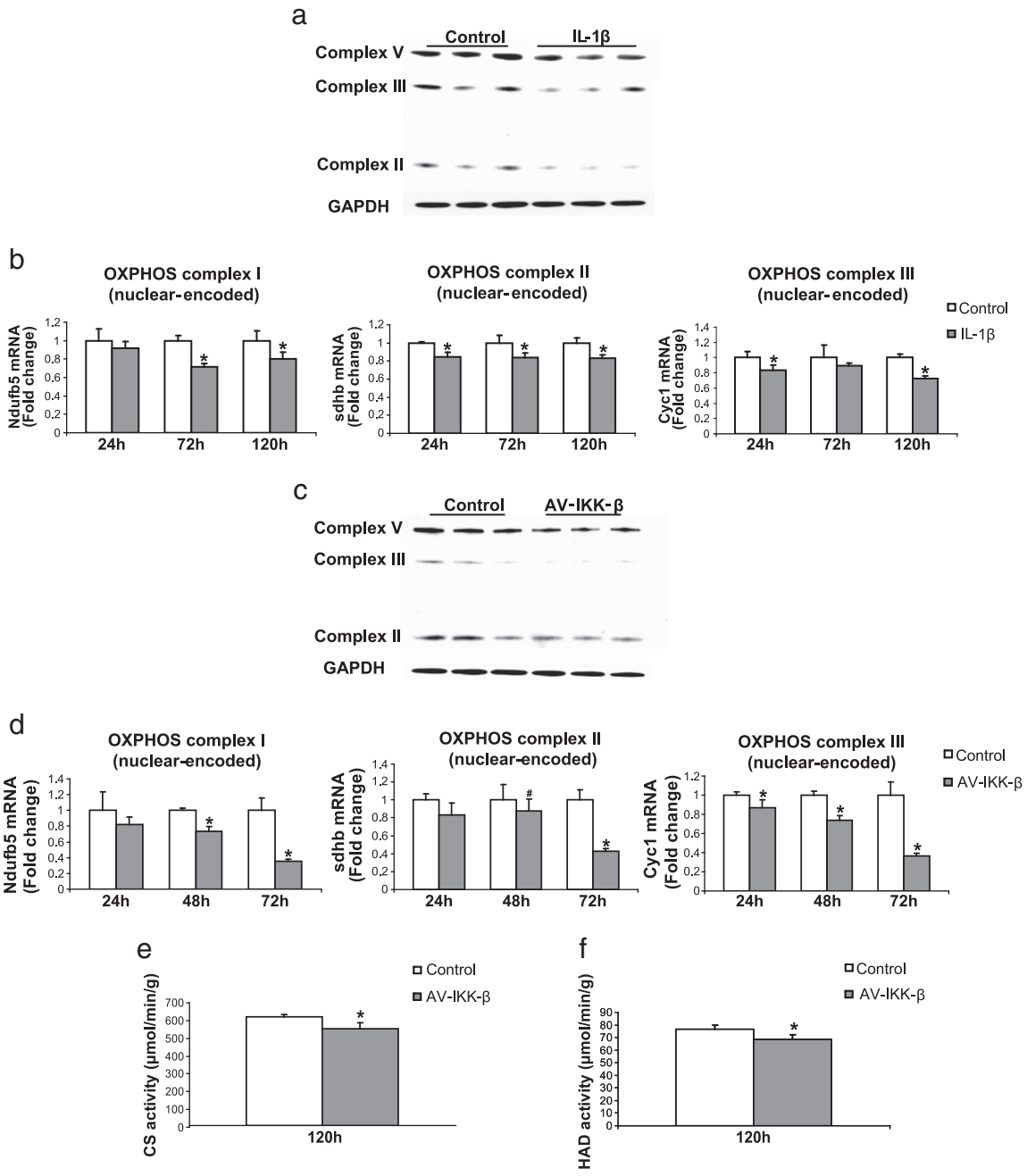


Fig. 1. Classical NF-κB activation impairs expression and function of mitochondrial proteins C2C12 myoblasts were differentiated for 5 days in differentiation medium into fully mature myotubes. a, b) Myotubes were stimulated with vehicle (bovine serum albumin; control) or interleukin-1β (IL-1β; 10 ng/ml) for 120 h (a) or for the indicated time (b). c–f) Myotubes were infected with an adenoviral (AV) construct expressing IKK-β or an AV-GFP construct as control for 120 h (c, e, f) or for the indicated time (d). Cells were harvested at the indicated time-points and oxidative phosphorylation (OXPHOS) sub-unit mRNA and protein expression and β-hydroxy-acyl CoA dehydrogenase (HAD) and citrate synthase (CS) enzyme activities were determined. Western blots were corrected for total protein and GAPDH was used as a loading control. Gene expression data were corrected by using a GeNorm normalisation factor calculated from the expression of 4 housekeeping genes (cyclophilinA, β2-microglobuline, hypoxanthine-guanine phosphoribosyltransferase and glyceraldehyde-3-phosphate dehydrogenase). Enzyme activity levels were corrected for total protein content. Values are expressed as mean ± SD from triplicate samples (experiments $n = 3$). Significance compared to control: * $p \leq 0.05$, # $p \leq 0.1$.

contrast to wild-type C2C12 cells, mitochondrial fusion and fission gene transcript levels increased rather than decreased in response to TNF-α stimulation in IκBα-SR myotubes (Fig. 3b, c).

3.4. Activation of classical NF-κB signalling impairs slow MyHC expression

IL-1β stimulation of C2C12 myotubes decreased MyHC slow protein and MyHC I mRNA levels while MyHC fast protein and MyHC IIx mRNA levels were unaffected (Fig. 4a–c). Similarly, AV-IKK-β delivery to C2C12 myotubes potently reduced MyHC slow protein and MyHC I mRNA levels without altering MyHC fast protein or MyHC IIx mRNA abundance (Fig. 4d–g). In line with these data, a constitutive active form of IKK-β (IKK-β c.a.) was sufficient to diminish MyHC I promoter activation, while transfection of a kinase-dead isoform of IKK-β (IKK-β KD) had no significant effect (Fig. 4h). Importantly, we verified that both inflammatory cytokine-induced and

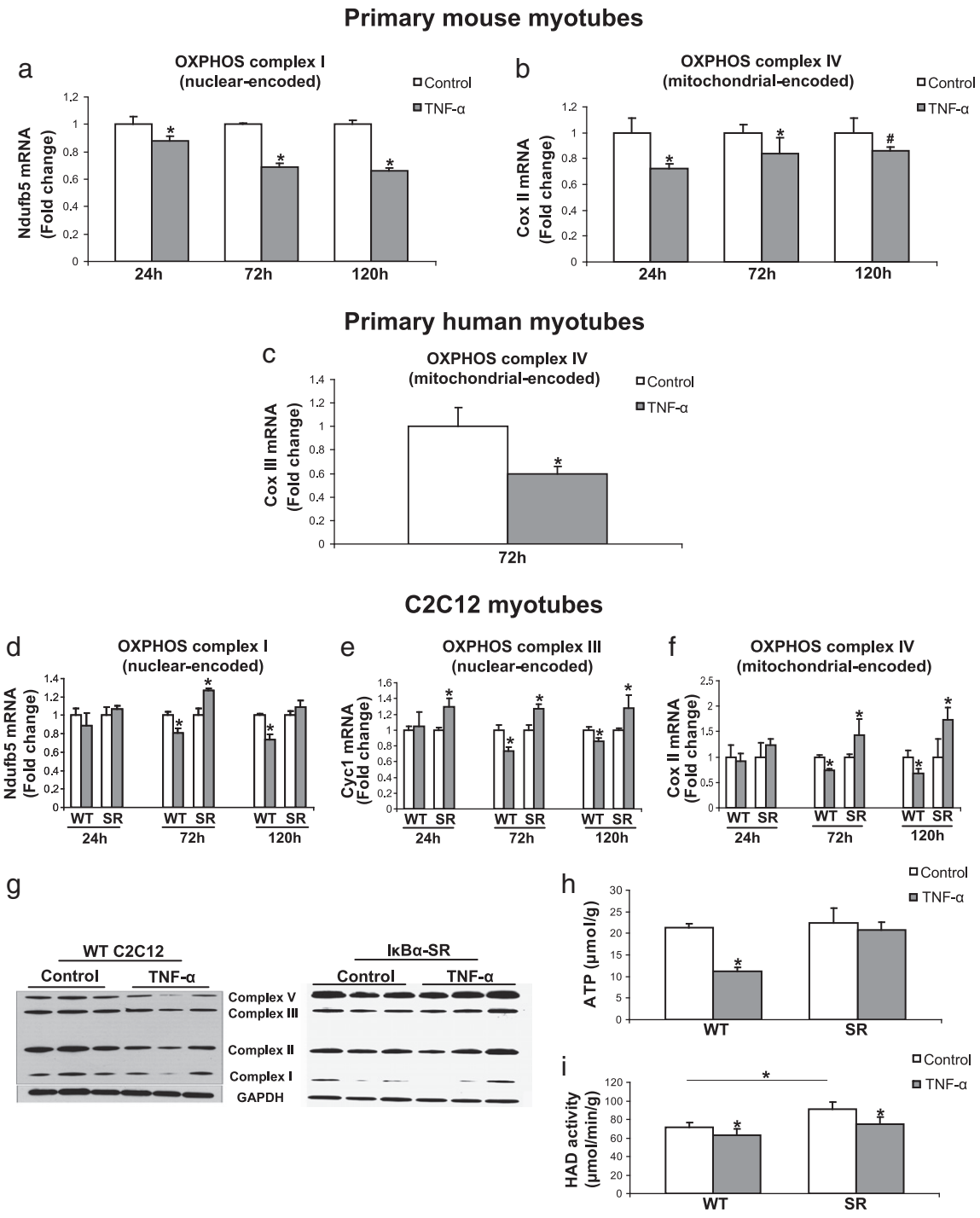
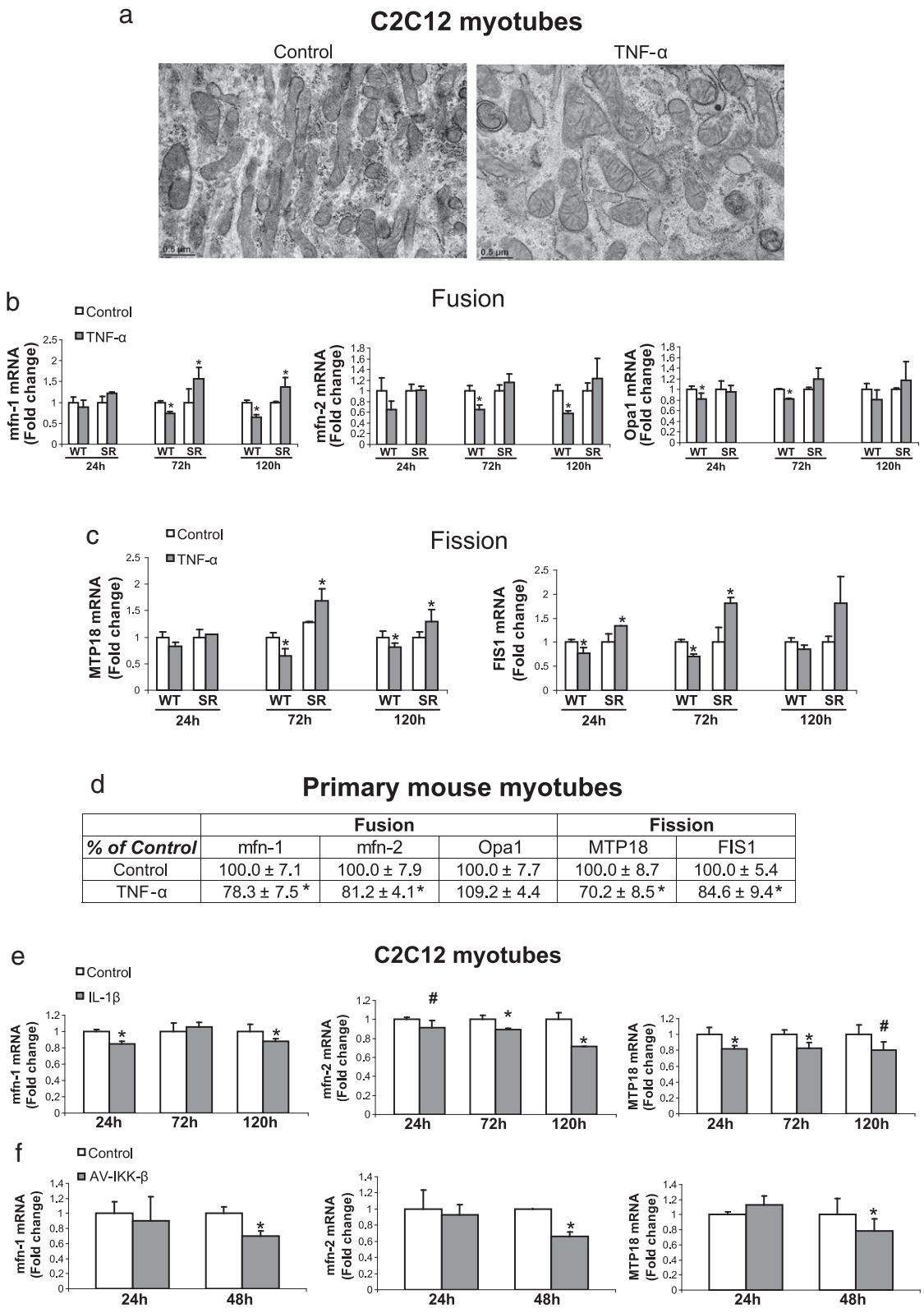


Fig. 2. Classical NF-κB activation is required for TNF-α-induced reductions in expression of mitochondrial proteins. a–c) Mouse and human primary myoblasts were differentiated for 5 days in differentiation medium into fully mature myotubes. Myotubes were subsequently stimulated with vehicle (bovine serum albumin; control) or tumour necrosis factor α (TNF-α; 10 ng/ml) for the indicated time and oxidative phosphorylation (OXPHOS) sub-unit mRNA expression was determined by Q-PCR. d–i) Wild-type (WT) C2C12 or C2C12-IκBα-super-repressor (SR) myoblasts were differentiated for 5 days in differentiation medium into fully mature myotubes. Myotubes were stimulated with vehicle (bovine serum albumin; control) or TNF-α (10 ng/ml) for 120 h or for the indicated times and OXPHOS sub-unit mRNA and protein expression as well as β-hydroxy-acyl CoA dehydrogenase (HAD) enzyme activity and ATP content were determined. Western blots were corrected for total protein and GAPDH was used as a loading control. Gene expression data were corrected by using a GeNorm normalisation factor calculated from the expression of 4 housekeeping genes (cyclophilinA, β2-microglobuline, hypoxanthine-guanine phosphoribosyltransferase and glyceraldehyde-3-phosphate dehydrogenase). Enzyme activity levels and ATP content were corrected for total protein content. Values are expressed as mean ± SD from triplicate samples (experiments $n = 3$). Significance compared to control: * $p \leq 0.05$, # $p \leq 0.1$.

genetically-induced classical NF-κB activation decreased expression levels of type I, slow MyHC in mouse- and human primary myotubes (Fig. S3A–C).

In wild-type C2C12 myotubes, TNF-α-induced reductions in MyHC I mRNA and MyHC slow protein became apparent after 24 h and 48 h of TNF-α stimulation respectively (Fig. S3D, E and Fig. 4j).



	Fusion			Fission	
% of Control	mfn-1	mfn-2	Opa1	MTP18	FIS1
Control	100.0 ± 7.1	100.0 ± 7.9	100.0 ± 7.7	100.0 ± 8.7	100.0 ± 5.4
TNF-α	78.3 ± 7.5 *	81.2 ± 4.1*	109.2 ± 4.4	70.2 ± 8.5 *	84.6 ± 9.4*

Fig. 3. Classical NF-κB activation perturbs mitochondrial morphology. a) C2C12 myoblasts were differentiated for 5 days in differentiation medium into fully mature myotubes. Myotubes were subsequently stimulated with vehicle (bovine serum albumin; control) or tumour necrosis factor α (TNF-α; 10 ng/ml) for 120 h and transmission electron microscopy was applied to visualise mitochondrial morphology. b, c) Wild-type (WT) C2C12 or C2C12-IκBα-super-repressor (SR) myoblasts were differentiated for 5 days in differentiation medium fully mature myotubes. Myotubes were stimulated with vehicle (bovine serum albumin; control) or TNF-α (10 ng/ml) for the indicated times. mRNA levels of mitochondrial fission and fusion genes were determined by Q-PCR. d) Mouse primary myoblasts were differentiated into mature myotubes and treated with vehicle (bovine serum albumin; control) or TNF-α (10 ng/ml) for 72 h. mRNA levels of mitochondrial fission and fusion genes were determined by Q-PCR. e, f) C2C12 myoblasts were differentiated for 5 days in differentiation medium into fully mature myotubes. Myotubes were subsequently stimulated with vehicle (bovine serum albumin; control) or interleukin-1β (IL-1β; 10 ng/ml) or infected with an adenoviral (AV) construct expressing IKK-β or an AV-GFP construct as control for the indicated time. Gene expression data were corrected by using a GeNorm normalisation factor calculated from the expression of 4 housekeeping genes (cyclophilinA, β2-microglobuline, hypoxanthine-guanine phosphoribosyltransferase and glyceraldehyde-3-phosphate dehydrogenase). Values are expressed as mean ± SD from triplicate samples (experiments $n = 3$). Significance compared to control: * $p \leq 0.05$, # $p \leq 0.1$.

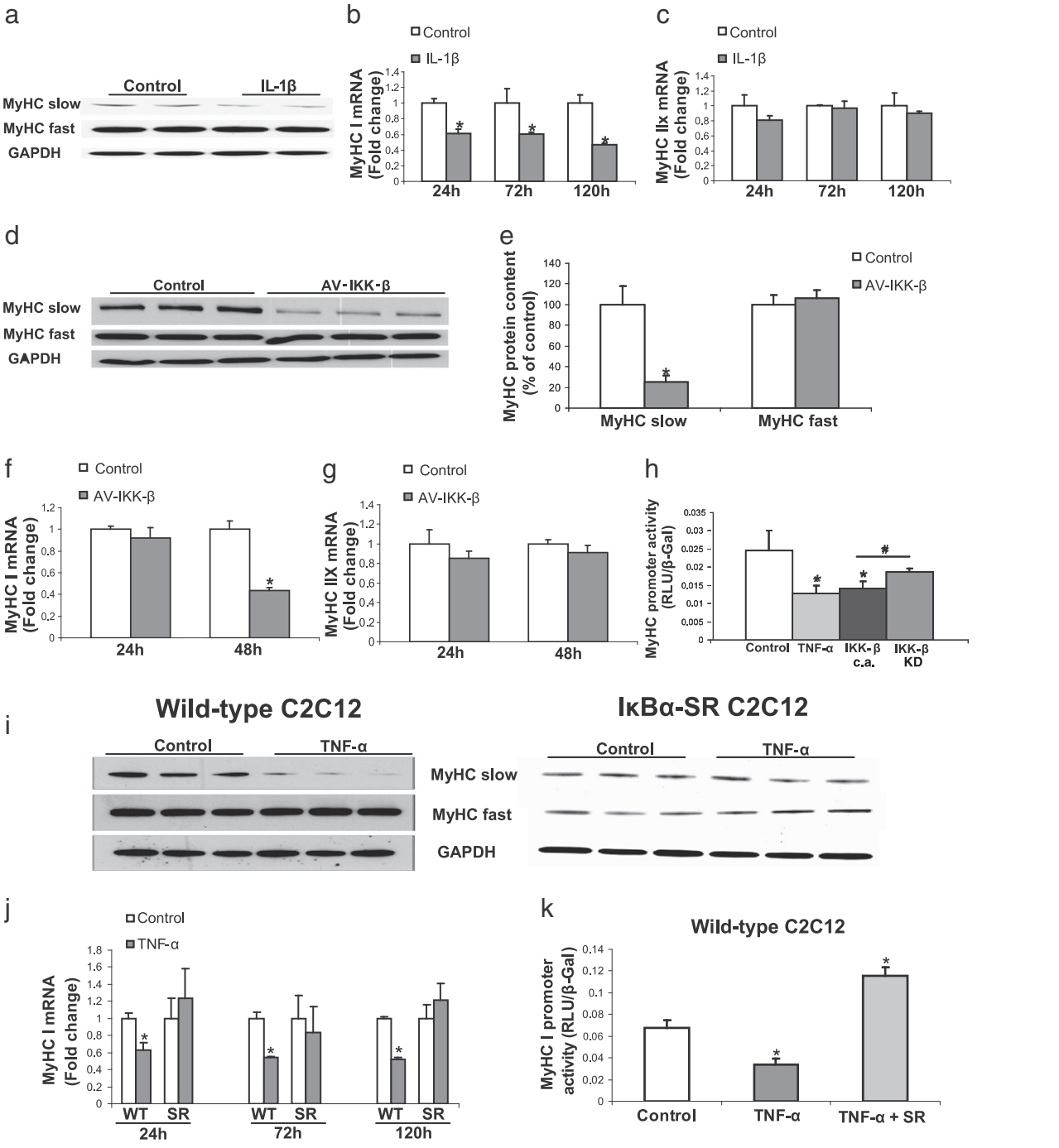


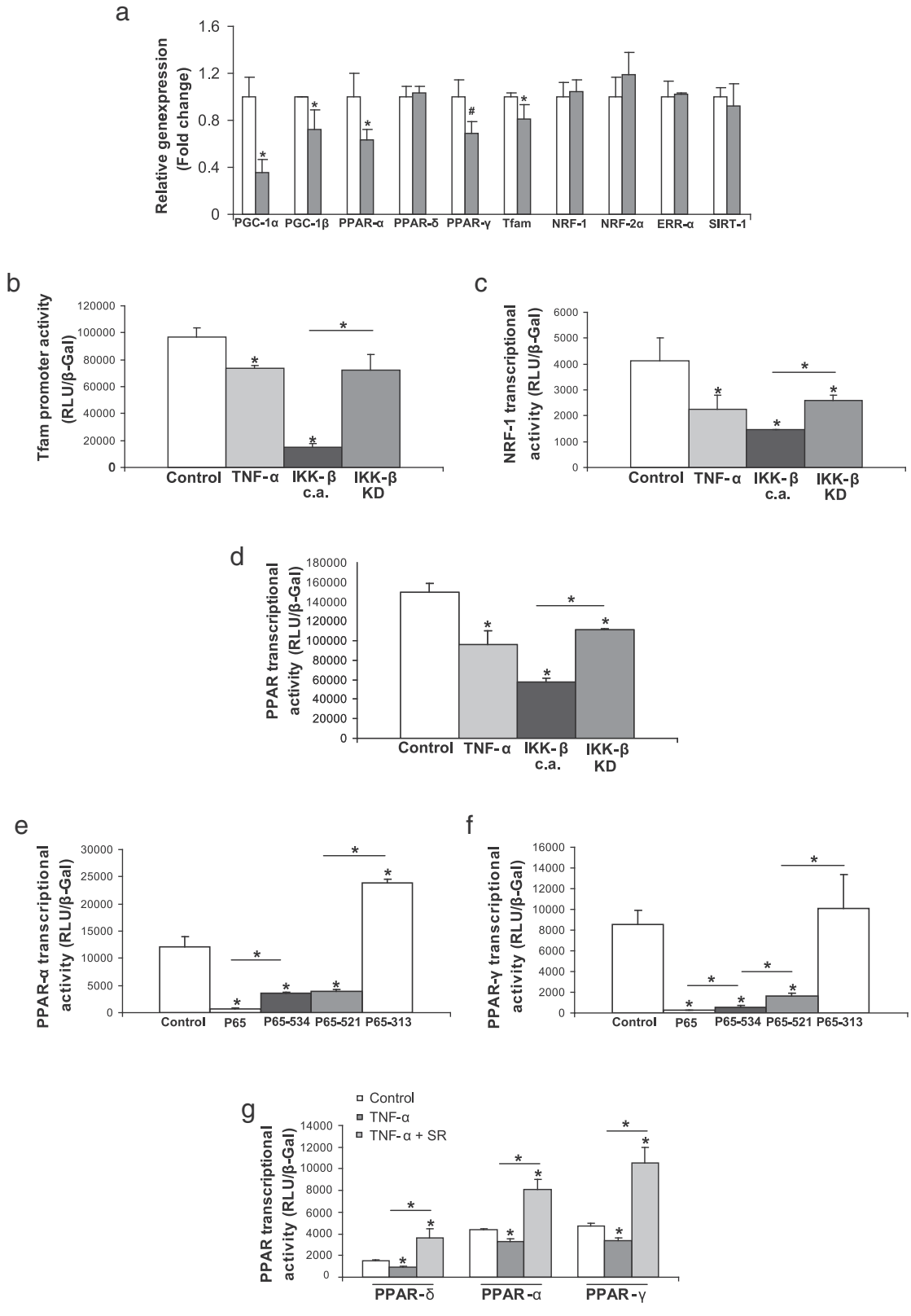
Fig. 4. Classical NF-κB activation impairs slow, type I MyHC expression. a–g) C2C12 myoblasts were differentiated for 5 days in differentiation medium into fully mature myotubes. Myotubes were subsequently stimulated with vehicle (bovine serum albumin; control) or interleukin-1β (IL-1β; 10 ng/ml) or infected with an adenoviral (AV) construct expressing IKK-β or an AV-GFP construct as control for 120 h or the indicated time. MyHC slow and MyHC fast protein and MyHC I and IIx mRNA levels were determined by Western blotting and Q-PCR respectively. h) C2C12 myoblasts were transiently transfected with a myosin I slow promoter reporter construct. Simultaneously, plasmids encoding empty vector (PcDNA3.1) or plasmids encoding constitutive active IKK-β (c.a. IKK-β) or a kinase-dead IKK-β isoform (IKK-β KD) were co-transfected. Cells were stimulated with TNF-α (10 ng/ml) or vehicle (bovine serum albumin). Twenty-four hours after transfection cells were lysed, luciferase activity was determined and normalised for β-galactosidase activity. i, j) Wild-type (WT) C2C12 or C2C12-IκBα-super-repressor (SR) myoblasts were differentiated for 5 days in differentiation medium into fully mature myotubes. Myotubes were stimulated with vehicle (bovine serum albumin; control) or TNF-α (10 ng/ml) for 120 h or the indicated time. MyHC slow and MyHC fast protein and MyHC I mRNA levels were determined by Western blotting and Q-PCR respectively. k) C2C12 myoblasts were transiently transfected with a myosin slow, I promoter reporter construct and PcDNA3.1 as an empty vector or a plasmid expressing the IκBα-super repressor (IκBα-SR). Twenty-four hours post-transfection cells were treated with vehicle (bovine serum albumin; control) or TNF-α (10 ng/ml) for 24 h. Subsequently, cells were lysed, luciferase activity was determined and normalised for β-galactosidase activity. Gene expression data were corrected by using a GeNorm normalisation factor calculated from the expression of 4 housekeeping genes (cyclophilinA, β2-microglobuline, hypoxanthine-guanine phosphoribosyltransferase and glyceraldehyde-3-phosphate dehydrogenase). Western blots were corrected for total protein and GAPDH was used as a loading control. Values are expressed as mean ± SD from triplicate samples (experiments $n = 3$). Significance compared to control: * $p \leq 0.05$.

No significant reductions were found when shorter stimulation times were applied (data not shown). Compared to wild-type cells, TNF-α-induced reductions in MyHC slow protein and MyHC I mRNA levels were completely abrogated in C2C12 myotubes with blocked classical NF-κB signalling (Fig. 4i, j). In line with protein and mRNA data, TNF-α reduced MyHC type I promoter activity which was prevented upon transient expression of non-degradable mutant SR-IκBα protein (Fig. 4k).

3.5. Classical NF-κB activation impairs the regulation of muscle oxidative phenotype

Multiple molecules have been implicated in the regulation of skeletal muscle OXPHEN [19,37]. As depicted in Fig. 5a, TNF-α significantly reduced mRNA expression levels of PGC-1α, PGC-1β, PPAR-α and Tfam while expression levels of other known regulators of muscle OXPHEN (NRF-1, PPAR-δ, NRF-2α, ERR-α and SIRT-1) were unaltered.

As shown in Fig. 5b, C, activation of classical NF-κB signalling by TNF-α or transient over-expression of c.a. IKK-β in C2C12 cells potently reduced Tfam promoter activity as well as NRF-1 transcriptional activity, both of which depend on PGC-1 mediated co-activation



[38]. Transfection of kinase-dead IKK-β on the other hand resulted in significant sparing of reporter activity compared to transfection of the IKK-β c.a. isoform. In addition, TNF-α reduced general PPAR transcriptional activity as well as basal and agonist-induced transcriptional activity of individual PPAR isoforms (Fig. 5d, g and Fig. S4A). Similar to the observations using Tfam and NRF-1 reporter constructs, over-expression of c.a. IKK-β diminished PPAR transcriptional activity which was significantly attenuated when a kinase-dead IKK-β isoform was over-expressed (Fig. 5d). As p65 is the DNA-binding protein down-stream of IKK-β in the classical NF-κB pathway [25], we next investigated whether p65 directly interferes with the regulation of muscle OXPHEN. As shown in Fig. 5e, f, over-expression of wild-type p65 potently decreased PPAR-α and PPAR-γ transcriptional activity and, interestingly, serial deletion of 1, 2 or all 3 p65 trans-activation domains increasingly restored PPAR transcriptional activity (Fig. 5e, f). TNF-α-induced reductions in transcriptional activity of the specific PPAR isoforms were abrogated when classical NF-κB activity was blocked by over-expression of the IκBα-SR construct (Fig. 5g).

3.6. Classical NF-κB activation reduces PGC-1 expression

TNF-α decreased both PGC-1α and PGC-1β mRNA levels over-time in mouse primary myotubes as it did in C2C12 myotubes (Fig. 6a, b). A more detailed time-course investigation revealed that TNF-α-induced reductions in PGC-1α mRNA in C2C12 myotubes became apparent after 8 h and persisted over time while reductions in PGC-1β mRNA levels only occurred after 72 h of TNF-α exposure (Fig. 6c, d). Moreover, TNF-α-induced reductions in PGC-1α but not PGC-1β expression levels consistently depended on intact classical NF-κB signalling (Fig. 6c, d). In order to verify whether TNF-α reduces muscle PGC-1α mRNA levels *in vivo* and whether this depended on classical NF-κB activation, we injected WT mice and MISR mice (genetically blocked NF-κB signalling by stable muscle-specific expression of the IκBα-SR construct) intra-muscularly with TNF-α. TNF-α-induced p65 DNA binding and inflammatory gene expression was quantified 7 h after intra-muscular injection and found to be abrogated in MISR mice compared to WT animals (Fig. S5A, B). Injection of TNF-α significantly decreased PGC-1α, not PGC-1β (data not shown), mRNA levels in WT animals but not in MISR mice (Fig. 6e). In addition, to investigate whether activation of classical NF-κB is sufficient to reduce PGC-1 expression levels, we used IL-1β or AV-IKK-β as NF-κB activating stimuli. In C2C12 myotubes, both IL-1β stimulation and AV-IKK-β delivery, like TNF-α, decreased PGC-1α and PGC-1β mRNA levels (Fig. 6f, g). Interestingly, pre-treatment of differentiated C2C12 myotubes with a RNA polymerase inhibitor, actinomycin D, before TNF-α treatment abolished TNF-α-induced reductions in PGC-1α mRNA (Fig. 6h) and PGC-1α-regulated genes (MyHC I and Cox II) (Fig. S6A, B). Knock-down of PGC-1α decreased mRNA levels of MyHC I, OXPHOS sub-units and mitochondrial fusion and fission genes, recapitulating the effects of TNF-α stimulation (Fig. S6C–E).

4. Discussion

In the present study, we unravelled a molecular mechanism underlying the detrimental effects of TNF-α on skeletal muscle OXPHEN. A detailed investigation of key markers and regulators of muscle OXPHEN and a dissection of inflammatory signalling revealed that intact classical NF-κB signalling is required for TNF-α-induced impairments in muscle OXPHEN and that activation of classical NF-κB signalling is sufficient to impair multiple aspects of skeletal muscle OXPHEN. Furthermore, as a potential underlying mechanism, NF-κB activation proved to be required and sufficient for reducing PGC-1α mRNA expression levels *in vitro* and *in vivo* and for disturbing PGC-1-co-activated signalling events that signify key steps in OXPHEN regulation.

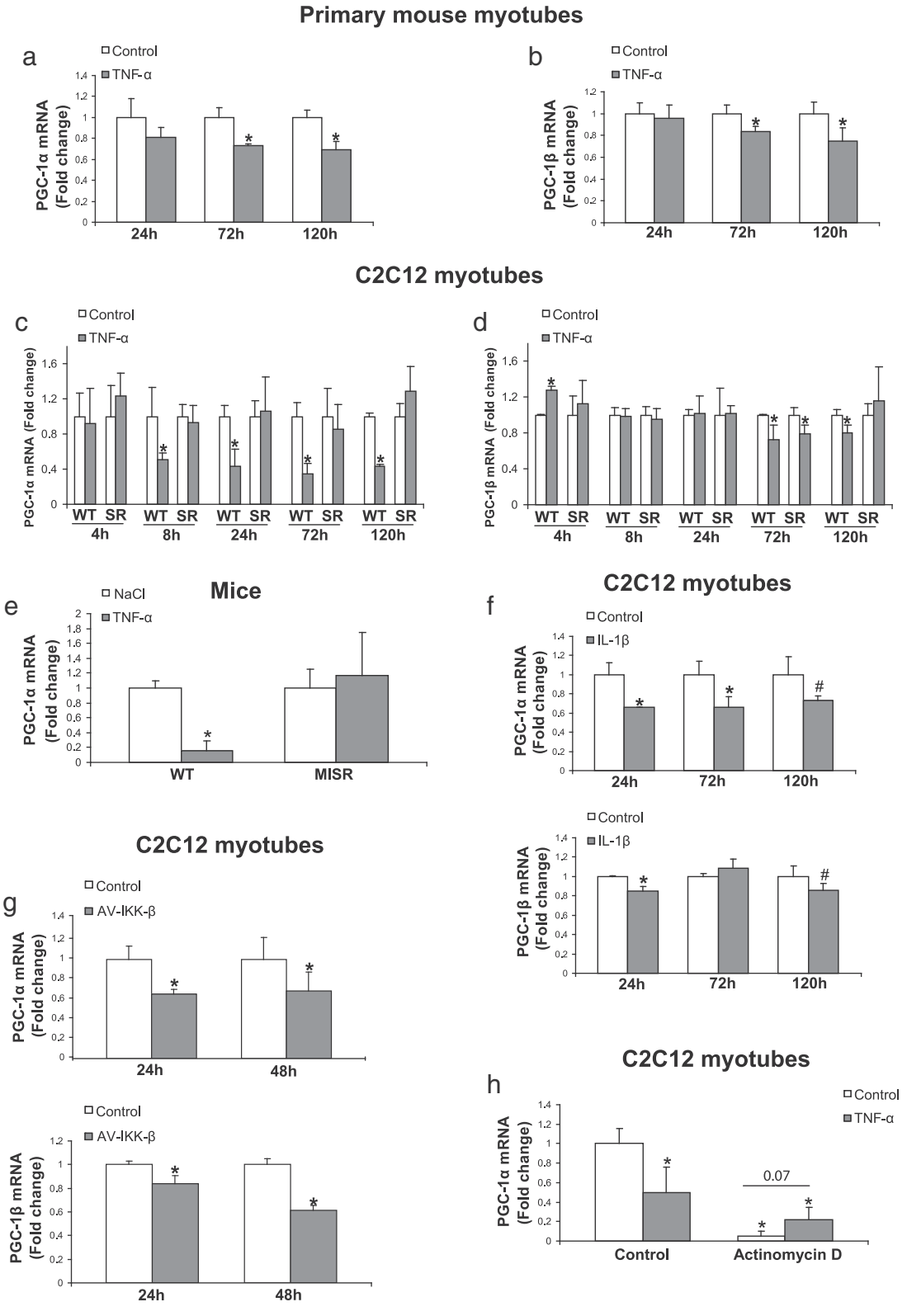
Interestingly, similar to our findings in skeletal muscle, TNF-α also depresses mitochondrial function and content in other cell types. In cardiac myocytes, reductions in expression levels of OXPHOS complex I, decreases in ATP content and impairments in mitochondrial function upon TNF-α exposure have been described *in vitro* and *in vivo* [39–41]. Furthermore, blockade of TNF-α proved to be sufficient to ameliorate cardiac mitochondrial dysfunction in a mouse model of experimental heart failure [42]. In addition, in white and brown adipocytes, TNF-α impaired mitochondrial biogenesis, decreased expression and activity levels of constituents of fatty acid β-oxidation, the Krebs cycle as well as OXPHOS events with a subsequent decrease in cellular ATP content [43–45]. These findings imply that TNF-α-induced disturbances in OXPHEN are not restricted to skeletal muscle and may involve a mechanism conserved within various tissues.

The molecular mechanisms by which TNF-α impairs mitochondrial function however have not been properly addressed yet. In muscle, we now convincingly show that classical NF-κB activation is required for TNF-α-induced impairment of all constituents of cellular OXPHEN that were examined. In line with our data, Cogswell et al., using lymphoma and fibrosarcoma cell lines, demonstrated that NF-κB blockade prevented TNF-α-induced reductions in the expression of mitochondrial-encoded OXPHOS sub-units [46]. In addition to the requirement of intact NF-κB signalling, we tested whether classical NF-κB activation, in absence of inflammatory cytokines, was sufficient to impair cellular OXPHEN. To this end, we applied IL-1β rather than TNF-α to myotube cultures as an alternative NF-κB activating stimulus. Although TNF-α and IL-1β feed into the classical NF-κB pathway via different receptors, classical NF-κB activation by both of these inflammatory cytokines converges at the level of the IKK complex [47]. Similar to TNF-α, when IL-1β was used, an impaired muscle OXPHEN was observed. Consistent with these data, IL-1β was shown to inhibit mitochondrial respiration and ATP synthesis in primary hepatocytes and hepatocyte cell lines [48–50]. It has to be noted however that the impact of IL-1β on muscle OXPHEN in our study was less pronounced when compared to TNF-α, which may be due to a smaller degree of NF-κB activation upon chronic stimulation in our system. In addition to experiments with IL-1β or TNF-α as NF-κB activators genetic activation of classical NF-κB by IKK-β

Fig. 5. Classical NF-κB activation impairs the regulation of muscle oxidative phenotype a) WT C2C12 myoblasts were differentiated for 5 days into mature myotubes. Myotubes were stimulated with vehicle (bovine serum albumin; control) or TNF-α (10 ng/ml) for 72 h. PGC-1α, PGC-1β, PPAR-α, PPAR-δ, PPAR-γ, Tfam, NRF-1, NRF-2α, ERR-α and SIRT-1 mRNA levels were determined by Q-PCR. Gene expression data were corrected by using a GeNorm normalisation factor calculated from the expression of 4 housekeeping genes (cyclophilinA, β2-microglobuline, hypoxanthine-guanine phosphoribosyltransferase and glyceraldehyde-3-phosphate dehydrogenase). b, c) C2C12 myoblasts were transiently transfected with either b) a Tfam promoter reporter construct or c) a NRF-1 transcriptional activity reporter plasmid. Simultaneously, plasmids encoding empty vector (PcDNA3.1) or plasmids encoding constitutive active IKK-β (c.a. IKK-β) or a kinase-dead IKK-β isoform (IKK-β KD) were co-transfected. Cells were stimulated with TNF-α (10 ng/ml) or vehicle (bovine serum albumin). Twenty-four hours after transfection cells were lysed, luciferase activity was determined and normalised for β-galactosidase activity. d) C2C12 myoblasts were transiently transfected with a general PPAR reporter plasmid. Simultaneously, plasmids encoding empty vector (PcDNA3.1) or plasmids encoding constitutive active IKK-β (c.a. IKK-β) or a kinase-dead IKK-β isoform (IKK-β KD) were co-transfected. Cells were stimulated with TNF-α (10 ng/ml) or vehicle (bovine serum albumin). Twenty-four hours after transfection cells were harvested. e, f) C2C12 myoblasts were transiently transfected with either a PPAR-α or a PPAR-γ transcriptional reporter construct. Simultaneously, plasmids encoding empty vector (PcDNA3.1) or plasmids encoding wild-type p65 or p65 with 1 (-534), 2 (-521) or 3 (-313) mutated trans-activation domains were co-transfected. Twenty-four hours post transfection, cells were lysed, luciferase activity was determined and normalised for β-galactosidase activity. g) C2C12 myoblasts were transiently transfected with specific PPAR reporters and empty vector (PcDNA 3.1) or an IκBα-SR-expressing plasmid (SR). 24 h post-transfection cells were treated with vehicle (bovine serum albumin; control) or TNF-α (10 ng/ml) for 24 h. Subsequently, cells were lysed, luciferase activity was determined and normalised for β-galactosidase activity. Values are expressed as mean ± SD from triplicate samples (experiments $n = 3$). Significance compared to control: * $p \leq 0.05$, # $p \leq 0.1$.

over-expression proved to be sufficient to potently impair muscle OXPHEN. Collectively, these data demonstrate that intact NF-κB signalling is required for TNF-α-induced impairments in muscle OXPHEN and that activation of classical NF-κB signalling is sufficient for impairing structural as well as enzymatic components of muscle OXPHEN.

Mitochondria are dynamic organelles that constantly undergo fusion and fission. These opposing processes work in concert to maintain the shape, size, and number of mitochondria and their physiological function [51]. Interestingly, mitochondrial morphology appeared altered upon TNF-α-induced NF-κB activation in our system, manifested by swollen and less elongated organelles compared to vehicle-treated myotubes. Similar effects on mitochondrial morphology have been observed in adipocytes and cardiomyocytes chronically exposed to TNF-α [40,44]. Altered mitochondrial morphology induced by TNF-α in our



study was associated with decreased expression levels of mitochondrial fusion and fission genes, a process that required intact NF-κB signalling. Mitochondrial fission and fusion events have been implicated in the process of mitophagy, which is a quality control process in which tubular networks of mitochondria are degraded to segregate dysfunctional mitochondria which are then selectively degraded by autophagy [51]. Whether or not inflammation may lead to mitophagy and how this subsequently affects muscle OXPHEN requires further studies as no data is present in the literature. On the other hand, it is well-known that mitochondrial fusion and fission genes are under control of PGC-1-co-activated signalling events, which is in line with our own data. Therefore, the decrease in mitochondrial fusion and fission gene expression upon TNF-α exposure might well just be a direct consequence of the general decrease in mitochondrial protein expression and impairments in the PGC-1 signalling cascade caused by classical NF-κB activation.

The fact that all parameters of cellular OXPHEN were indiscriminately down-regulated by inflammatory cytokine- and genetically-induced classical NF-κB activation suggests that activation of the classical NF-κB pathway impedes on the key regulatory pathways determining muscle OXPHEN. PGC-1 molecules are essential co-activators of proteins involved in the regulation of mitochondrial biogenesis (Tfam, NRF-1), uptake and oxidation of substrates, expression of oxidative fibres (PPAR-α, PPAR-δ) and control of mitochondrial fusion and fission events [19,52,53]. In our study, inflammatory cytokine-induced reductions in expression and activity levels of mitochondrial proteins were associated with decreased mRNA levels of both PGC-1α and PGC-1β. In addition, TNF-α not only reduced PGC-1 isoform mRNA expression itself but also potently inhibited PGC-1-co-actived signalling events. These findings are consistent with observations in other cell types as TNF-α-mediated reductions in PGC-1α expression levels have been described in cardiomyocytes [54] and adipocytes [43]. Furthermore, both our *in vitro* and *in vivo* data show that TNF-α-induced decreases in PGC-1α mRNA expression depended on intact classical NF-κB activation which is in coherence with our data on phenotypic OXPHEN markers. Similarly, TNF-α-induced reductions in PGC-1α mRNA expression in cardiac cells were shown to be attenuated by the NF-κB inhibitor parthenolide [54]. On the other hand, activation of classical NF-κB in our study (by over-expression of IKK-β) potently reduced PGC-1α and PGC-1β expression and impaired PGC-1-controlled signalling events similar to TNF-α which was largely mediated through IKK-β enzymatic activity. The observation that reductions in PGC-1α expression in our study occurred rapidly after TNF-α stimulation and temporally preceded changes in cellular OXPHEN suggest that reductions in PGC-1α expression and function underlie NF-κB-induced impairments in muscle OXPHEN. Importantly, a swift reduction in PGC-1α mRNA levels upon TNF-α exposure was confirmed *in vivo* by intra-muscular TNF-α injection. It is well described that muscle-specific knock-out of PGC-1α impairs muscle OXPHEN *in vivo* [55,56] and knock-down of PGC-1α in our system mimicked TNF-α-induced reductions in oxidative gene expression as well as mitochondrial fission and fusion genes. Collectively, this body of data supports the notion that reductions in PGC-1α expression and subsequent inhibition of PGC-1-co-activated signalling events by activation of classical NF-κB may well underlie inflammation-induced impairments in cellular OXPHEN and alterations in mitochondrial fission and fusion gene expression. The exact mechanisms underlying reductions in PGC-1 expression and function however remain to be elucidated.

Interestingly, Eisele et al. recently reported that PGC-1 molecules inhibit activation of the classical NF-κB pathway [57]. This implies that activation of the PGC-1 signalling cascade might not only improve muscle OXPHEN directly but may also alleviate muscle inflammatory status. Furthermore, the reciprocal interaction of these signalling pathways in muscle may not only control muscle OXPHEN but may also apply to muscle atrophy or dystrophy-associated damage which are both induced by inflammation and prevented or attenuated by ectopic PGC-1α expression [57].

Intriguingly, PGC-1α is present in the nucleus as well as in mitochondria and can bind to the genomes of both organelles [25,58]. In addition, NF-κB constituents: (i.e. p65, the IKK proteins and IκBα) have also been found to be present in mitochondria. Stimulation of mitochondria with TNF-α subsequently resulted in activated NF-κB signalling in these organelles (evidenced by IκBα degradation) and reduced expression of mitochondrial-encoded OXPHOS sub-units [46]. As p65 has been shown to be able to physically bind to PGC-1α [59], p65 may exert physical binding-mediated repression on PGC-1α-co-activated signalling events, both in the nuclear and mitochondrial compartment, thereby compromising adequate regulation of cellular OXPHEN. Our finding that TNF-α decreases both nuclear-encoded and mitochondrial-encoded OXPHOS sub-units is in line with this suggestion. Several groups confirmed p65 mitochondrial localisation and recruitment to the mitochondrial genome where it repressed mitochondrial gene expression, oxygen consumption, and cellular ATP levels [60,61]. Interestingly, it was also shown that trans-activation domains of p65 were required for the p65-mediated decrease in expression levels of mitochondrial-encoded OXPHOS sub-units. In line with this notion, in our study, intact p65 trans-activation domains were required for p65-mediated inhibition of signalling events known to be co-activated by PGC-1 molecules. This suggests that active transcription of a NF-κB target gene by P65 is required for inhibition of PGC-1-mediated regulation of muscle OXPHEN by classical NF-κB signalling. This is in coherence with our data that blocking of *de novo* transcription dissipates the inhibitory effect of TNF-α on PGC-1α transcript levels and PGC-regulated gene expression. Although speculative in nature, P65-mediated transcription of miRNA molecules encoded within classical NF-κB target genes might be involved in PGC-1 inhibition upon NF-κB activation. Recently, miR-696 was shown to negatively regulate PGC-1α abundance in mouse skeletal muscle [62]. However, this was shown to be mediated at the post-translational level by modulation of PGC-1α protein abundance. In addition, miR-23 was identified as a putative negative regulator of muscle PGC-1α abundance [63]. It is however unclear whether or not miR-696 and miR-23 are targets of classical NF-κB signalling and if these molecules are involved in classical NF-κB activation-induced repression of PGC-1-mediated signalling events in the control of muscle OXPHEN. In addition, an NF-κB DNA binding site was shown to be present within the PGC-1α promoter [64] suggesting that modulation of PGC-1α transcript levels by classical NF-κB activation might be mediated at the level of promoter activation.

Fig. 6. Classical NF-κB activation reduces PGC-1 mRNA expression. a, b) Mouse primary myoblasts were differentiated for 5 days into mature myotubes. Myotubes were stimulated with vehicle (bovine serum albumin; control) or TNF-α (10 ng/ml) for the indicated times and PGC-1α and PGC-1β mRNA levels were determined by Q-PCR. c, d) Wild-type (WT) C2C12 or C2C12-IκBα-super-repressor (SR) myoblasts were differentiated for 5 days in differentiation medium into fully mature myotubes. Myotubes were stimulated with vehicle (bovine serum albumin; control) or TNF-α (10 ng/ml) for the indicated times. PGC-1α and PGC-1β mRNA levels were determined by Q-PCR. e) Wild-type (WT) or MISR (muscle-specific expression of the IκBα-SR construct) mice received an intra-muscular (*m. gastrocnemius*) injection with NaCl ($n = 3$) or TNF-α (10 ng/ml) ($n = 3$). Mice were sacrificed 7 h post-injection and PGC-1α and PGC-1β mRNA expression was evaluated by Q-PCR. f, g) WT C2C12 myoblasts were differentiated for 5 days in differentiation medium into fully mature myotubes. Myotubes were stimulated with vehicle (bovine serum albumin; control) or IL-1β (10 ng/ml) (f) or infected with AV-GFP (control) or AV-IKK-β for the indicated times (g). PGC-1α and PGC-1β mRNA expression were determined by Q-PCR. h) C2C12 myoblasts were differentiated for 5 days in differentiation medium into fully mature myotubes. Myotubes were subsequently pre-treated with actinomycin D (50 ng/ml) or vehicle (DMSO) for 2 h after which myotubes were stimulated with vehicle (BSA; control) or TNF-α (10 ng/ml) for 24 h. PGC-1α mRNA levels were determined by Q-PCR. Gene expression data were corrected by using a GeNorm normalisation factor calculated from the expression of 4 housekeeping genes (cyclophilinA, β2-microglobuline, hypoxanthine-guanine phosphoribosyltransferase and glyceraldehyde-3-phosphate dehydrogenase). Values are expressed as mean ± SD from triplicate samples (experiments $n = 3$). Significance compared to control: * $p \leq 0.05$, # $p \leq 0.01$.

5. Conclusion

Our findings uncover a new mechanism in which p65 (as part of classical NF-κB signalling) impairs the regulation of skeletal muscle OXPHEN under inflammatory conditions. The molecular mechanisms by which classical NF-κB activation reduces PGC-1 transcript levels and impairs PGC-1-mediated signalling events in muscle OXPHEN regulation however warrant further investigation. In this regard, it is interesting to note that Guttridge et al. recently identified an important role for IKK-α, as part of the alternative NF-κB pathway, in the regulation of muscle OXPHEN by PGC-1 molecules [65]. Future research is needed to unravel whether or not IKK-α-mediated control of muscle OXPHEN is modulated by inflammation-induced classical NF-κB activation as a potential novel mechanism underlying inflammation-induced impairments of muscle OXPHEN.

Collectively, this research is relevant for chronic diseases with severe systemic consequences of (inflammation-induced) loss of muscle OXPHEN, including COPD, CHF, T2DM and perhaps also cancer. In light of our data, inhibition of chronic NF-κB activation in muscle may be desirable to improve muscle OXPHEN in these diseases. Novel inhibitors of classical NF-κB are currently in development [66,67]. Recently, systemic delivery of a NF-κB blocking peptide and genetic NF-κB blockade by adenoviral delivery proved to be efficient in attenuating muscle NF-κB signalling and improved muscle function and muscle regeneration in an mdx mouse model of muscular dystrophy (which is characterised by chronic muscle inflammation) [66,67]. Future studies are needed to establish whether blockade of classical NF-κB can restore skeletal muscle OXPHEN in the context of chronic disorders associated with a sustained low-grade inflammatory status.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.03.018.

Conflict of interest

The authors declare that they have no conflict of interest

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