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Classical NF-κB activation impairs skeletal muscle oxidative phenotype by reducing IKK-α expression

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ABSTRACT

Background: Loss of quadriceps muscle oxidative phenotype (OXPHEN) is an evident and debilitating feature of chronic obstructive pulmonary disease (COPD). We recently demonstrated involvement of the inflammatory classical NF-κB pathway in inflammation-induced impairments in muscle OXPHEN. The exact underlying mechanisms however are unclear. Interestingly, IκB kinase α (IKK-α: a key kinase in the alternative NF-κB pathway) was recently identified as a novel positive regulator of skeletal muscle OXPHEN. We hypothesised that inflammation-induced classical NF-κB activation contributes to loss of muscle OXPHEN in COPD by reducing IKK-α expression. Methods: Classical NF-κB signalling was activated (molecularly or by tumour necrosis factor α: TNF-α) in cultured myotubes and the impact on muscle OXPHEN and IKK-α levels was investigated. Moreover, the alternative NF-κB pathway was modulated to investigate the impact on muscle OXPHEN in absence or presence of an inflammatory stimulus. As a proof of concept, quadriceps muscle biopsies of COPD patients and healthy controls were analysed for expression levels of IKK-α, OXPHEN markers and TNF-α. Results: IKK-α knock-down in cultured myotubes decreased expression of OXPHEN markers and key OXPHEN regulators. Moreover, classical NF-κB activation (both by TNF-α and IKK-β over-expression) reduced IKK-α levels and IKK-α over-expression prevented TNF-α-induced impairments in muscle OXPHEN. Importantly, muscle IKK-α protein abundance and OXPHEN was reduced in COPD patients compared to controls, which was more pronounced in patients with increased muscle TNF-α mRNA levels. Conclusion: Classical NF-κB activation impairs skeletal muscle OXPHEN by reducing IKK-α expression. TNF-α-induced reductions in muscle IKK-α may accelerate muscle OXPHEN deterioration in COPD.

Abbreviations: ACTB, Beta Cytoskeletal Actin; ALAS1, Delta-aminolevulinate synthase 1; ATP, Adenosine triphosphate; Ad, Adenoviral; B2M, β2m, Beta 2 microglobulin; BMI, Body mass index; COPD, Chronic obstructive pulmonary disease; CA, Constitutively active; COXIV, Cytochrome *c* oxidase 4; CS, Citrate synthase; DMEM, Dulbecco's Modified Eagle Medium; FEV1, Forced expiratory volume in one second; FVC, Forced vital capacity; GUSB, Glucuronidase, bèta; GAPDH, Gapdh, Glyceraldehyde-3-phosphate dehydrogenase; Gfp, Green fluorescent protein; HAD, β-hydroxyacyl-CoA dehydrogenase; HBSS, Hank's Balanced Salt solution; HCBP, Human carnitine-palmitoyl transferase B; HMBS, Hydroxymethylbilane Synthase; HPRT, Hprt, Hypoxanthine phosphoribosyltransferase 1; Icam-1, Intra-cellular adhesion molecule 1; IKK-α, Ikk-α, IκB kinase alpha; Ikk-β, IκB kinase beta; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; Il-1β, interleukin 1β; Mlc, Myosin light chain; Myhc, Myosin heavy chain; Nrf, Nuclear respiratory factor; NS, Not significant; NF-κB, Nuclear factor kappa B; OXPHEN, Oxidative phenotype; Oxphos, Oxidative phosphorylation; PBS, Phosphate-buffered saline; PGC-1, Pgc-1, Peroxisome proliferator-activated receptor gamma co-activator 1; PPAR, Ppar, Peroxisome proliferator-activated receptor; PPIA, peptidylprolyl isomerase A (cyclophilin A); RPLO, 50S ribosomal subunit protein L15; *RPL13A*, 60S ribosomal protein L13a; SD, Standard deviation; SEM, Standard equality of the mean; SR, Super repressor; TFAM, Tfam, Mitochondrial transcription factor A; TNF-α, Tnf-α, Tumour necrosis factor alpha; UBC, Ubiquitin C; WT, Wild-type; YWHAZ, 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

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1. Introduction

Loss of quadriceps muscle oxidative phenotype (OXPHEN) is a well-recognised feature of chronic obstructive pulmonary disease (COPD) and significantly impairs muscle endurance, exercise capacity and quality of life in patients [1,2]. Moreover, loss of muscle OXPHEN has been postulated as a driver of muscle wasting and cardiovascular/metabolic risk in COPD, both of which contribute significantly to morbidity and mortality of the disease [3,4]. Importantly, the muscle phenotype is not uniform [5] so understanding aetiological drivers of key subsets is necessary for therapeutic advances.

Collectively, loss of muscle OXPHEN in COPD includes a fibre-type shift from type I (slow, oxidative) to type II (fast, glycolytic) fibres, decreased muscle mitochondrial content and reduced activity levels of metabolic enzymes involved in substrate oxidation pathways [6–8]. Moreover, decreased expression levels of constituents of the PGC-1/PPAR signalling pathway, a key pathway regulating cellular OXPHEN, have been shown in the musculature of COPD patients [9,10].

The exact triggers and molecular mechanisms leading to loss of muscle OXPHEN in COPD remain obscure. However, chronic (systemic) inflammation, frequently associated with COPD [11], has been implicated in muscle OXPHEN deterioration observed in COPD [3]. Indeed, we previously showed that expression levels of key OXPHEN constituents were reduced in quadriceps muscle of COPD patients with elevated levels of the pro-inflammatory cytokine tumour necrosis factor α (TNF-α) in muscle compared to healthy controls and compared to patients with normal muscle TNF-α levels. Stimulation of cultured myotubes with TNF-α subsequently revealed that this cytokine directly impairs muscle OXPHEN and reduces Pgc-1/Ppar signalling [12]. Furthermore, we recently showed that inflammation-induced loss of muscle OXPHEN requires activation of the classical NF-κB pathway [13], a key inflammatory signalling pathway which is of particular importance as increased classical NF-κB activation has been demonstrated in skeletal muscle of patients with severe COPD [14,15]. Under normal physiological conditions, the classical 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 initiate the rapid phosphorylation and degradation of IκBα through Ikk-β kinase activity. This liberates Rela to move into the nucleus and initiate transcription of NF-κB target genes [16].

The down-stream effector and the mechanisms of classical NF-κB activation-mediated impairment of skeletal muscle OXPHEN are unknown. Interestingly, Bakkar *et al.* recently identified IκB kinase α (Ikk-α), a key kinase in the alternative NF-κB pathway, as a novel positive regulator of skeletal muscle OXPHEN. These authors showed that Ikk-α improves muscle OXPHEN through direct transcriptional control of Pgc-1-mediated signalling [17]. It however remains to be determined whether or not the role of the alternative NF-κB pathway in the regulation of muscle OXPHEN is compromised by (inflammation-induced) classical NF-κB activation and whether or not impairments in the alternative NF-κB pathway contribute to loss of muscle OXPHEN observed in COPD.

Therefore, in the current translational study, we hypothesised that (inflammation-induced) classical NF-κB activation impairs skeletal muscle OXPHEN by reducing Ikk-α expression and that loss of muscle OXPHEN in COPD is associated with decreased levels of muscle IKK-α protein and an increased muscle inflammatory status. We therefore investigated whether classical NF-κB activation by TNF-α or Ikk-β over-expression impedes on Ikk-α-mediated regulation of muscle OXPHEN in cultured myotubes and explored muscle OXPHEN as well as IKK-α and TNF-α levels in quadriceps muscle biopsies from a large group of COPD patients and healthy controls.

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 [18] and C2C12-IκBα-SR cells (displaying blocked classical NF-κB signalling) were kindly provided by Dr. Guttridge (Ohio State University, OH, USA). The stable Ppar reporter cell line was constructed and cultured as described previously [12]. C2C12 myoblasts were cultured and differentiated into mature multi-nucleated myotubes as described previously [18]. Myotubes were differentiated for 5 days after which hormonal (TNF-α) or molecular interventions (Ad-Gfp, Ad-Ikk-β, Ad-Ikk-α or SiRNA) were initiated for the indicated time. Additional detail regarding culture conditions is provided in the online data supplement.

2.2. Transfections and plasmids

For the assessment of NF-κB transcriptional activity or Ppar transcriptional activity, C2C12 cells were stably transfected with a 6κB-TK luciferase plasmid (NF-κB reporter) or a HCBP (Human CPT-I B promoter) reporter plasmid (Ppar reporter) as previously described [12,18]. Details regarding transfection procedures and reporter assays are provided in the online data supplement.

2.3. Western blot & 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 data supplement.

2.4. Enzyme activity assay

Activity of β-hydroxyacyl-CoA dehydrogenase (Had) (EC 1.1.1.35) was measured as described previously [19]. Enzyme activity levels were measured at 37°C and at PH 7.3 and were corrected for total protein content which was determined using a detergent compatible protein determination assay (Bio-Rad Hercules, CA, USA).

2.5. ATP assay

Details about the ATP assay are provided in the online data supplement.

2.6. Subjects

For the human study, we analysed remaining quadriceps muscle biopsies of clinically stable COPD patients and healthy controls obtained from 2 cohorts previously published as an ENIGMA study [12] and a study within the framework of the Dutch Top Institute Pharma (TIP) [20]. Written consent was obtained from all subjects and the study was approved by the ethical review boards of the different European centres. These studies have been carried out in accordance with the declaration of Helsinki. Data within each cohort (lung function and muscle gene expression) were expressed as a percentage of their respective control group (to reduce the risk for any potential selection bias) and data from these 2 cohorts were pooled. Additional details regarding inclusion and exclusion criteria, collection and processing of muscle biopsies and methodology for assessment of lung function are provided in the online data supplement.

2.7. Statistics

Data was analysed according to the guidelines of Altman et al. using SPSS (SPSS Inc., Chicago, IL, USA) [21]. Unpaired, independent student t-tests (corrected for unequal variances if appropriate) or one-way ANOVA analyses with an LSD *post hoc* correction were used when appropriate. Linear regression was applied to correct for any inter-cohort differences in the human muscle biopsy analyses. The Pearson correlation test was used to explore relevant correlations. Where appropriate data was transformed as log (x) to correct for non-linearity. In addition, if natural zero values were observed and log transformation was appropriate data was transformed as log (x + 1). Data are represented as the mean ± SD or mean ± SEM as indicated. A p-value of less than 0.05 was considered to be significant.

3. Results

3.1. Loss of Ikk-α expression disturbs skeletal muscle oxidative phenotype

To confirm and extend the dependency of muscle cell OXPHEN on Ikk-α, Ikk-α was silenced in cultured myotubes. SiRNA molecules targeting Ikk-α effectively reduced both Ikk-α mRNA and protein levels by approximately 70% (Fig. S1A–C). Ikk-α knock-down subsequently resulted in reduced protein and mRNA levels of sub-units of mitochondrial oxidative phosphorylation (Oxphos) complexes, reduced activity of Had (β-hydroxyacyl-CoA dehydrogenase: the rate-limiting enzyme in fatty acid β-oxidation) and a decreased intra-cellular ATP content (Fig. 1A–F). Furthermore, knock-down of IKK-α resulted in significantly decreased protein levels of type I, slow myosin heavy chain (Myhc) while type II, Myhc fast protein levels remained unaffected. Protein levels of the fast isoforms of myosin light chain (Mlc) 3 and Mlc 1 tended to increase upon Ikk-α knock-down (Fig. 1G, H). In addition, mRNA levels of the slow Myhc I and Myhc IIa isoforms decreased while mRNA levels of the fast isoform Myhc IIb increased in response to Ikk-α knock-down (Fig. 1I–K). Myhc IIx mRNA levels were largely unaltered (data not shown). Ikk-α knock-down reduced Pgc-1α as well as Pgc-1β mRNA transcript levels and decreased Ppar transcriptional activity (Fig. 1L, M). mRNA expression levels of Ppar-δ, Tfam, Nrf-1 and Nrf-2α were unaltered in response to loss of Ikk-α protein. Collectively, this data shows that loss of Ikk-α protein reduces muscle OXPHEN and impedes on the molecular regulation of muscle OXPHEN by interfering with the Pgc-1/Ppar signalling pathway.

3.2. Classical NF-κB Decreases Muscle Ikk-α Expression

We previously convincingly demonstrated that TNF-α impairs muscle OXPHEN [12]. In addition, as indicated in Fig. S2, TNF-α stimulation of C2C12 myotubes that were differentiated for 5 days potently reduced mRNA expression levels of markers and regulators of muscle OXPHEN in absence of a change in mitochondrial DNA (mtDNA) content (Fig. S2A–E). In light of the data presented in Fig. 1, we next investigated Ikk-α protein levels in cultured myotubes in response to TNF-α stimulation. As depicted in Fig. 2A, Ikk-α protein content was significantly reduced in wild-type C2C12 myotubes chronically stimulated with TNF-α (Fig. 2A). To verify whether this represented a specific effect on Ikk-α, Ikk-β protein content was determined after TNF-α exposure and found not to be significantly altered (Fig. S3A). We previously demonstrated that TNF-α-induced deterioration of muscle OXPHEN depends on activation of the inflammatory signalling pathway classical NF-κB [13]. In the present study, increased expression of a known target gene of the classical NF-κB pathway (intra-cellular adhesion molecule 1: Icam-1) and potent nuclear translocation of the main transcriptionally active sub-unit: Rela (Fig. S4A–B) reflected classical NF-κB activation following TNF-α stimulation of cultured myotubes. To investigate whether intact classical NF-κB signalling is required for TNF-α-induced loss of muscle Ikk-α expression, we blocked the classical NF-κB pathway by transient or stable transfection of a non-degradable form of the repressor of classical NF-κB signalling IκBα (IκBα-SR). Effectiveness of stable NF-κB blockade in C2C12 myotubes is shown in Fig S4 A-D. Interestingly, C2C12 myotubes with blocked classical NF-κB signalling were refractory to TNF-α-induced reductions in Ikk-α protein (Fig. 2B). Also, although TNF-α reduced Ikk-α mRNA levels in wild-type C2C12 myotubes, myotubes from the IκBα-SR C2C12 cell line were refractory to TNF-α-induced reductions in Ikk-α mRNA levels (Fig. 2C). In addition, TNF-α reduced Ikk-α promoter activity in wild-type C2C12 cells but not in IκBα-SR C2C12 cells (Fig. 2D). In coherence, TNF-α-induced reductions in Ikk-α promoter activity were abrogated in C2C12 cells transiently expressing a non-degradable IκBα construct (Fig. 2E).

To investigate whether activation of classical NF-κB signalling, in absence of inflammatory cytokines, is sufficient to reduce Ikk-α expression, classical NF-κB signalling was activated by adenoviral (Ad) delivery of Ikk-β, a key kinase in the classical NF-κB pathway. As illustrated in Fig. 3, Ad-Ikk-β delivery to C2C12 myotubes resulted in potent Ikk-β over-expression (Fig. 3A) and activation of NF-κB transcriptional activity (Fig. 3B). Ikk-β over-expression subsequently decreased both Ikk-α protein and mRNA levels in C2C12 myotubes (Fig. 3C, D). In line with protein and mRNA data, over-expression of a constitutive active form of Ikk-β diminished Ikk-α promoter activation (Fig. 3E).

Collectively this data shows that activation of classical NF-κB signalling is required and sufficient for (TNF-α-induced) loss of muscle Ikk-α expression.

3.3. Ikk-α Over-Expression Prevents TNF-α-Induced Impairments in Muscle OXPHEN

We next investigated whether over-expression of Ikk-α protein could prevent TNF-α-induced impairments in muscle OXPHEN (as we have previously reported [12,13]). As depicted in Fig. 4, Ikk-α was potently over-expressed and TNF-α-induced decreases in Ikk-α protein levels were restored by adenoviral over-expression of Ikk-α (Fig. 4A, B). Ikk-α over-expression increased Myhc slow protein content and TNF-α-induced reductions in Myhc slow protein content were attenuated by Ikk-α over-expression (Fig 4A, C). Similarly, Ikk-α over-expression increased Myhc I mRNA levels and TNF-α-induced reductions in Myhc I mRNA transcript abundance were abrogated when Ikk-α was over-expressed (Fig 4D). Cyc1 and Sdhb mRNA levels, as representative OXPHOS markers, were increased upon Ikk-α over-expression. In addition, both Cyc1 and Sdhb mRNA levels decreased upon TNF-α exposure whereas this reduction was not observed when exogenous Ikk-α was expressed (Fig 4E, F). Furthermore, Ikk-α over-expression increased Pgc-1α, not Pgc-1β, mRNA levels and prevented TNF-α-induced reductions in Pgc-1α and Pgc-1β mRNA levels (Fig 4G, H).

3.4. Skeletal muscle IKK-α protein abundance is reduced in COPD

As expected, COPD patients had an impaired muscle oxidative phenotype evidenced by reduced muscle gene expression levels of HAD, Citrate synthase (CS), Cytochrome *c* oxidase sub-unit IV (COX IV), PPAR-α and PGC-1α (key markers/regulators of muscle OXPHEN) compared to controls (Table 1). Furthermore, IKK-α protein levels were significantly lower in quadriceps muscle biopsies from COPD patients compared to healthy controls (Fig. 5A). Muscle TNF-α mRNA levels were significantly higher in patients compared to healthy subjects (Table 1). Regression analysis revealed that differences in levels of TNF-α, IKK-α, HAD, COX IV, PGC-1α and PPAR-α observed between COPD patients and controls were independent of which of the two studies the biopsies came from.

The subset of patients with high levels of TNF-α mRNA in muscle not only displayed lower levels of CS, HAD, COX IV and PPAR-α but also significantly lower levels of muscle IKK-α protein compared to patients with normal muscle TNF-α levels (Table 1) (Fig. 5B). Additionally, in the whole study population (n = 81) muscle TNF-α levels correlated

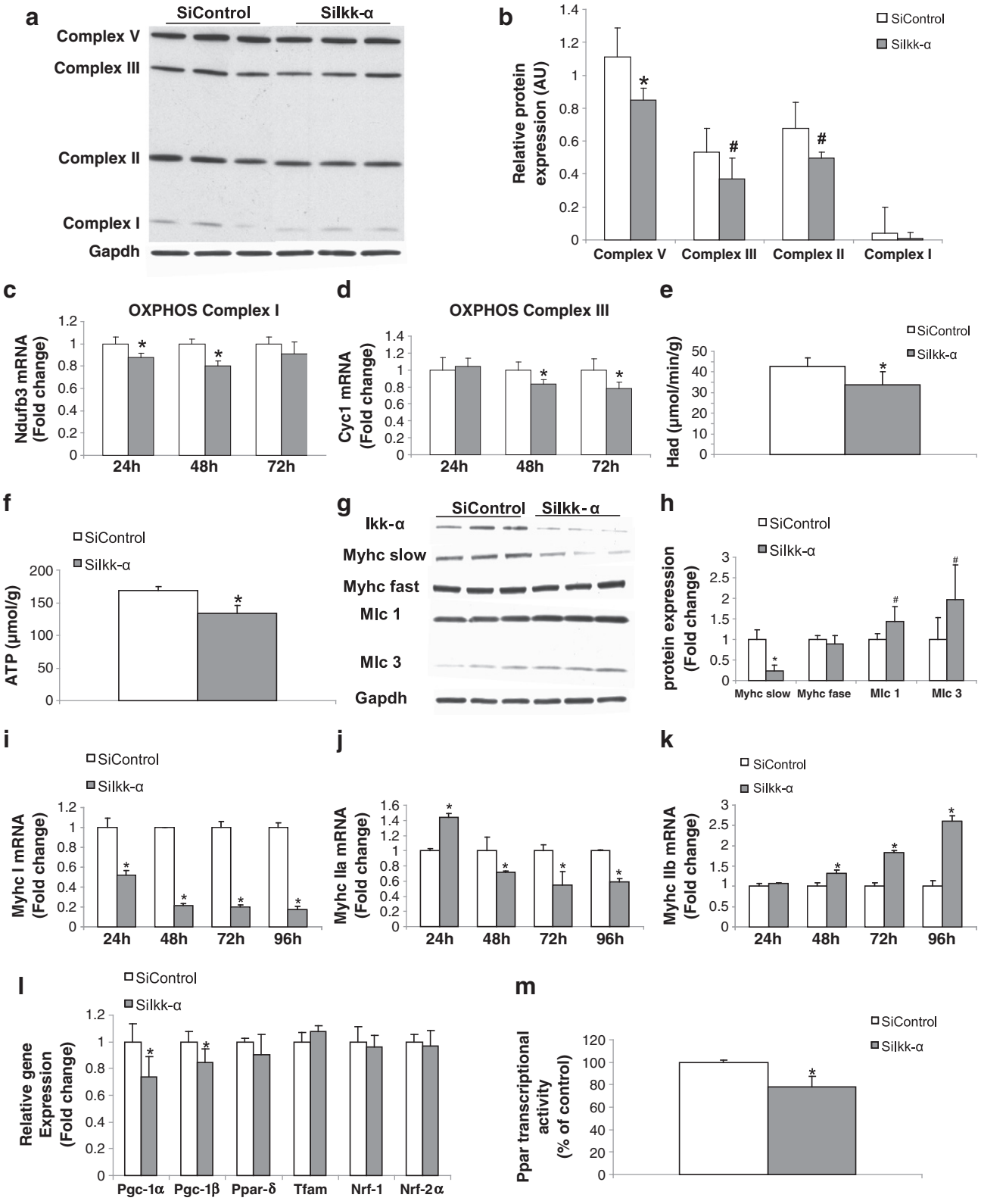

Fig. 1. Loss of Ikk-α impairs skeletal muscle oxidative phenotype. C2C12 myotubes (differentiated for 5 days) were subjected to knock-down of Ikk-α by use of a specific siRNA targeting Ikk-α for 120 h or the indicated time. As a control, myotubes were treated with a non-specific scrambled control siRNA construct. a, b, g, h) Whole-cell lysates were obtained and Myhc slow, Myhc fast, Mlc 3 and 1 and OXPHOS protein content were determined by western blotting. Western blots were corrected for total protein and Gapdh was used as a loading control. c, d, i–l) mRNA levels of Myhc I, IIb, IIa as well as OXPHOS sub-units and Pgc-1α, Pgc-1β, Ppar-δ, Tfam, Nrf-1 and Nrf-2α were determined by Q-PCR. e) HAD enzyme activity was determined and corrected for total protein content f) ATP content was determined and corrected for total protein content. m) Ppar transcriptional activity was determined by luciferase measurement and corrected for total protein content. Values are expressed as mean ± SD from triplicate samples (experiments n = 3). Significance compared to control: * $p \leq 0.05$, # $p \leq 0.1$.

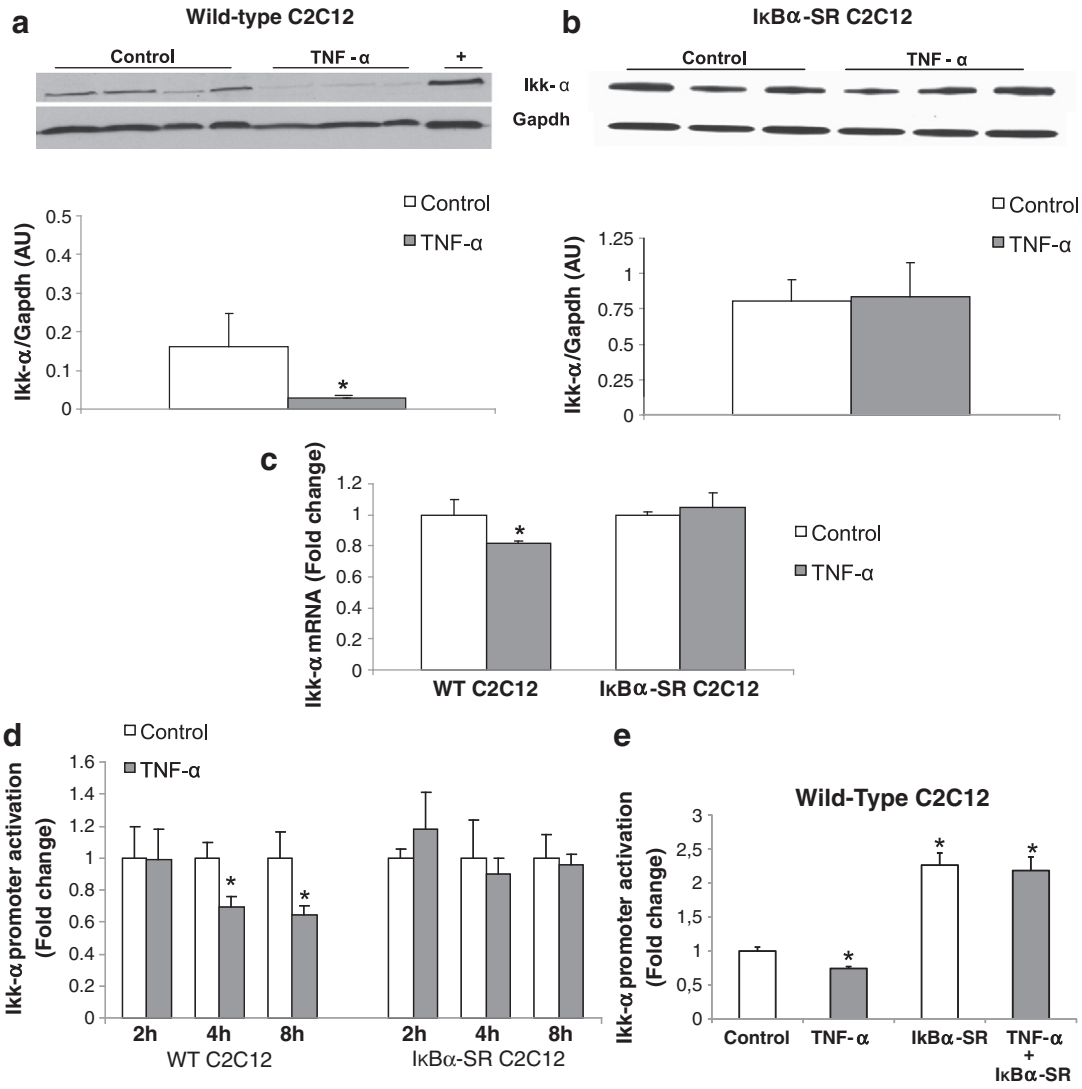

Fig. 2. TNF-α-induced classical NF-κB activation decreases muscle Ikk-α expression. a–c) Wild-type (WT) C2C12 or C2C12-IκBα-super-repressor (IκBα-SR) myotubes were differentiated for 5 days and subsequently stimulated with vehicle (bovine serum albumin; Control) or TNF-α (10 ng/ml) for 120 h (a, b) or 72 h (c). Whole cell lysates and RNA lysates were obtained and Ikk-α protein and mRNA levels were determined by western blotting and Q-PCR respectively. Western blots were corrected for total protein and Gapdh was used as a loading control. As a positive control C2C12 myoblasts were transfected with an Ikk-α expression plasmid for 48 h after which cells were harvested. d) Wild-type (WT) C2C12 or C2C12-IκBα-super-repressor (SR) myoblasts were transfected with an Ikk-α promoter reporter construct and subsequently stimulated with TNF-α (10 ng/ml) for the indicated time after which cells were lysed. e) Wild-type (WT) C2C12 myoblasts were transfected with an Ikk-α promoter reporter construct and an empty vector as a control or an IκBα-SR expression plasmid and subsequently stimulated with TNF-α (10 ng/ml) or vehicle (bovine serum albumin; Control) for 24 h. For all reporter assays, 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: * $p \leq 0.05$.

inversely with muscle IKK-α protein content ($r = -0.309$, $p = 0.005$) and when COPD patients only ($n = 59$) were considered in isolation ($r = -0.279$, $p = 0.032$). Also, in both the whole study population and also in COPD patients considered alone, IKK-α protein levels correlated positively with HAD ($r = 0.440$ and $r = 428$ respectively; $p \leq 0.001$) and with parameters of lung function as FEV_1 ($r = 0.473$ and $r = 0.427$, $p \leq 0.001$). Hence, muscle TNF-α levels correlated inversely with FEV_1 ($r = -0.417$ and $r = -0.369$, $p \leq 0.01$) and inversely with HAD ($r = -0.484$ and $r = -0.485$; $p \leq 0.001$) (Fig. 6). Differences in OXPHEN markers between COPD patients with high muscle TNF-α levels and normal TNF-α levels remained statistically significant after inclusion of study group as an independent variable. However, although IKK-α protein levels were significantly lower in patients with high muscle TNF-α levels compared to patients with normal TNF-α levels and healthy controls in an ANOVA test, this statistically significant difference was dissipated when the study source was included as an independent variable in our regression model.

4. Discussion

The present study is the first to reveal that TNF-α-induced classical NF-κB activation reduces Ikk-α expression levels in skeletal muscle. Loss of Ikk-α protein subsequently resulted in an impaired muscle OXPHEN as restoring Ikk-α levels proved to be sufficient to prevent TNF-α-induced impairments in muscle OXPHEN. Moreover, analysis of human skeletal muscle biopsies revealed that muscle IKK-α protein content was significantly lower in COPD patients compared to healthy controls, which was accompanied by an impaired muscle OXPHEN and, interestingly, was more pronounced in patients with high muscle TNF-α levels. Collectively, these data suggest a role for loss of IKK-α expression in inflammation-induced impairment of muscle OXPHEN with relevance for a significant subset of patients with COPD.

We previously demonstrated that inflammatory cytokines as TNF-α potently reduce muscle OXPHEN [12]. As it is known that inflammatory cytokines can inhibit myogenic differentiation [18] and we previously

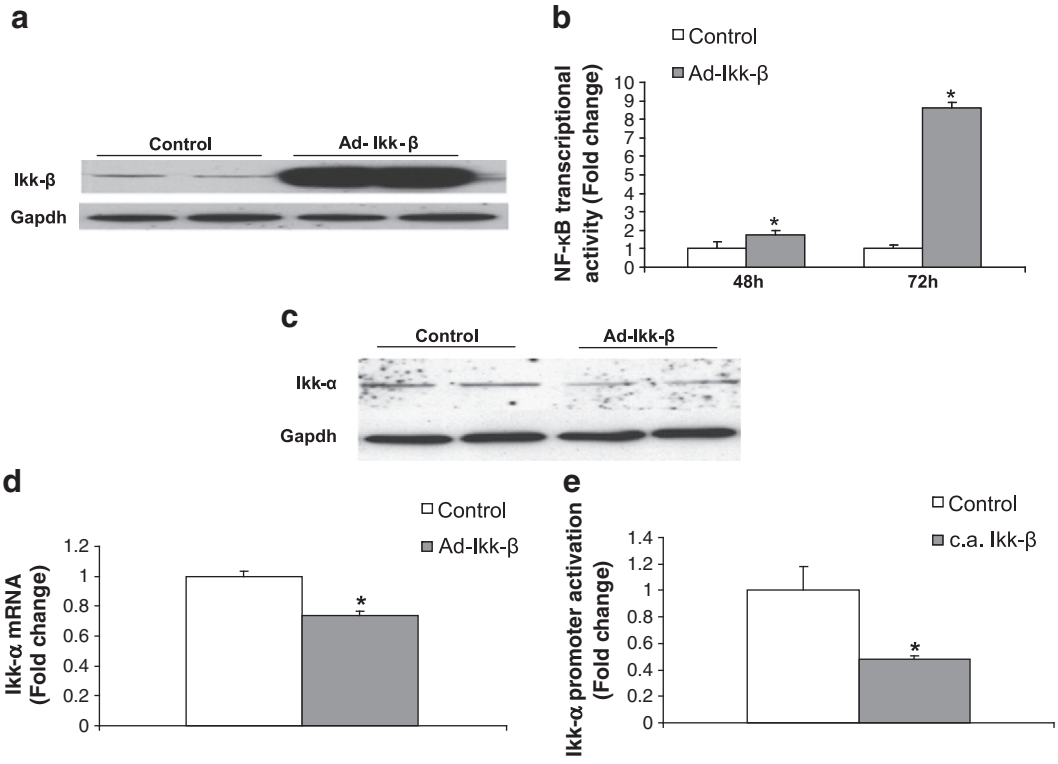

Fig. 3. Classical NF-κB activation decreases muscle Ikk-α expression. a) C2C12 myotubes (differentiated for 5 days) were infected with an adenoviral (Ad) construct expressing Ikk-β or an Ad-Gfp construct (control) for 120 h (2×10^7 PFU/ml). Ikk-β over-expression was verified by western blot analysis. Western blot data was corrected for total protein content and Gapdh was used as a loading control. b) C2C12 myoblasts stably expressing a NF-κB responsive luciferase construct (6-κB TK luciferase) were differentiated for 5 days in differentiation medium into fully mature myotubes. Myotubes were stimulated with Ad-Ikk-β or Ad-Gfp for the indicated time. Luciferase activity was determined and normalised for total protein. c, d) Wild-type (WT) C2C12 myotubes were infected with an adenoviral construct expressing Ikk-β or an Ad-Gfp construct as control for 120 h (c) or 72 h (d). Cells were harvested at the indicated time-points and Ikk-α protein and mRNA were determined by western blotting and Q-PCR respectively. Western blots were corrected for total protein and Gapdh was used as a loading control. e) WT C2C12 myoblasts were transfected with an Ikk-α promoter reporter construct and an empty vector as a control or a constitutive active (c.a.) Ikk-β expression construct. 24 h after transfection, cells were harvested, 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: * $p \leq 0.05$.

showed that myogenic differentiation is associated with a well-defined shift towards a more oxidative metabolic profile [22], we verified that TNF-α-induced decreases in expression levels of markers and regulators of muscle OXPHEN represented a true decline in cellular OXPHEN and not a delayed development of muscle OXPHEN due to impaired myogenesis. This is in line with our previous findings which demonstrated that TNF-α stimulation of myotubes that have undergone the myogenic differentiation programme for several days potently decreased cellular OXPHEN but failed to affect myogenic index and creatine kinase activity (as markers for myogenic differentiation) [23]. In addition, we further characterised the impact of TNF-α on cultured myotube OXPHEN by assessing TNF-α-induced alterations in mtDNA content. Although TNF-α clearly impaired myotube OXPHEN, no significant changes were observed in mtDNA content upon chronic TNF-α stimulation. This is in line with a recent report from Rowe *et al.* which demonstrated that muscle OXPHEN was heavily impaired in absence of alterations in mitochondrial content in mice lacking both Pgc-1α and Pgc-1β in muscle [24]. By applying electron microscopy, these authors uncovered that muscle mitochondria of mice lacking both Pgc-1 molecules were less densely packed with components of the respiratory electron transport chain which explains the loss of oxidative capacity without an apparent decrease in mitochondrial DNA content.

Inflammatory cytokines as TNF-α are potent activators of the classical NF-κB pathway [16]. In the present study, similar to TNF-α-induced classical NF-κB activation, activation of classical NF-κB signalling by Ikk-β over-expression (in absence of inflammatory cytokines) decreased Ikk-α protein and mRNA expression and Ikk-α promoter activation showing that activation of classical NF-κB *per se* is sufficient to reduce muscle Ikk-α expression. Given the novel role of Ikk-α in the regulation of skeletal muscle OXPHEN [17], which we have confirmed and extended in this study, this observation is coherent with our previous findings. Indeed, we recently demonstrated that activation of classical NF-κB signalling by TNF-α, but also by interleukin 1β (Il-1β) or by over-expression of Ikk-β, is sufficient to impair muscle OXPHEN evidenced by reduced expression levels of mitochondrial proteins, altered mitochondrial morphology and by decreases in expression levels of genes involved in mitochondrial biogenesis and mitochondrial fusion and fission [13]. In light of these observations, inflammatory cytokines other than TNF-α known to induce activation of classical NF-κB signalling (as *e.g.* IL-1β) may well impair muscle OXPHEN in an Ikk-α-mediated

Fig. 4. Ikk-α over-expression prevents TNF-α-induced impairments in muscle OXPHEN. a-c) Wild-type (WT) C2C12 myotubes (differentiated for 5 days) were infected with an adenoviral (Ad) construct expressing Ikk-α or an Ad-Gfp construct as control for 48 h (2×10^7 PFU/ml). After 48 h myotubes were stimulated with vehicle (bovine serum albumin; Control) or TNF-α (10 ng/ml) for an additional 72 h. Cells were harvested and Myhc slow, Myhc fast and Ikk-α protein content was determined by western blotting. Western blots were corrected for total protein and Gapdh was used as a loading control. d–h) Wild-type (WT) C2C12 myotubes were infected with an Ad-Ikk-α or an Ad-Gfp construct for 48 h. After 48 h myotubes were stimulated with vehicle (bovine serum albumin; Control) or TNF-α (10 ng/ml) for an additional 48 h. mRNA expression levels of Myhc I and OXPHOS complexes II and III as well as Pgc-1α and Pgc-1β were determined by Q-PCR. Values are expressed as mean ± SD from triplicate samples (experiments n = 3). Significance compared to control: * $p \leq 0.05$.

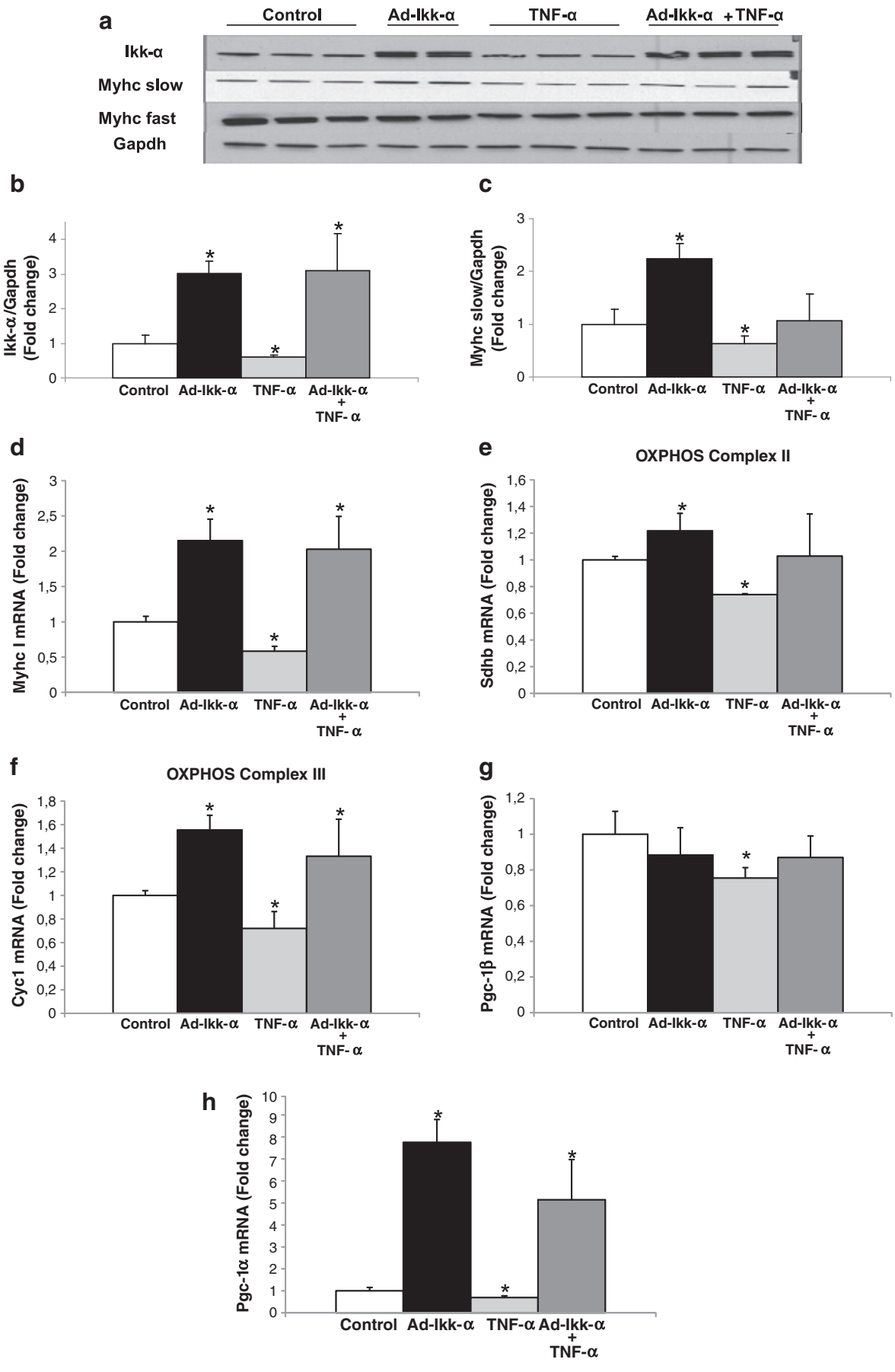
a
Control
Ad-Ikk-α
TNF-α
Ad-Ikk-α +TNF-α
Ikk-α
Myhc slow
Myhc fast
Gapdh
b
Ikk-α/Gapdh (Fold change)
c
Myhc slow/Gapdh (Fold change)
d
Myhc I mRNA (Fold change)
e
OXPHOS Complex II
Sdhb mRNA (Fold change)
f
OXPHOS Complex III
Cyc1 mRNA (Fold change)
g
Pgc-1β mRNA (Fold change)
h
Pgc-1α mRNA (Fold change)
Control
Ad-Ikk-α
TNF-α
Ad-Ikk-α + TNF-α

Table 1
Subject characteristics. All markers of muscle oxidative phenotype as well as TNF-α levels are expressed as gene expression data. FEV_1: Forced expiratory volume in 1s. FVC: Forced vital capacity. BMI: Body mass index. CS: Citrate synthase. HAD: β-hydroxyacyl-CoA dehydrogenase. COX IV: Cytochrome *c* oxidase sub-unit IV. PPAR-α: Peroxisome proliferator-activated receptor α. PGC-1α: PPAR-γ co-activator 1α. TNF-α: Tumour necrosis factor α. Values are expressed as mean ± SD except for gene expression data which is presented as mean ± SEM. Significance compared to healthy controls: * $p \leq 0.05$; Significance compared to the "normal TNF-α" COPD subgroup: # $p \leq 0.05$.

	Controls (n = 22)	COPD (n = 59)	COPD Normal TNF-α (n = 36)	COPD High TNF-α (n = 23)
Age (years)	61.5 ± 9.1	64.1 ± 7.5	64.4 ± 7.9	63.7 ± 6.9
Sex (M/F)	13/9	42/17	22/14	20/3
Lung function				
FEV_1 (% predicted)	102.7 ± 20.4	46.9 ± 18.4*	51.4 ± 18.6*	39.8 ± 16.0* #
FVC (% predicted)	110.1 ± 21.3	90.0 ± 26.0*	94.9 ± 22.6	82.3 ± 6.2* #
Body composition				
Weight (kg)	73.1 ± 16.0	70.6 ± 12.4	70.1 ± 11.0	71.3 ± 14.6
BMI	25.5 ± 4.2	24.8 ± 4.0	25.1 ± 4.1	24.3 ± 4.0
Muscle oxidative phenotype				
CS (% of control)	100.0 ± 5.0	84.5 ± 4.2*	93.9± 4.7	69.8 ± 6.9* #
HAD (% of control)	100.0 ± 4.0	76.9 ± 3.6*	85.1 ± 3.9*	64.2 ± 6.1* #
COX IV (% of control)	100.0 ± 5.2	88.1 ± 3.1*	92.8 ± 4.0	80.7 ± 4.4* #
PGC-1α (% of control)	100.0 ± 8.6	76.1 ± 5.2*	77.1 ± 6.1*	74.6 ± 9.4*
PPAR-α (% of control)	100.0 ± 5.5	84.9 ± 3.5*	92.8 ± 4.8	72.6 ± 3.9* #
Muscle inflammation				
TNF-α (% of control)	100.0 ± 15.7	202.6 ± 24.4 *	83.8 ± 9.0	388.7 ± 35.3* #

mechanism similar to TNF-α. Also, in the present study we show that TNF-α-induced reductions in Ikk-α expression were convincingly dissipated upon abrogation of classical NF-κB signalling, indicating that activation of classical NF-κB activation is a prerequisite for inflammatory mediator-induced loss of Ikk-α expression. Again, this is in line with previous findings from our group showing that TNF-α-induced impairments in muscle OXPHEN were abrogated in myotubes with defective classical NF-κB signalling [12,13]. Moreover, over-expression of Ikk-α protein prevented TNF-α-induced reductions in the expression of slow Myhc and OXPHOS complexes, demonstrating that loss of Ikk-α expression is causally related to TNF-α-induced impairments in skeletal muscle OXPHEN.

The molecular mechanism by which classical NF-κB activation reduces Ikk-α expression levels remains to be established. Our data shows that activation of classical NF-κB signalling decreases Ikk-α promoter activation. The classical NF-κB transcriptionally active sub-unit Rela has been described to be able to mediate promoter repression upon binding to a target sequence [25]. However, no NF-κB responsive elements have been described in the Ikk-α promoter arguing against direct binding of NF-κB sub-units to the Ikk-α promoter region mediating inhibition [26]. In addition, transcription of the Ikk-α promoter is negatively regulated by P53 [27] and multiple P53 responsive elements are present in the Ikk-α promoter reporter construct we used [28]. Interestingly, P53 was recently shown to positively affect muscle OXPHEN [29]. Whether or not P53 is involved in classical NF-κB-induced inhibition of Ikk-α promoter activity remains to be determined. Additionally, recently, miR-223, miR-15a and miR-16 were identified as potent negative regulators of Ikk-α protein abundance in macrophages [30]. miR-223, miR-15a and miR-16 are expressed in skeletal muscle [31–33] and miR-223 and miR-16 have been shown to be up-regulated in conditions in which TNF-α levels are elevated [34,35]. Moreover, miR-16 is under direct transcriptional control of the classical NF-κB pathway [36]. Therefore, increased levels of these miRNA molecules might well be involved in post-transcriptional regulation of Ikk-α protein content in skeletal muscle under chronic inflammatory conditions.

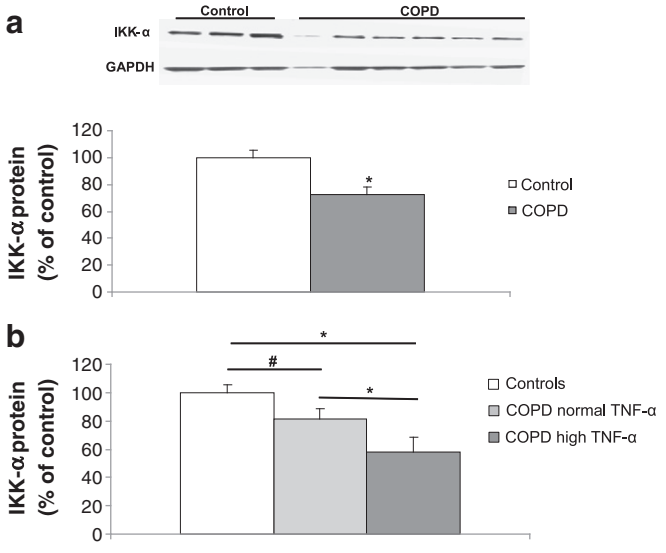

Fig. 5. Skeletal muscle IKK-α protein content is reduced in COPD. Skeletal muscle biopsies were obtained from a group of COPD patients (n = 59) and a group of healthy controls (n = 22). a, b) IKK-α protein was determined by western blotting. Western blots were corrected for total protein and GAPDH was used as a loading control. A representative Western blot is shown. Values are expressed as mean ± SEM. Significance: * $p \leq 0.05$; # $p \leq 0.1$.

Although the exact molecular mechanism remains to be elucidated, our data clearly shows that (TNF-α-induced) classical NF-κB activation reduces Ikk-α protein with a subsequent detrimental impact on muscle OXPHEN. Intriguingly, the alternative NF-κB pathway (of which Ikk-α is an essential constituent) and the classical NF-κB pathway appear to play opposing roles during the regulation of myogenic differentiation in which development of cellular OXPHEN is crucial to meet the metabolic demands of newly formed muscle fibres. More specifically, activity of the classical NF-κB pathway was shown to diminish during myogenesis while activity of the alternative pathway was potently induced [37,38]. This is suggestive of an antagonistic relationship between classical and alternative NF-κB signalling in the regulation of muscle OXPHEN, which is in line with our data. Conversely, Ikk-α also is a known negative regulator of the classical NF-κB pathway. A recent study reported that Ikk-α inhibits classical NF-κB signalling by phosphorylating multiple substrates that together coordinate repression of classical NF-κB signalling [39]. In this context, it can be speculated that loss of Ikk-α protein relieves negative feed-back on the classical NF-κB pathway thereby potentiating detrimental effects of activation of this pathway on skeletal muscle OXPHEN as we previously reported [12,13].

Bakkar *et al.* recently showed that muscle-specific knock-out of Ikk-α *in vivo* impaired muscle OXPHEN and decreased levels of Pgc-1β, not Pgc-1α, which was in line with their findings that identified Pgc-1β as a

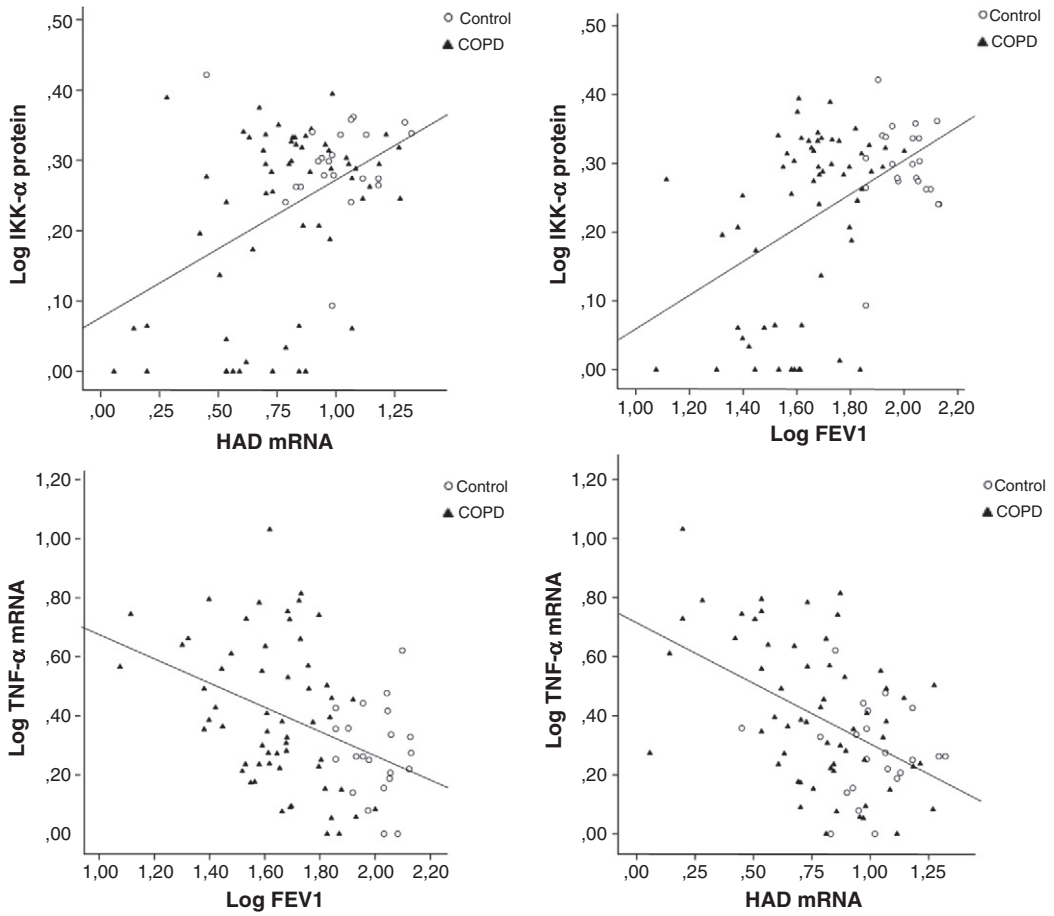

Fig. 6. Correlations between muscle IKK-α, TNF-α, HAD and FEV1. Correlations between muscle IKK-α, muscle TNF-α and muscle HAD mRNA expression as well as FEV1 were explored with a Pearson correlation test.

direct transcriptional target of Ikk-α-mediated signalling [17]. In contrast with findings from Bakkar *et al.* loss of Ikk-α protein in our study was associated with decreased mRNA transcript levels of both Pgc-1α and Pgc-1β. Moreover, Ikk-α over-expression potently increased Pgc-1α, not Pgc-1β, mRNA levels and TNF-α-induced reductions in Pgc-1α and Pgc-1β mRNA levels were both prevented by over-expression of Ikk-α. Collectively, this suggests that Ikk-α is involved in the regulation of muscle OXPHEN through control over both Pgc-1α and Pgc-1β. The reason for the apparent contradiction with results from Bakkar *et al.* may be explained by differences in the applied methodology. In the present study, we modulated Ikk-α C2C12 myotubes that were differentiated for 5 days which, as our data shows, display a near fully developed OXPHEN. In contrast, Bakkar *et al.* modulated Ikk-α protein content in C2C12 myoblasts after which the myogenic program was initiated. As the expression of both Pgc-1α and Pgc-1β are induced during myogenic differentiation [17,22], the observed effects of Ikk-α modulation on expression of the Pgc-1 molecules in the latter approach might reflect differential regulation of Pgc-1α and Pgc-1β by Ikk-α modulation during myogenesis rather than regulation of the Pgc-1 molecules by Ikk-α in myotubes with a fixed, fully developed OXPHEN. In addition, Bakkar *et al.* applied adeno-associated viral (AAV) Ikk-α expression constructs to over-express Ikk-α *in vivo* and subsequently investigate effects on the regulation of muscle OXPHEN by Pgc-1 molecules, whereas we applied adenoviral expression (Ad) constructs which may yield higher levels of over-expression as compared to AAV constructs.

Both muscle OXPHEN as well as PGC-1/PPAR signalling are compromised in skeletal muscle of COPD patients [9,40,41]. Interestingly, our own work and that of Bakkar *et al.* shows that loss of muscle Ikk-α expression *in vitro* and *in vivo*, recapitulates many of the alterations observed in COPD-associated loss of muscle OXPHEN. Indeed, loss of muscle Ikk-α protein content resulted in decreased expression levels of mitochondrial proteins and impairments in the Pgc-1/Ppar pathway, similar as to what is observed in COPD [9]. In fact, in the present manuscript we show, for the first time, that an impaired muscle OXPHEN and decreased muscle PGC-1/PPAR signalling in COPD are indeed associated with a reduced muscle IKK-α protein content. In addition, knock-down of Ikk-α in cultured myotubes in our hands resulted in significantly decreased levels of Myhc I and IIA (slow) isoforms while expression the Myhc IIb (fast) isoform was significantly induced. Although in humans MYHC IIb is only expressed at very low levels in muscle, human skeletal muscle does express high levels of MYHC I and MYHC IIa. As muscle of COPD patients is often characterised by a reduction in the proportion of type I (slow, oxidative) fibres, loss of IKK-α, may well contribute to the fibre-type shift towards a more glycolytic distribution that is often observed in this disease [7].

In addition to an impaired muscle OXPHEN [7,42,43] and disturbed PGC-1 signalling [9,12], increased activation of inflammatory signalling (classical NF-κB) has been described in the musculature of severe COPD patients [44]. In our study, reductions in IKK-α protein as well as impairments in muscle OXPHEN appeared more pronounced in COPD patients with high levels of muscle TNF-α expression. Moreover, IKK-α levels in muscle correlated inversely with muscle TNF-α in the total group of investigated subjects as well as in the COPD patient group only. In concert with our *in vitro* data, this strongly suggests that TNF-α-induced reductions in muscle IKK-α may accelerate muscle OXPHEN deterioration in COPD. Although we observed that loss of Ikk-α *in vitro* resulted in decreased expression levels of both Pgc-1β and Pgc-1α and that COPD patients with high levels of muscle TNF-α

displayed lower levels of IKK-α, PGC-1α expression was not significantly different in this sub-set of patients compared to patients with normal TNF-α levels despite a significantly more impaired muscle OXPHEN. The reason for this is unclear but may be related to the multi-factorial nature of the disease or to inherent differences between cell culture models and intact human muscle *in vivo*. As we show that TNF-α-induced decreases in Ikk-α protein levels in mature myotubes represent a true decline in OXPHEN (and its regulation by Pgc-1 signalling) and not a delayed development of muscle OXPHEN due to impaired myogenesis, the relevance of the work described in the present paper lies in the direct effects of (TNF-α-induced) classical NF-κB activation on mature skeletal muscle oxidative phenotype in COPD. Unfortunately, as a limitation of the present study, limited quantities of biopsy tissue were available from the 2 previous reported clinical studies which precluded robust assessment of classical NF-κB activity in muscle. However, increased activation of the classical NF-κB pathway has been described by others in skeletal muscle of severe COPD patients [14].

5. Conclusion

In conclusion, in the current manuscript we show that classical NF-κB activation potently induces loss of skeletal muscle Ikk-α protein abundance which subsequently results in an impaired muscle OXPHEN. This implies that reductions in Ikk-α levels underlie inflammation-induced impairment of muscle OXPHEN. Moreover, our finding that COPD patients are not only characterised by an impaired muscle OXPHEN but also by decreased levels of IKK-α in muscle, which both were more pronounced in patients with high levels of muscle TNF-α, indicates that TNF-α-induced loss of muscle IKK-α levels may well be of relevance to acute or chronic inflammation-induced impairments of muscle OXPHEN in COPD. Future research into the exact implications of both NF-κB pathways in the regulation of skeletal muscle OXPHEN in COPD may be paramount in the development of new therapeutic strategies.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.11.001.

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