TNF-alpha-Induced NF-kappa B Activation Stimulates Skeletal Muscle Glycolytic Metabolism Through Activation of HIF-1 alpha

TNF-α-Induced NF-κB Activation Stimulates Skeletal Muscle Glycolytic Metabolism Through Activation of HIF-1α

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A shift in quadriceps muscle metabolic profile toward decreased oxidative metabolism and increased glycolysis is a consistent finding in chronic obstructive pulmonary disease (COPD). Chronic inflammation has been proposed as a trigger of this pathological metabolic adaptation. Indeed, the proinflammatory cytokine TNF-α impairs muscle oxidative metabolism through activation of the nuclear factor-κB (NF-κB) pathway. Putative effects on muscle glycolysis, however, are unclear. We hypothesized that TNF-α-induced NF-κB signaling stimulates muscle glycolytic metabolism through activation of the glycolytic regulator hypoxia-inducible factor-1α (HIF-1α). Wild-type C2C12 and C2C12-IκBα-SR (blocked NF-κB signaling) myotubes were stimulated with TNF-α, and its effects on glycolytic metabolism and involvement of the HIF pathway herein were investigated. As proof of principle, expression of HIF signaling constituents was investigated in quadriceps muscle biopsies of a previously well-characterized cohort of clinically stable patients with severe COPD and healthy matched controls. TNF-α increased myotube glucose uptake and lactate production and enhanced the activity and expression levels of multiple effectors of muscle glycolytic metabolism in a NF-κB-dependent manner. In addition, TNF-α activated HIF signaling, which required classical NF-κB activation. Moreover, the knockdown of HIF-1α largely attenuated TNF-α-induced increases in glycolytic metabolism. Accordingly, the mRNA levels of HIF-1α and the HIF-1α target gene, vascular endothelial growth factor (*VEGF*), were increased in muscle biopsies of COPD patients compared with controls, which was most pronounced in the patients with high levels of muscle TNF-α. In conclusion, these data show that TNF-α-induced classical NF-κB activation enhances muscle glycolytic metabolism in a HIF-1α-dependent manner. (***Endocrinology*** **156: 1770–1781, 2015)**

Adequate functioning of skeletal muscle tissue relies on its intrinsic capacity to derive energy from metabolizing substrates such as carbohydrates and fat (1). Although most ATP in muscle is produced aerobically through oxidative phosphorylation in the mitochondria, anaerobic glycolysis in the cytosol contributes to skeletal muscle ATP generation, albeit much less efficiently, by metabolizing glucose to lactate. Interestingly, glycolysis and subsequent lactate production can also occur in the presence of adequate amounts of O_2 (2, 3).

Illustrative of their significance in chronic disease prevention, impairments in (lower limb) muscle oxidative metabolism have been linked to the development of insulin resistance, metabolic inflexibility, and progression of the metabolic syndrome (4–7). In addition, in chronic obstructive pulmonary disease (COPD), it is well established that loss of muscle oxidative metabolism and a fiber-type

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Abbreviations: CA9, carbonic anhydrase 9; COPD, chronic obstructive pulmonary disease; β-gal, β-galactosidase; GLP, glycogen phosphorylase; Glut, glucose transporter; GS, glycogen synthase; HIF, hypoxia-inducible factor; HK II, hexokinase II; HRE, HIF-responsive element; NF-κB, nuclear factor-κB; PDK-4, pyruvate dehydrogenase kinase 4; PFK, phosphofructokinase; PGC-1α, peroxisome proliferator-activated receptor 1α; Q-PCR, quantitative PCR; RNAi, RNA interference; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.

shift toward a larger proportion of glycolytic fibers modulates disease progression and health-related quality of life via adverse effects on exercise capacity (4–11). In these patients, whole-body glucose production was found to be increased and lactate accumulated faster and at a lower work load during exercise (12–14). Collectively, these metabolic alterations in the musculature of COPD patients are indicative of a pathologically increased reliance of the muscle on less efficient glycolytic metabolism as a source of energy production (13, 15–17). Importantly, although physical inactivity elicits similar morphological, structural, and biochemical abnormalities as seen in skeletal muscles of COPD patients, physical inactivity does not sufficiently explain muscle dysfunction seen in these patients, suggesting the involvement of pathological mechanisms other than muscle disuse (18).

We recently provided evidence that the inflammatory cytokine TNF-α directly impairs skeletal muscle oxidative metabolism by signaling through the inflammatory nuclear factor-κB (NF-κB) pathway (19). Moreover, we showed that increased TNF-α expression in muscle of COPD patients was associated with not only reduced expression of genes involved in oxidative metabolism but also with increased expression levels of the glycolytic enzyme hexokinase II (HK II) compared with patients with normal levels of muscle TNF-α (19). In addition, one in vitro study previously reported increased lactate levels in cell supernatant of L6 myocytes exposed to TNF-α (20). Although, collectively, these studies suggest a potential involvement of TNF-α in the observed shift toward glycolytic metabolism in muscle of COPD patients, a comprehensive analysis of the impact of TNF-α on muscle glycolysis is currently lacking, and the signaling pathways involved remain to be identified. Furthermore, whether the observed shift toward glycolytic metabolism in COPD is secondary to a loss of oxidative muscle metabolism or may represent an autonomous effect of TNF-α on muscle glycolysis is unclear. We hypothesized that TNF-α-induced NF-κB activation directly stimulates skeletal muscle glycolytic metabolism through activation of the glycolytic regulator hypoxia-inducible factor (HIF)-1α. To address this, we exposed cultured myotubes to TNF-α, investigated cellular glycolytic metabolism, classical NF-κB signaling and constituents of the HIF pathway, and addressed their causal involvement in TNF-α-induced changes in myotube metabolism. Additionally, we assessed the expression of HIF signaling constituents in quadriceps muscle biopsies of COPD patients and healthy matched controls to investigate associations with muscle TNF-α expression and effectors of glycolytic metabolism.

Materials and Methods

Cell culture

The murine C2C12 skeletal muscle cell line was obtained from the American Type Culture Collection (ATCC CRL1772). The stable C2C12 HIF transcriptional activity reporter cell line was constructed by stable transfection of a HIF reporter plasmid bearing five consecutive HIF-responsive elements (HREs) from the human vascular endothelial growth factor (*VEGF*) A promoter (5HRE/hCMVmp-luc) (21). C2C12-IκBα-SR cells (displaying blocked classical NF-κB signaling) were kindly provided by Dr D. Guttridge (Ohio State University, Columbus, Ohio). Additional detail regarding culture conditions is provided in the online data supplement.

Chemicals, reagents, and plasmids

TNF-α (Calbiochem) was dissolved in 0.1% BSA, which also served as a vehicle control (0.005% final concentration) to a stock of 200 ng/mL. Small interfering RNA (siRNA) constructs targeting HIF-1α or peroxisome proliferator-activated receptor 1α (PGC-1α) and a scrambled control siRNA construct were purchased from Invitrogen. Plasmids encoding P65 or P50 were kind gifts from Dr Michael Karin (University of California, San Diego, La Jolla, California). The HIF reporter expresses the luciferase gene controlled by a promoter bearing five consecutive HREs from the human *VEGFA* promoter (5HRE/hCMVmp-luc) (21) and was kindly provided by Dr T. van den Beucken (Maastricht University, Maastricht, the Netherlands). 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.

Reporter assays

The HIF reporter C2C12 cell line was plated in 35-mm dishes and allowed to grow to 90% confluence. After induction of differentiation, cells were differentiated into myotubes for 5 days after which cells were stimulated. Cells were harvested by washing two times with cold PBS and subsequent lysis by adding (100 μL) 1$\times$ reporter lysis buffer (Promega) and incubation on ice for 10 minutes. Cell lysates were centrifuged (14 000 rpm, 1 min), and supernatants were snap frozen and stored at -80°C for later analysis. Luciferase activity was measured according to the manufacturer's instructions (Promega) and corrected for total protein content (Bio-Rad Laboratories). For transient transfections, C2C12 cells were transfected with the HIF reporter plasmid and expression plasmids of interest. Luciferase activity in transient transfection experiments was corrected for β-gal activity.

RNA interference (RNAi)-mediated suppression of target genes

HIF-1α or PGC-1α were silenced in C2C12 myotubes cultured in 35-mm dishes (BD Biosciences). Myotubes were transiently transfected using Stealth RNAi siRNAs (Invitrogen) according to the manufacturer's instructions. The transfection mix was prepared in Opti-MEM reduced serum medium, and Lipofectamine RNAiMAX (both from Invitrogen) was used as a transfection agent. The transfection mixture, for both universal control siRNA (low percentage of guanine plus cytosine) and target siRNA (final concentration both at 10 nM), was incubated

at room temperature for 20 minutes, after which it was added to the culture dishes containing fresh differentiation medium.

RNA isolation, cDNA synthesis, and quantitative PCR (Q-PCR)

For in vitro work, total RNA was extracted using an on-column RNA isolation kit (QIAGEN) according to the manufacturer's instructions. RNA concentration was determined using a spectrophotometer, and 0.4 μg of RNA per sample was reverse transcribed into cDNA using a Transcriptor cDNA synthesis kit (Roche). cDNA was diluted 1:50 and Q-PCR amplified with Sensimix SYBR green and fluorescein mix (GC Biotech) on a Bio-Rad PCR apparatus. Primers were designed to generate a PCR amplification product of 100–150 bp. Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. Gene expression levels were normalized by calculating an average value of four housekeeping genes using GeNorm software (Primerdesign). For human muscle biopsies, RNA isolation, cDNA synthesis, and Q-PCR analyses were performed as described previously (19).

Western blotting

For Western blotting, 0.4 μg (HIS-3) or 10 μg [RelA, glucose transporter (Glut)-1, HK I, HK II, phosphoglycogen synthase (GS)] was loaded. Details regarding the preparation of whole-cell lysates and nuclear extracts as well as procedures regarding Western blot analysis are provided in the online data supplement.

Enzyme activity assays

Activity of phosphofructokinase (PFK) was measured spectrophotometrically as described previously (22). Enzyme activity levels were corrected for total protein content, which was determined using a detergent-compatible protein determination assay (Bio-Rad Laboratories).

Glucose uptake and lactate production

Glucose uptake was determined by measuring levels in cell supernatant using a standard glucose meter (Bayer). Lactate levels in the cell supernatant were determined by electrospray ionization tandem mass spectrometry with multiple reaction monitoring (multiple reaction monitoring mode). Integration of the data was performed by use of Masslynx software, and data was quantified based on internal standards labeled with a stable isotope.

Glycogen content

Myotubes were washed twice with ice-cold PBS. Subsequently, glycogen was extracted by lysing the cells in 200 μL 30% KOH and heating the samples at 70°C for 30 minutes. Two hundred microliters of Na_2SO_4 and 600 μL 100% ethanol were added for precipitation of the lysate. Samples were incubated on a rotating platform (4°C) for 60 minutes and subsequently centrifuged (14 000 rpm) for 5 minutes. Glycogen in the pellet was subsequently hydrolyzed to glucose by dissolving the precipitate in 165 μL of HCl (1 M) and boiling them at 100°C for 120 minutes. Finally, 75 μL of NaOH (2 M) was added and glucose was determined according to manufacturers' instructions (Sigma-Aldrich).

Subjects

For the human proof-of-concept study, we analyzed remaining mRNA isolated from quadriceps muscle biopsies of a cohort of clinically stable COPD patients and healthy age-matched smoking controls who were previously characterized (19). A high TNF-α COPD subgroup was defined as having muscle TNF-α mRNA levels, corrected for a normalization factor calculated on expression levels of five housekeeping genes, greater than the mean + 2 × SD of that of the control group as previously described (19). Experiments involving human tissue are in accordance with the principles set out in the Declaration of Helsinki. Informed consent was obtained from the participants and an institutional human research committee approved the investigations.

Statistics

Data were analyzed using SPSS (SPSS Inc). Unpaired *t* tests (in vitro) or ANOVA analyses with least significant differences post hoc correction (human data) were used. The Pearson correlation test was used to explore relevant correlations. Data are represented as the mean ± SD unless specified otherwise. A value of $P < .05$ was considered to be significant.

Results

TNF-α-induced NF-κB signaling enhances skeletal muscle glycolytic metabolism

To investigate the impact of TNF-α-induced NF-κB activation on muscle glycolytic metabolism, we stimulated wild-type C2C12 myotubes and myotubes with blocked NF-κB signaling (IκBα-SR C2C12) with TNF-α and investigated multiple effectors of myotube glycolytic metabolism. Effectiveness of blockade of classical NF-κB signaling in IκBα-SR C2C12 myotubes is depicted in Supplemental Figure 1. Treatment of wild-type myotubes with TNF-α resulted in decreased glucose levels and increased lactate levels in cell culture medium, which largely depended on intact NF-κB signaling (Figure 1, A and B). Furthermore, increased uptake of glucose upon exposure of myotubes to TNF-α was accompanied by enhanced mRNA and protein levels of the glucose transporter molecule Glut-1 and increased mRNA transcript abundance and enzymatic activity of PFK, which was not observed in IκBα-SR myotubes (Figure 1, C–F). Although PFK enzyme activity was slightly induced in IκBα-SR C2C12 myotubes exposed to TNF-α, this was less pronounced compared with inductions observed in wild-type myotubes (Figure 1F).

Also, mRNA expression levels of the glycolytic enzymes HK II and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3) as well as mRNA transcript abundance of lactate dehydrogenase 1 and the lactate transporter monocarboxylate transporter molecule 4 increased upon TNF-α exposure in a NF-κB-dependent manner (Figure 1, G–J). In addition to being directed toward lactate production, glucose can also be oxidized in

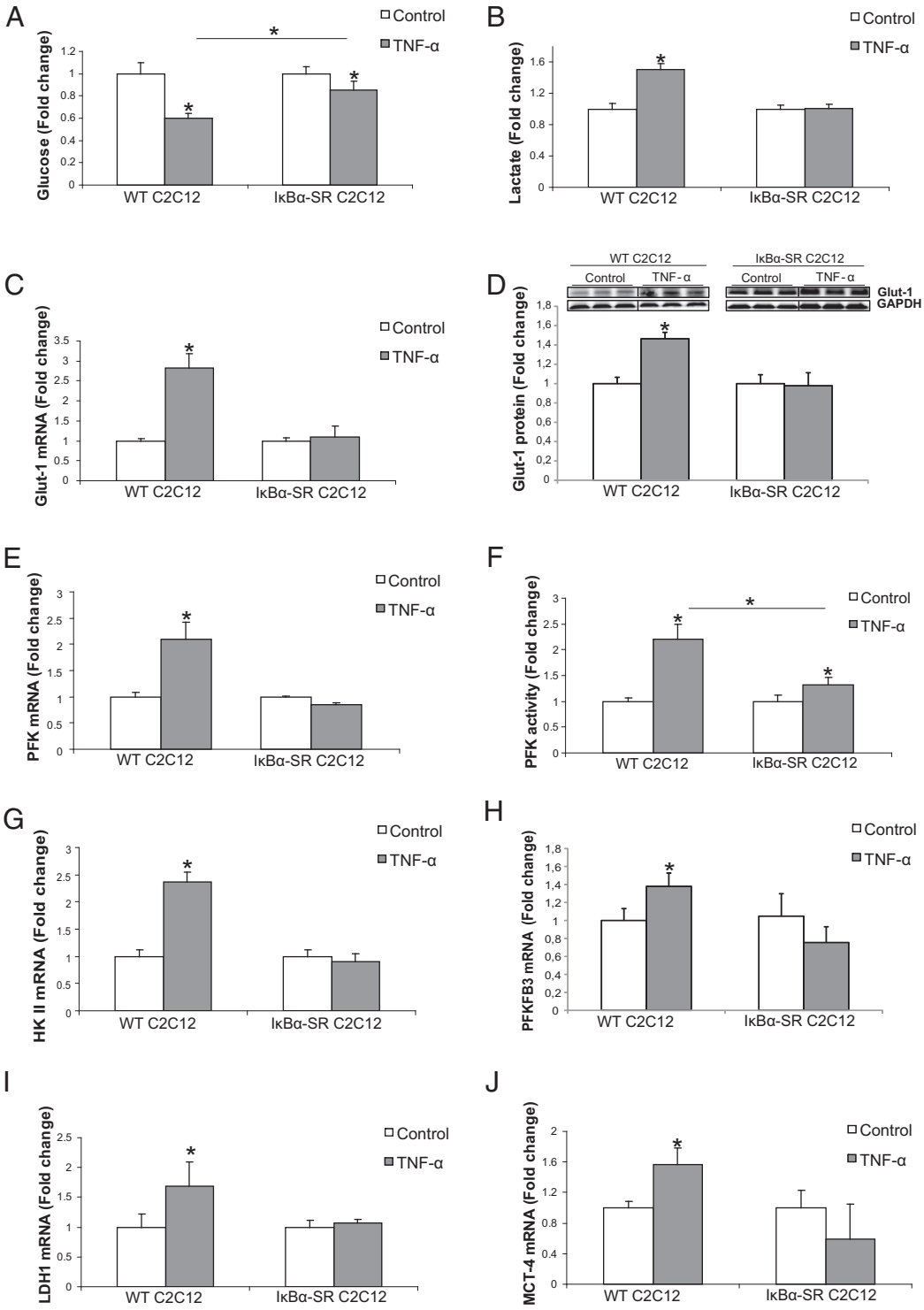

Figure 1. TNF-α-induced increases in muscle glycolytic metabolism depend on intact classical NF-κB signaling. C2C12 myoblasts and C2C12-IκBα-SR myoblasts were induced to differentiate into multinucleated myotubes. At day 5 of differentiation, myotubes were stimulated with TNF-α (10 ng/mL) or vehicle (control) for 96 hours (A, B, and F) or 48 hours (C–E and G–J). Glucose uptake and lactate export (A and B) as well as PFK enzyme activity (F), Glut-1 protein abundance (D), and mRNA expression levels of genes involved in glycolytic metabolism (C, E, and G-J) were determined. Enzyme activity levels were corrected for total protein content and Western blots were corrected for GAPDH as a loading control. For both cell lines, Western blot triplicates for each treatment condition were cut from the same immunoblot as indicated by the black boxes. Values are expressed as mean ± SD (as fold change compared with control) from triplicate samples (experiments n = 3). *, Significance compared with control ($P \leq .05$). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; WT, wild type.

the mitochondria or stored as glycogen. To explore these potential fates of glucose in our experiments, we measured the phosphorylation status of GS (rate-limiting in glycogen synthesis), mRNA expression levels of glycogen phosphorylase (GLP; glycogenolysis), and cellular glycogen content in response to TNF-α. In addition, transcript abundance of pyruvate dehydrogenase kinase 4 (PDK-4; inhibitor of glucose oxidation) was investigated. We observed that phosphorylated GS protein as well as GLP mRNA abundance decreased upon TNF-α exposure in a NF-κB-dependent manner, whereas glycogen content was unaltered in wild-type myotubes exposed to TNF-α (Figure 2, A–C). Furthermore, PDK-4 mRNA levels decreased upon TNF-α exposure in a NF-κB-dependent manner (Figure 2D).

We previously demonstrated that (TNF-α-induced) NF-κB activation potently impairs myotube oxidative metabolism (23). To investigate whether increased glycolysis in response to TNF-α results from an autonomous effect of TNF-α on myotube glycolytic metabolism or rather reflects an adaptive response to an impaired cellular oxidative phenotype, we inhibited oxidative metabolism by knockdown of PGC-1α and investigated several markers of myotube glycolytic metabolism. As depicted in Figure 3A, PGC-1α was successfully knocked down. PGC-1α knockdown subsequently resulted in decreased mRNA expression of a subunit of complex III of the oxidative phosphorylation chain and increased PDK-4 expression levels, providing evidence for inhibition of myotube oxidative metabolism. Furthermore, PGC-1α knockdown decreased mRNA levels of Glut-1 and PFK, whereas HK II mRNA transcript abundance was unaltered and lactate levels in cell supernatant were unchanged (Figure 3, B–D).

Collectively these data suggest that TNF-α-induced activation of myotube glycolytic metabolism is dependent on intact NF-κB signaling and most likely represents an autonomous effect on glycolysis rather than an adaptive response to an impaired oxidative metabolism.

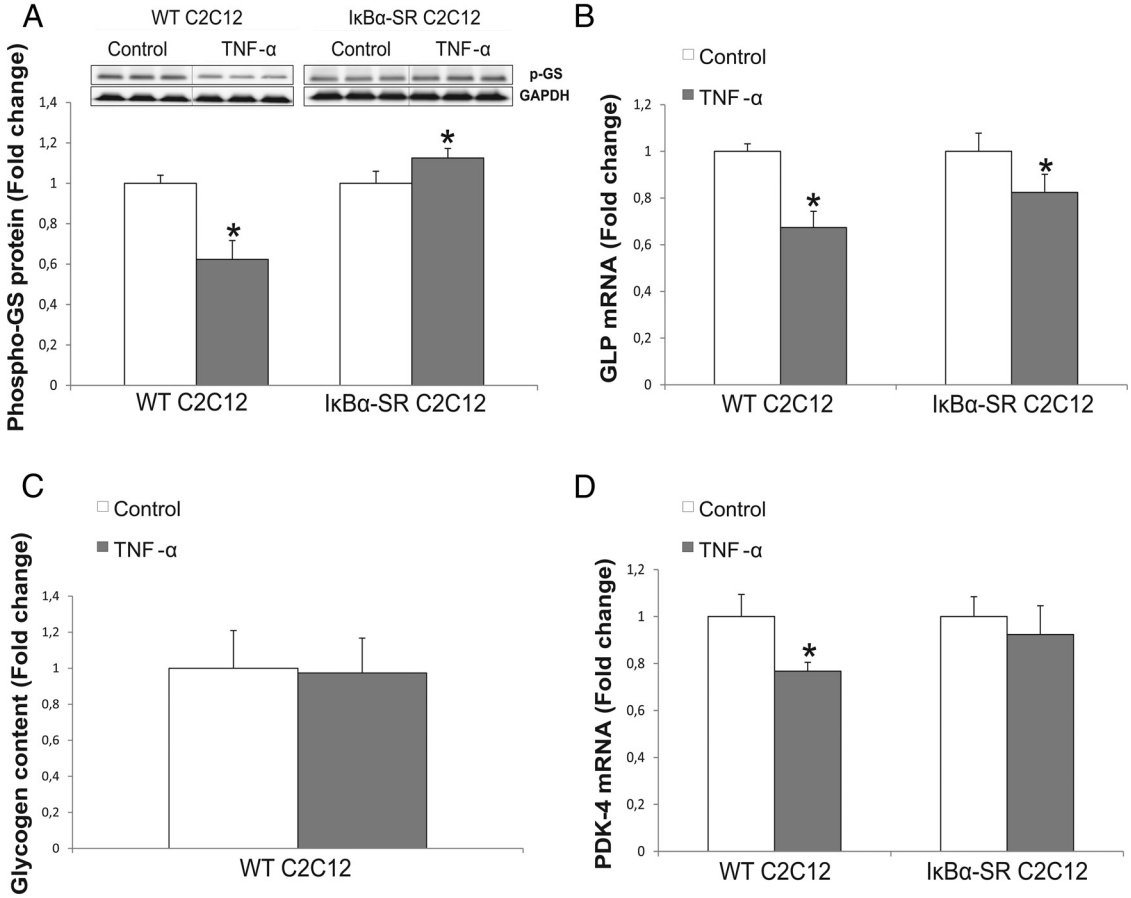

Figure 2. TNF-α does not alter cellular glycogen content but decreases PDK-4 expression in a NF-κB-dependent manner. C2C12 myoblasts and C2C12-IκBα-SR myoblasts were induced to differentiate into multinucleated myotubes. At day 5 of differentiation, myotubes were stimulated with TNF-α (10 ng/mL) or vehicle (control) for 48 hours. Phosphorylated GS protein (A), GLP mRNA abundance (B), and cellular glycogen content (C) as well as PDK-4 mRNA levels (D) were assessed. Western blots were corrected for GAPDH as a loading control. For both cell lines, Western blot triplicates for each treatment condition were cut from the same immunoblot as indicated by the black boxes. Values are expressed as mean ± SD (as fold change compared with control) from triplicate samples (experiments n = 3). *, Significance compared with control ($P \leq .05$). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; WT, wild type.

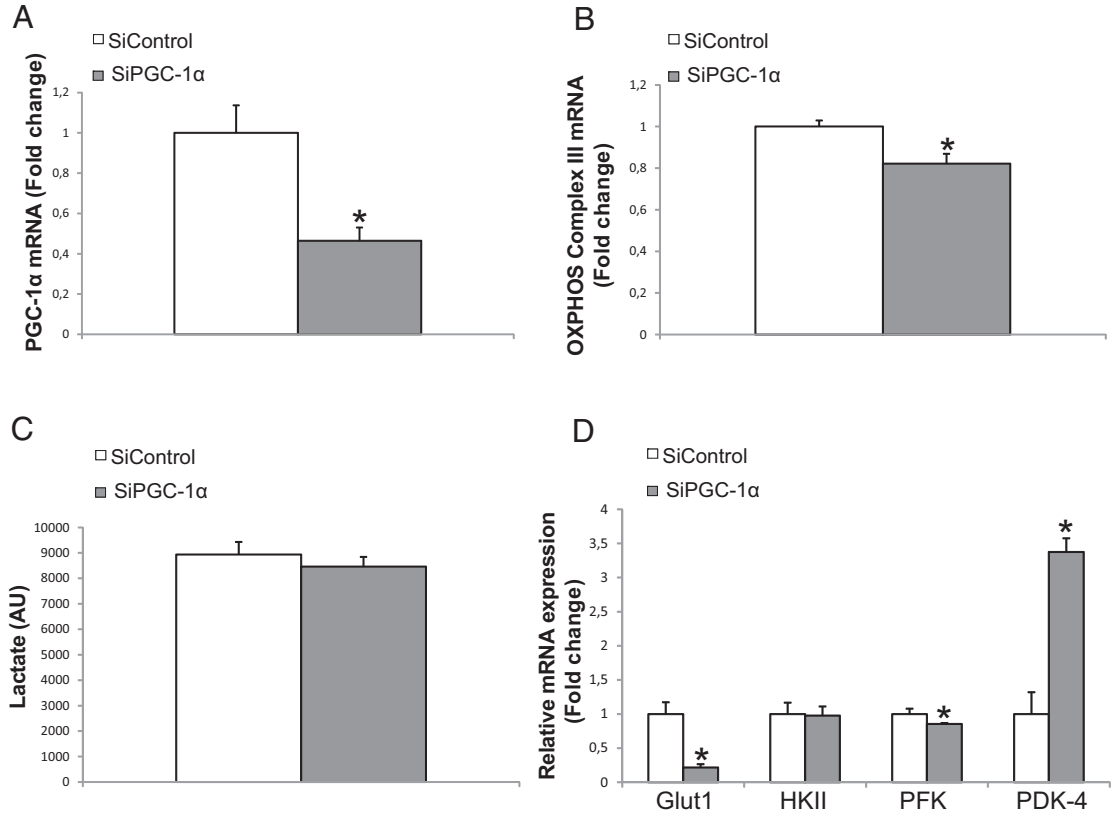

Figure 3. Knockdown of PGC-1α fails to induce glycolytic gene expression and cellular lactate production. C2C12 myoblasts were differentiated for 5 days into fully mature myotubes. Myotubes were subjected to knockdown of PGC-1α by use of a specific siRNA targeting PGC-1α for 48 hours. As a control, myotubes were treated with a nonspecific scrambled control siRNA construct. PGC-1α and oxidative phosphorylation complex III mRNA as well as lactate levels in the supernatant and expression levels of genes involved in glycolytic metabolism were determined (A–D). Values are expressed as mean ± SD from triplicate samples (experiments n = 3). *, Significance compared with control ($P \leq .05$).

TNF-α-induced classical NF-κB activation activates muscle HIF-1α signaling

HIF-1α is a known positive regulator of muscle glycolytic metabolism (24–26). Therefore, we investigated the effect of TNF-α-induced NF-κB activation on the HIF signaling pathway. As illustrated in Figure 4A, exposure of C2C12 myotubes to TNF-α induced an increase in HIF transcriptional activity, which was largely mediated through HIF-1α (Figure 4B). Furthermore, mRNA expression levels of HIF-1α, but not HIF-2α, as well as HIF-1α target genes *VEGF* and carbonic anhydrase 9 (*CA9*) increased significantly upon exposure of cultured C2C12 myotubes to TNF-α in a NF-κB-dependent manner (Figure 4, C–F). Conversely, overexpression of the classical NF-κB signaling constituents P50 and P65, which we previously showed to result in a strong induction of NF-κB transcriptional activity in C2C12 cells (29), potently increased HIF transcriptional activity (Figure 4G).

TNF-α-induced increases in muscle glycolytic metabolism depend on HIF-1α

Next, we investigated whether TNF-α-induced activation of myotube glycolytic metabolism is mediated through HIF-1α. As illustrated in Supplemental Figure 2, HIF-1α knockdown suppressed HIF transcriptional activity and decreased mRNA expression of HIF-1α as well as transcript abundance of the HIF-1α target genes *VEGF* and *CA9*. Also, HIF-1α knockdown abrogated TNF-α-induced increases in the mRNA transcript abundance of these markers (Supplemental Figure 2, A–D).

Knockdown of HIF-1α decreased basal myotube glucose uptake and attenuated TNF-α-induced increases in glucose uptake (Figure 5A). HIF-1α knockdown also reduced the basal levels of Glut-1 mRNA and protein as well as mRNA abundance and enzymatic activity of PFK (Figure 5, B–E). Basal PFKFB3 mRNA levels were similarly decreased (Figure 5F). Basal HK II mRNA and HK I protein levels, however, were unaltered upon HIF-1α knockdown, whereas HK II protein levels increased (Figure 5, G–I). Moreover, TNF-α-induced increases in Glut-1 mRNA expression levels were significantly attenuated by knockdown of HIF-1α (Figure 5B) as were TNF-α-induced increases in PFK, PFKFB3, and HK II mRNA abundance (Figure 5, D, F, and G). In contrast, PFK enzyme activity as well as Glut-1 protein abundance was signifi-

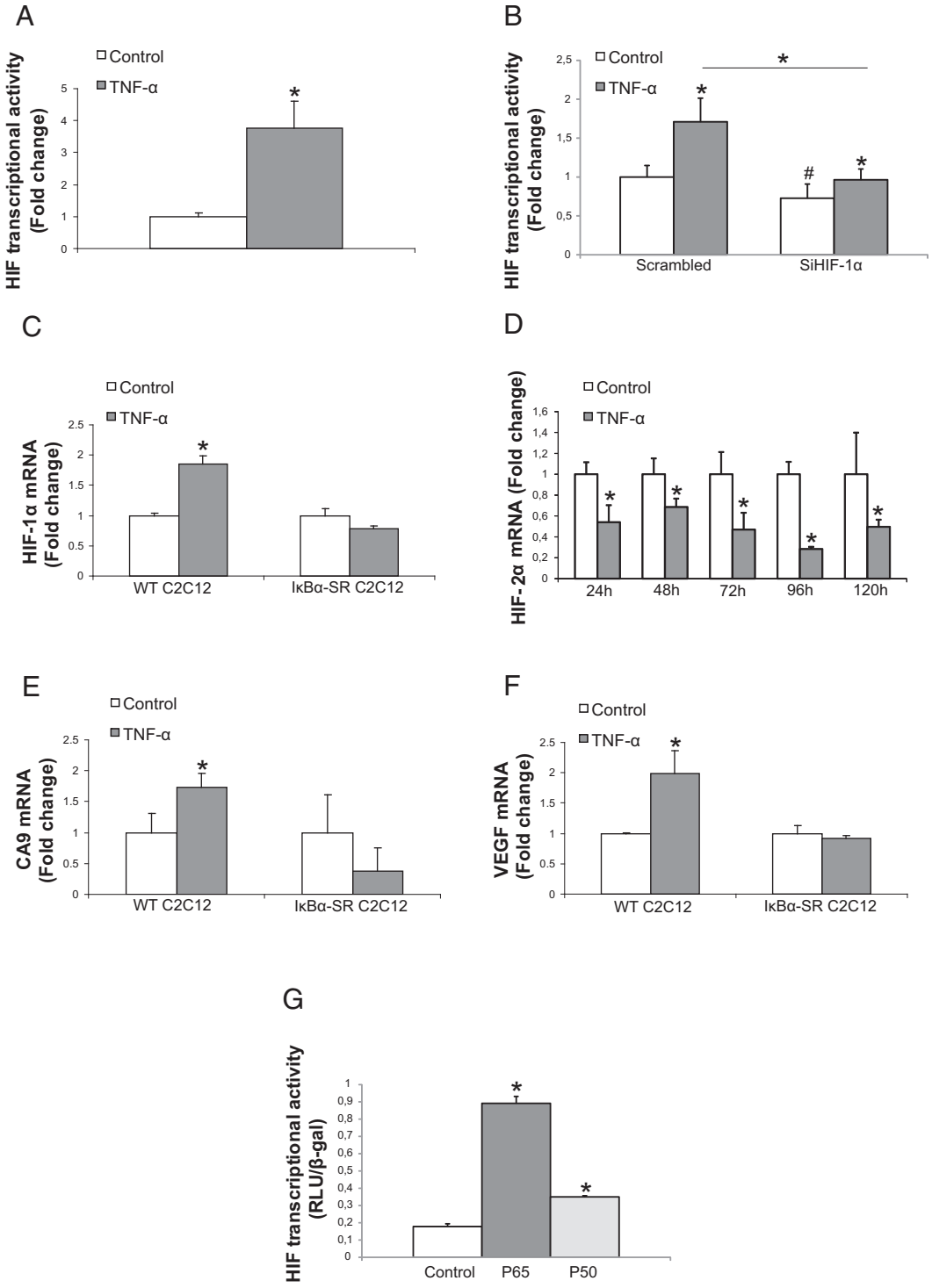

Figure 4. TNF-α-induced classical NF-κB activation increases muscle HIF-1α signaling. C2C12 myoblasts were stably transfected with a HIF transcriptional reporter construct, differentiated into myotubes for 5 days, and subsequently stimulated with TNF-α (10 ng/mL) or vehicle (control) for 24 hours (A) or HIF-1α was knocked down by specific siRNA constructs for 24 hours followed by 24 hours of TNF-α stimulation (10 ng/mL) (B). As a control, a scrambled nonspecific siRNA construct and vehicle was applied. Luciferase readout was corrected for total protein content. C–F, C2C12 myoblasts and C2C12-IκBα-SR myoblasts were induced to differentiate into multinucleated myotubes. At day 5 of differentiation, myotubes were stimulated with TNF-α (10 ng/mL) or vehicle (control) for 48 hours. HIF-1α (C), HIF-2α (D), CA9 (E), and VEGF (F) mRNA levels were determined. G, C2C12 myoblasts were transiently cotransfected with a HIF transcriptional reporter construct and expression plasmids encoding P65 or P50 or a PcDNA3.1 empty vector (control) for 48 hours. Luciferase activity was determined and corrected for β-gal. Values are expressed as mean ± SD (as fold change compared with control) from triplicate samples (experiments n = 3). *, Significance compared with control ($P \leq .05$); #, significance compared with C2C12 myotubes receiving vehicle control and scrambled nonspecific siRNA ($P \leq .05$). WT, wild type.

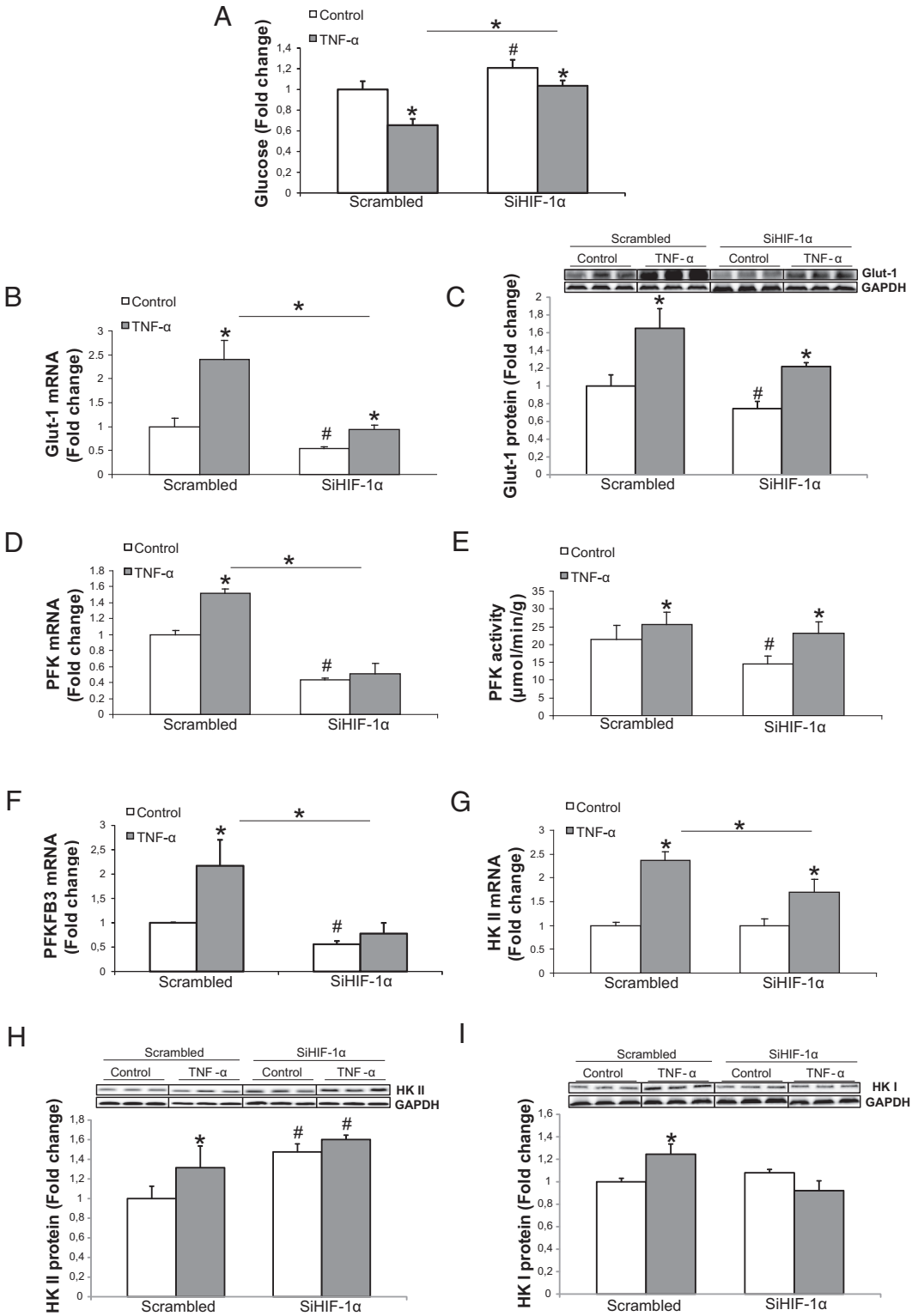

Figure 5. TNF-α-induced increases in glycolytic metabolism depend on HIF-1α. C2C12 myoblasts were differentiated into myotubes for 5 days. HIF-1α was knocked down by specific siRNA constructs for 24 hours. As a control, a scrambled nonspecific siRNA construct was applied. Myotubes were subsequently stimulated with TNF-α (10 ng/mL) or vehicle (control) for 48 hours. Glucose uptake, PFK enzyme activity, and mRNA expression levels as well as protein abundance of glycolytic constituents were assessed. Enzyme activity levels were corrected for total protein content and Western blots were corrected for GAPDH as a loading control. Western blot triplicates for each treatment condition were cut from the same immunoblot as indicated by the black boxes. Values are expressed as mean ± SD (as fold change compared with scrambled control) from triplicate samples (experiments n = 3). *, Significance compared with control ($P \leq .05$); #, significance compared with C2C12 myotubes receiving vehicle control and scrambled nonspecific siRNA ($P \leq .05$). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

cantly induced in response to TNF-α in both wild-type myotubes and in myotubes in which HIF-1α was knocked down (Figure 5, C and E). TNF-α-induced increases in HK II and HK I protein abundance, on the other hand, were abrogated when HIF-1α was knocked down (Figure 5, H and I). Also, basal lactate dehydrogenase 1 and monocarboxylate transporter molecule-4 mRNA transcript levels decreased upon HIF-1α knockdown and TNF-α-induced increases in mRNA levels of these markers were abrogated in response to loss of HIF-1α (Supplemental Figure 3, A and B), further evidencing that TNF-α-induced activation of myotube glycolytic metabolism largely depends on HIF-1α.

HIF-1α and VEGF mRNA expression is increased in muscle of COPD patients

We previously demonstrated that COPD patients with high levels of TNF-α expression in muscle were characterized by increased expression levels of the glycolytic enzyme HK II compared with patients with normal muscle TNF-α expression (19). In these patients, TNF-α correlated positively with HK II mRNA expression ($r = 0.628$; $P \leq .001$) and inversely with markers of muscle oxidative metabolism (Supplemental Table 1). In this cohort of COPD patients, we explored whether increased muscle TNF-α levels were associated with elevated expression of constituents of HIF-1α signaling. HIF-1α mRNA expression levels correlated positively with VEGF transcript abundance ($r = 0.633$; $P \leq .001$). As depicted in Figure 6A, COPD patients displayed significantly higher HIF-1α and VEGF mRNA transcript levels in muscle compared with healthy controls. Moreover, when patients were stratified according to normal or high muscle TNF-α transcript abundance, increases in HIF-1α and VEGF mRNA levels were most pronounced in patients with high levels of muscle TNF-α (Figure 6B).

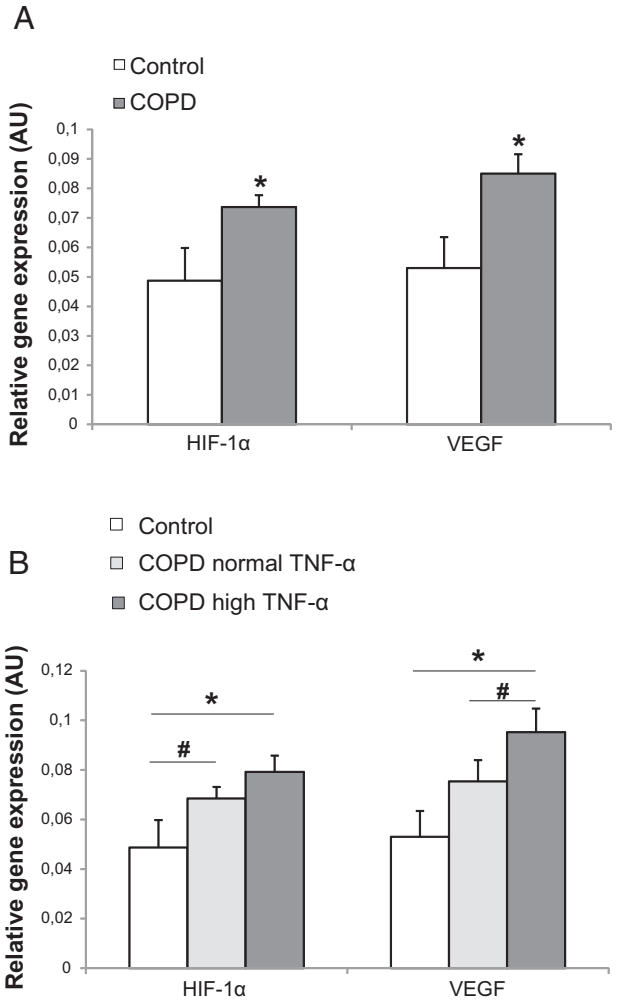

Figure 6. HIF signaling is activated in COPD patients with high muscle TNF-α levels. Skeletal muscle biopsies were obtained from a group of COPD patients (n = 29) and a group of healthy controls (n = 7). A, HIF-1-α and VEGF mRNA levels were determined by QPCR. B, COPD patients were stratified according to muscle TNF-α mRNA levels. HIF-1-α and VEGF mRNA transcript abundance was compared among healthy controls (n = 7), COPD patients with normal TNF-α levels (n = 15), and COPD patients with high muscle TNF-α levels (n = 14). Values are expressed as mean ± SEM. *, $P \leq .05$; #, $P \leq .1$.

Discussion

In the current study, we show that TNF-α-induced classical NF-κB activation stimulates skeletal muscle glycolytic metabolism through activation of HIF-1α. Moreover, we describe that, in addition to previously reported increased glycolytic gene expression, muscle tissue of COPD patients was characterized by increased mRNA expression levels of HIF-1α and the HIF-1α target gene *VEGF*, which was most pronounced in patients with high levels of muscle TNF-α.

Although impairment of cellular oxidative metabolism by TNF-α has been described in muscle (19, 27) and other tissue/cell types (28–30), a comprehensive analysis of the effects of TNF-α on muscle glycolytic metabolism, as we provide in the present study, was lacking. Previously, Spitzer et al (31) reported that endotoxemia in mice was associated with dramatically increased levels of endogenous circulating TNF-α and resulted in augmented muscle glycolytic activity. Similar findings have been described in peripheral muscle of a mouse model of cancer cachexia, which was associated with a chronic proinflammatory state (2). Moreover, iv administration of TNF-α to mice decreased serum glucose levels and increased muscle levels of fructose 2,6-biphosphate, a positive allosteric regulator of glycolysis, indicative of enhanced peripheral muscle glycolytic activity (32).

Although suggestive of a causal link between inflammation (TNF-α) and the induction of glycolytic muscle metabolism in vivo, these studies do not allow to discriminate whether the activation of glycolytic muscle metabolism in these models represents a direct effect of TNF-α on the musculature or may arise indirectly as a consequence of inflammation-induced alterations in, for example, circulating glucocorticoids or insulin sensitivity (33–35). In this context, one in vitro study reported increased lactate levels in cell supernatant of L6 myocytes exposed to TNF-α, which does suggest a direct effect of TNF-α on muscle glycolytic metabolism rather than an indirect mechanism (20). However, glycolytic metabolism in this study was assessed solely by glucose uptake and cellular lactate production. In the present study, we now comprehensively demonstrate that TNF-α directly enhances muscle glycolytic metabolism as evidenced by increased glucose uptake and lactate production and a coordinated increase in mRNA and protein levels and enzymatic activity of a multitude of molecules involved in glycolytic metabolism. In addition, TNF-α decreased PDK-4 expression, suggesting that some of the glucose that is taken up is directed toward oxidation in the mitochondria. Although phosphorylation of GS was decreased, no changes in myotube glycogen content were observed upon stimulation of myotubes with TNF-α, suggestive of an increased glycogen turnover.

Having established that TNF-α enhances muscle glycolysis, we subsequently explored potential underlying molecular mechanisms. TNF-α-induced increases in glycolytic muscle metabolism in our study were completely abrogated when classical NF-κB signaling was blocked. Conversely, it was shown that genetic activation of classical NF-κB signaling (in absence of inflammatory cytokines) up-regulated mRNA expression levels of glucose transporters, increased glucose uptake, and enhanced glycolytic flux in mouse embryonic fibroblasts (36). In addition, we previously demonstrated that TNF-α-induced loss of muscle oxidative metabolism completely depended on intact classical NF-κB signaling (23). Collectively, this suggests that (TNF-α-induced) activation of the classical NF-κB pathway in muscle reprograms metabolic circuitry toward glycolysis rather than oxidative substrate metabolism.

An important question that subsequently arises is whether the impact of TNF-α-induced NF-κB activation on myotube glycolytic metabolism represents an autonomous effect of this pathway on glycolysis or arises indirectly as a compensatory response to TNF-α-induced impairments in cellular oxidative metabolism. To address this, we knocked down PGC-1α, a master regulator of mitochondrial oxidative metabolism. Loss of PGC-1α strongly reduces skeletal muscle oxidative metabolism in vivo (37) but also in C2C12 myotubes as we showed previously (19, 23). Our data clearly showed that loss of PGC-1α fails to induce glycolytic gene expression or cellular lactate production and thereby suggests that the increase in myotube glycolytic metabolism upon TNF-α-induced NF-κB activation likely constitutes an autonomous rather than an adaptive mechanism.

To further elucidate the molecular mechanisms governing the observed increase in muscle glycolytic metabolism upon TNF-α-induced NF-κB activation, we subsequently focused on the HIF signaling pathway, a well-known regulator of cellular glycolytic metabolism (38). Activation of HIF signaling in muscle, which is observed in response to tissue hypoxia, has been shown to up-regulate glycolytic metabolism by increasing the expression of Glut-1 and increasing activity levels and mRNA transcript abundance of several glycolytic enzymes including PFK, the rate-limiting glycolytic enzyme (24–26). We observed not only that TNF-α potently activated HIF signaling, as shown by enhanced HIF transcriptional activity and increased mRNA levels of HIF-1α and known HIF-1α target genes, but also that TNF-α-induced activation of myotube glycolytic metabolism is largely mediated through HIF-1α. This is in line with reports in several cell types demonstrating that TNF-α can activate the HIF signaling pathway under normoxic conditions, as evidenced by increased HIF DNA-binding concomitant with increased HIF-1α mRNA or protein levels (39, 40). Moreover, TNF-α-induced activation of the HIF pathway in our study required intact classical NF-κB signaling and overexpression of p50 or p65, key constituents of the NF-κB pathway, potently increased HIF transcriptional activity in absence of inflammatory cytokines. Taken together, this shows that transcriptional activation of NF-κB is a prerequisite for TNF-α-induced activation of the HIF pathway and that classical NF-κB activation per se is sufficient to activate HIF transcriptional activity.

This is in line with the current understanding of these pathways as interactions between the inflammatory NF-κB pathway and the hypoxia-sensitive HIF-1α pathway have been described previously (41). Indeed, overexpression of p65, the transcriptionally active NF-κB subunit, resulted in an accumulation of HIF-1α protein, whereas inhibition of NF-κB prevented TNF-α-induced increases in HIF-1α protein levels in kidney cells (42). Furthermore, NF-κB has been shown to be important in regulating basal levels of HIF-1α gene expression (43, 44), and this NF-κB dependence of HIF-1α expression has also been demonstrated to occur in vivo (44). Moreover, the promoter region of the gene encoding *HIF-1α* contains a canonical NF-κB binding site, the mutation of which leads

to loss of hypoxia-induced HIF-1α up-regulation (43). This is supported by evidence that NF-κB family members physically bind to the *HIF-1α* promoter (43, 45).

As described in the introduction, different approaches have provided evidence for enhanced glycolytic metabolism in lower limb muscle of patients with COPD (13, 15–17). We previously demonstrated that glycolytic gene expression was enhanced in COPD patients with elevated muscle TNF-α expression compared with controls and patients with normal muscular TNF-α levels (19). Combined with data described in the current manuscript, this suggests that TNF-α may be involved in the observed shift toward muscle glycolytic metabolism in COPD. Moreover, we now show that increased muscle TNF-α expression in these patients is also associated with enhanced expression levels of constituents of HIF-1α signaling, indicative of a role for TNF-α-induced enhanced HIF-1α signaling in the observed increase in muscle glycolytic metabolism in COPD. Only one study previously investigated HIF signaling in muscle of COPD patients and reported increased HIF-1α mRNA levels compared with controls (46), which is in line with our observations. Although our in vitro data show that TNF-α-induced enhancement of muscle glycolytic metabolism likely represents an autonomous rather than a compensatory mechanism, it remains to be determined whether enhanced muscle glycolytic metabolism in COPD musculature occurs in an autonomous fashion or arises secondary to loss of oxidative muscle metabolism. A recent study from our group demonstrated that loss of oxidative metabolism is already present in mild to moderate COPD (22). A comprehensive assessment of effectors and mediators of muscle glycolysis in this cohort of COPD patients might shed more light on this issue.

Taken together, in concert with current literature, our data illustrate that (TNF-α-induced) NF-κB activation enhances muscle glycolytic metabolism in an autonomous fashion by activating a NF-κB/HIF-1α signaling mechanism. Together with our previous findings that activation of classical NF-κB signaling potently impairs muscle oxidative metabolism (23), this points toward an important role for the classical NF-κB pathway in the regulation of skeletal muscle substrate metabolism. This is in line with the notion that, in addition to its well established role in regulating immune and inflammatory responses, the classical NF-κB pathway is increasingly being recognized as a central regulator of energy homeostasis in a variety of cell types via direct engagement of cellular networks governing glycolysis and respiration (47). Because an aberrant metabolic profile as well as increased activation of the NF-κB pathway has been shown in the musculature of patients with type 2 diabetes mellitus, chronic heart failure, and in obesity, the relevance of the NF-κB pathway in muscle metabolism extends beyond COPD toward multiple chronic conditions. We recognize that we could not assess activation of the classical NF-κB pathway in the muscle biopsy samples due to limited available material. However, increased activation of the classical NF-κB pathway has been described by others in the muscle of COPD patients (48), and, given that TNF-α is both an activator and a target gene of the classical NF-κB pathway (48), activation of NF-κB signaling, specifically in the subgroup of patients with elevated muscle TNF-α levels, is feasible. Given its apparent central role in the regulation of cellular energy metabolism, further elucidation of the role of NF-κB pathway in muscle metabolic regulation will pave the way for the development of novel therapeutic strategies to alleviate muscle metabolic abnormalities in chronic disease.

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