Elevated NF-κB Activation Is Conserved in Human Myocytes Cultured From Obese Type 2 Diabetic Patients and Attenuated by AMP-Activated Protein Kinase

Charlotte J. Green, Maria Pedersen, Bente K. Pedersen, and Camilla Scheele

OBJECTIVE—To examine whether the inflammatory phenotype found in obese and diabetic individuals is preserved in isolated, cultured myocytes and to assess the effectiveness of pharmacological AMP-activated protein kinase (AMPK) activation upon the attenuation of inflammation in these myocytes.

RESEARCH DESIGN AND METHODS—Muscle precursor cells were isolated from four age-matched subject groups: *1*) nonobese, normal glucose tolerant; *2*) obese, normal glucose tolerant; *3*) obese, impaired glucose tolerant; and *4*) obese, type 2 diabetes (T2D). The level of inflammation (nuclear factor-κB [NF-κB] signaling) and effect of pharmacological AMPK activation was assessed by Western blots, enzyme-linked immunosorbent assay, and radioactive assays ($n = 5$ for each subject group).

RESULTS—NF-κB-p65 DNA binding activity was significantly elevated in myocytes from obese T2D patients compared with nonobese control subjects. This correlated to a significant increase in tumor necrosis factor-α concentration in cell culture media. In addition, insulin-stimulated glucose uptake was completely suppressed in myocytes from obese impaired glucose tolerant and T2D subjects. It is interesting that activation of AMPK by A769662 attenuated NF-κB-p65 DNA binding activity in obese T2D cells to levels measured in nonobese myocytes; however, this had no effect on insulin sensitivity of the cells.

CONCLUSIONS—This work provides solid evidence that differentiated human muscle precursor cells maintain in vivo phenotypes of inflammation and insulin resistance and that obesity alone may not be sufficient to establish inflammation in these cells. It is important that we demonstrate an anti-inflammatory role for AMPK in these human cells. Despite attenuation of NF-κB activity by AMPK, insulin resistance in obese T2D cells remained, suggesting factors in addition to inflammation may contribute to the insulin resistance phenotype in muscle cells. *Diabetes* 60:2810–2819, 2011

It is increasingly recognized that chronic activation of inflammatory pathways in skeletal muscle is a major contributing factor in the pathophysiology of insulin resistance, obesity, and type 2 diabetes (T2D) (1–3). Subsequently, inflammation has been attributed to the increased morbidity and mortality associated with obesity. Skeletal muscle is responsible for 75–80% of glucose disposal in humans (4), and impaired insulin action in this tissue is considered the primary site of whole body insulin resistance. Therefore, development of strategies to reverse or prevent inflammation is important to successfully treat chronic diseases such as T2D. However, because the study of potential strategies in humans is difficult, it is essential that an optimal ex vivo model is established. Muscle precursor cell cultures, established from human muscle biopsies, have been shown to display numerous features of mature skeletal muscle (5) and have been used in a number of studies investigating muscle metabolism in T2D patients. It has been shown that myocytes isolated from individuals with T2D retain their donor phenotype, when differentiated into myocytes in vitro, in terms of deficient insulin signaling and phosphatidylinositol 3-kinase activity (6,7). However, the retention of other in vivo phenotypes such as obesity-associated inflammation has not been demonstrated in these cultures. It is important, therefore, to establish whether muscle precursor cells from obese and T2D volunteers retain their inflammatory phenotype in culture to establish this as a model of muscle inflammation and assess how this can be manipulated to treat metabolic diseases.

In obesity, accumulation of fatty acids serves to increase circulating levels of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), via the activation of nuclear factor-κB (NF-κB), which has been shown to impair numerous other signaling pathways regulating skeletal muscle insulin signaling and fatty acid oxidative capacity (2,3,8). NF-κB is activated by multiple proinflammatory stimuli including TNF-α, lipopolysaccharide, and free fatty acids (9–11). NF-κB signaling, therefore, serves as an indicator of inflammatory tone in skeletal muscle. Given the increasing evidence that there is a causal link between inflammation and metabolic diseases, there is considerable interest in developing anti-inflammatory strategies to counteract the suppression in insulin sensitivity induced by obesity-associated inflammation. Recently, AMP-activated protein kinase (AMPK) has been implicated as a modulator of inflammatory responses based on the fact that treatment with AMPK activators suppresses lipopolysaccharide and palmitate-induced NF-κB activity and expression of proinflammatory cytokines in multiple cell types (12–16). The anti-inflammatory role of AMPK in skeletal muscle, however, is poorly defined. In this article, we examine the level of basal inflammation in muscle cells isolated from lean and obese subjects (representative of the whole glucose tolerance continuum) and assess the role that direct pharmacological activation of AMPK plays in attenuating inflammation in these cells.

RESEARCH DESIGN AND METHODS

This study was performed in accordance with the Helsinki II Declaration and approved by the regional research ethics committee (H-KF-01–141/04). Skeletal

muscle biopsies from the vastus lateralis were obtained by the percutaneous needle biopsy method from 24 men and women. Impaired glucose tolerance (IGT) and T2D were diagnosed by an oral glucose tolerance test according to World Health Organization Classification of Diabetes Mellitus (The Decode Study Group, 2001). T2D subjects were all on antidiabetic medications, three of whom were prescribed metformin. None of the volunteers had any history of infections, fever, or inflammatory diseases. Obese volunteers were defined as having a BMI >30 kg/m^2; nonobese control subjects had a BMI <30 kg/m^2. Volunteers were divided, based on the preceding measurements, into non-obese with normal glucose tolerance (Non-Ob), obese with normal glucose tolerance (Ob-NGT), obese with IGT (Ob-IGT), and obese with T2D (Ob-T2D) groups. All volunteers gave their written consent before participation.

F10 nutrient mixture (HAM), Dulbecco's modified Eagle's medium (DMEM) low and high glucose, FBS, horse serum (HS), Fungizone antimycotic, and penicillin/streptomycin (P/S) were all from Invitrogen (Taastrup, Denmark). A769662 was purchased from Tocris Biosciences (Bristol, U.K.), and insulin (Actrapid) was from Novo Nordisk (Bagsværd, Denmark). Complete (mini) protein phosphatase inhibitor tablets were purchased from Boehringer-Roche Diagnostics (Copenhagen, Denmark), and protein protease inhibitor I and II and streptavidin–horseradish peroxidase were from Sigma-Aldrich (Brøndby, Denmark). ATP[^{32}P] and 2-deoxy-D-[^{3}H]glucose were from Perkin Elmer Life Sciences (Copenhagen, Denmark). Phospho–inhibitor of κB kinase (IKK)-α/β (Ser$^{180/181}$), anti–NF-κB-p65, phospho-Akt/protein kinase B (PKB) (Ser^{473}), anti–β-tubulin, phospho-ACC (Ser^{79}), phospho–stress-activated protein kinase/Jun NH$_2$-terminal kinase (SAPK/JNK) (Thr^{183}/Tyr^{185}), and anti-Akt/PKB antibodies were from Cell Signaling Technology (Boston, MA). Anti–inhibitor of κB (IκB)-α and anti–AMPK-α1/α2 were from Santa Cruz Biotechnology (Delaware, CA); AMPK-α2 antibody used to immunoprecipitate AMPK was a gift from Professor Hardie (Dundee, Scotland, U.K.). NF-κB-p65 DNA binding activity ELISA kit came from Active Motif (Carlsbad, CA). ELISA kit for TNF-α and IL-6 was purchased from Meso Scale Discovery (Gaithersburg, MD).

Human muscle satellite cell isolation, proliferation, and differentiation. Muscle precursor cells were isolated from vastus lateralis muscle biopsies as previously described (17). After removal of fat and connective tissue, the biopsy was digested in a 10 mL buffer containing trypsin and collagenase II for 15 min. To minimize fibroblast contamination, cells were preseeded in a culture dish for 3 h in F10/HAM, 20% FBS, 1% PS, and 1% Fungizone antimycotic. Unattached cells were then removed and seeded into a culture flask, precoated with Matrigel (BD Biosciences, San Jose, CA). After 4 days of incubation, the cell culture medium was changed and then every 2nd day thereafter. Cell cultures were expanded and then seeded on Matrigel-coated plates for differentiation. Next, 100% confluent cells were incubated with DMEM containing 1 g/L glucose, 10% FBS, and 1% P/S to allow cells to align; after 2 days, the medium was changed to DMEM containing 4.5 g/L glucose, 2% HS, and 1% P/S. Culture medium was changed to DMEM containing 1 g/L glucose, 2% HS, and 1% P/S 2 h prior to treatment with A769662. For all experiments, cells were used at day 6 or 7 of differentiation at either passage 5 or 6. All four cell groups were used on the same day of differentiation and at the same passage number for each experimental dataset.

Cell lysis. Cells were rinsed once in ice-cold PBS and lysed in 20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 0.1% Triton X-100, protease inhibitor (1 tablet/10 mL), and 1% phosphatase inhibitor cocktail. Whole cell lysates were centrifuged (12,000g at 4°C for 10 min), and supernatants were removed for storage at −80°C until required.

Immunoblotting. Protein concentrations were determined using the Bradford reagent (18). Whole cell lysates (20 μg) were subjected to SDS-PAGE using Invitrogen 4–12% precast gels and I-blot dry transfer machine according to the manufacturer's instructions. Polyvinylidene fluoride membranes were probed with primary antibodies raised against the protein of interest as indicated in the figure legends. Detection of primary antibodies was performed using appropriate peroxidize-conjugated IgG and protein signals visualized using

FEMTO-enhanced chemiluminescence and Bio-Rad Chemidoc XRS imager (Bio-Rad, Hercules, CA). Quantification of immunoblots was done using ImageJ (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

NF-κB-p65 DNA binding activity. Measurement of NF-κB-p65 DNA binding activity was carried out on 10-μg whole cell lysate using Active Motif ELISA kit according to the manufacturer's instructions.

Measurement of cytokines in cell culture media. Levels of TNF-α and interleukin (IL)-6 were measured 4 h post changing of cell culture media (100 μL) using Meso Scale Discovery proinflammatory ELISA kit according to the manufacturer's instructions.

Glucose uptake. Myocytes were serum starved in DMEM containing 1 g/L glucose for 2 h prior to assaying glucose uptake and incubated with reagents for times and at concentrations indicated in the figure legends. Cells were washed twice with HEPES-buffered saline (140 mmol/L NaCl, 20 mmol/L HEPES, 5 mmol/L KCl, 2.5 mmol/L MgSO$_4$, and 1 mmol/L CaCl$_2$, pH 7.4). Glucose uptake was assayed by incubation with 10 μmol/L 2-deoxy-D-[^{3}H] glucose (1 μCi/mL) for 10 min. Nonspecific tracer binding was determined by quantifying cell-associated radioactivity in the presence of 10 μmol/L cytochalasin B. Medium was aspirated before washing adherent cells twice with 0.9% ice-cold NaCl. Cells were subsequently lysed in 50 mmol/L NaOH, and radioactivity was quantified using a Perkin Elmer Tri-Carb 2810TR scintillation counter. Protein concentration was determined using the Bradford reagent.

Immunoprecipitation. Protein G-sepharose beads were washed once in PBS and incubated with anti–AMPK-α2 for 2 h at 4°C on an orbital platform shaker. Bead-antibody mix was then incubated with 500 μg of whole lysate protein for 2 h at 4°C before washing. The immunoprecipitates were washed twice in immunoprecipitation buffer (50 mmol/L Tris, 150 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L NaPPi, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.1 mmol/L benzamidine, 0.1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL soybean trypsin inhibitor, and 1% triton, pH 7.25) containing 0.5 mol/L NaCl, once in immunoprecipitation buffer containing 0.15 mol/L NaCl, and once in HEPES assay buffer (50 mmol/L HEPES, 1 mmol/L dithiothreitol, and 0.02% Brij-35, pH 7.0).

AMPK activity assay. AMPK-α2 immunoprecipitates were used to measure AMPK activity assayed in vitro by the phosphorylation of a synthetic peptide HMRSAMSGLHLVKRR (the "SAMS" peptide) in the presence of a saturating concentration of AMP (0.2 mmol/L). Kinase activity was assessed by a 15 min incubation at 30°C with reaction buffer ([^{32}P]ATP, 5 mmol/L MgCl$_2$, 200 μmol/L AMP, plus or minus 200 μmol/L SAMS peptide) on a shaking platform. The reaction was terminated by spotting 30 μL of the reaction mixture onto P81 phosphocellulose paper and washing in 1% phosphoric acid. Radioactivity was quantified using Perkin Elmer Tri-Carb 2810TR scintillation counter.

Statistical analyses. For multiple comparisons, statistical analysis was performed using one-way or two-way ANOVA with Bonferroni corrections. For data that were normalized to basal (i.e., fold changes), statistical analysis was performed using a one-sample t test. Data analysis was performed using GraphPad Prism (La Jolla, CA) software and considered statistically significant at $P < 0.05$.

RESULTS

Human skeletal muscle from Ob-T2D patients has a higher level of inflammation than skeletal muscle from Non-Ob subjects. The clinical characteristics of the subjects used in this study are shown in Table 1. All subjects were age-matched (54 ± 1 years) from a mixture of males and females. All obese subjects were matched for BMI (34.2 ± 0.9), which was statistically higher than Non-Ob control subjects ($P < 0.05$) (Table 1). We have previously shown that plasma markers of inflammation

TABLE 1
Clinical characteristics of subjects from whom muscle precursor cells were isolated

	Non-Ob ($N = 5$)	Ob-NGT ($N = 5$)	Ob-IGT ($N = 5$)	Ob-T2D ($N = 5$)
Age (years)	59 ± 3	51 ± 3	51 ± 2	56 ± 4
BMI (kg/m^2)	25.7 ± 0.7	34.7 ± 1.3**	34.1 ± 2.4**	33.7 ± 1.0**
Fasting glucose (mmol/L)	5.8 ± 0.2	6.1 ± 0.2	6.1 ± 0.3	8.7 ± 0.7*
OGTT 2-h glucose (mmol/L)	6.3 ± 0.6	7.1 ± 0.4	9.0 ± 0.3*	17.5 ± 1.5***
Sex (men:women)	4:2	4:2	3:3	3:3

Data are means ± SE. OGTT, oral glucose tolerance test. *$P < 0.05$ vs. Non-Ob control subjects. **$P < 0.005$ vs. Non-Ob control subjects. ***$P < 0.0005$ vs. Non-Ob control subjects.

(IL-6, TNF-α, and C-reactive protein) were significantly upregulated in both obese and diabetic subjects compared with healthy nonobese control subjects (19,20). However, inflammation in skeletal muscle isolated from these subjects was not investigated. Here we show that skeletal muscle from Ob-T2D patients has significantly lower protein expression of IκB-α (~70% lower) when compared with skeletal muscle from Non-Ob subjects (Fig. 1A and B). It is important that this lower expression of IκB-α in Ob-T2D muscle was sufficient to significantly increase NF-κB (p65 subunit) DNA binding activity (Fig. 1C). There was no significant difference in NF-κB-p65 subunit DNA binding activity between skeletal muscle from Non-Ob and either Ob-NGT or Ob-IGT muscle.

In vivo Ob-T2D–associated inflammation is conserved in cultured human myocytes. We wanted to assess whether this increased inflammation was conserved in our system
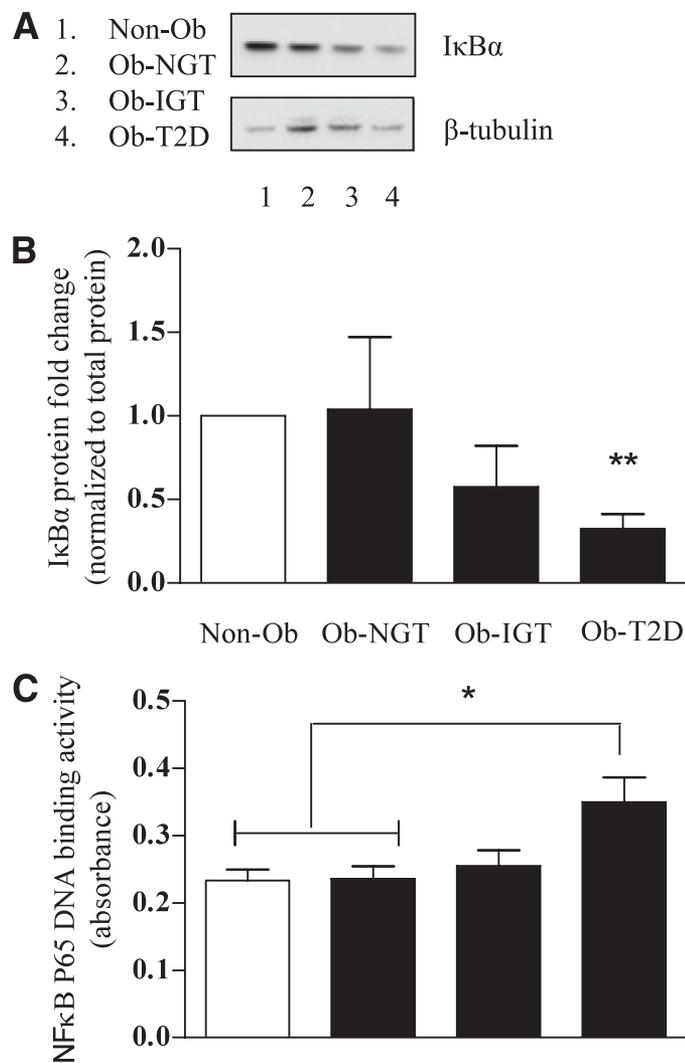
FIG. 1. NF-κB signaling in human skeletal muscle from Non-Ob and obese subjects. Muscle biopsies from vastus lateralis were taken from Non-Ob (BMI 23.4 ± 2.4), Ob-NGT, Ob-IGT, and Ob-T2D (BMI 35.1 ± 3.2) volunteers. Total protein lysate from biopsies was then used for (A) SDS-PAGE (20 μg total protein lysate) and immunoblotted for IκB-α and β-tubulin or (C) 10 μg total protein lysate to measure NF-κB-p65 subunit DNA binding activity in an ELISA assay. B: The effect of obesity (filled bars) on IκB-α abundance was quantified and expressed as a fold change from Non-Ob cells (open bar). Values shown are mean ± SEM from five individuals for each group. *P < 0.05 vs. Non-Ob. **P < 0.005 vs. Non-Ob.

of cultured muscle cells isolated from the same subjects. To assess this, we measured the phosphorylation of IKK-α/β and the associated loss in its downstream target, IκB-α, as a readout for NF-κB activation. We show that myocytes from Ob-T2D have threefold higher IKK-α/β phosphorylation and concomitant loss in IκB-α protein abundance of 45% (Fig. 2A and B) compared with Non-Ob myocytes. Importantly, we found that the level of IκB-α protein expression in skeletal muscle tissue and muscle precursor cells from the same individual had a significant correlation (P = 0.0034) with an r^2 value of 0.5 (Fig. 2C). To assess whether this loss in IκB-α protein was sufficient to upregulate NF-κB activity, the ability of the p65 subunit of NF-κB to bind to DNA was measured. Figure 2D shows that Ob-T2D myocytes have a significantly higher level of p65 DNA binding compared with Non-Ob myocytes. It is important that Ob-NGT myocytes had comparable levels of NF-κB signaling with Non-Ob control subjects, whereas Ob-IGT myocytes seemed to have an intermediate level of NF-κB signaling (between that of Non-Ob and Ob-T2D). To fully elucidate the level of inflammation in these cells, the concentrations of TNF-α and IL-6 in cell culture media were measured. TNF-α concentrations were significantly higher in Ob-T2D compared with Non-Ob control subject concentrations (Fig. 2E). Of interest, concentrations of IL-6 in cell culture media from Ob-IGT and Ob-T2D was significantly lower than that in Non-Ob cell media (Fig. 2F). This latter finding supports the idea that IL-6 exerts an inhibitory effect on TNF-α in muscle (21).

Insulin resistance is conserved in cultured human myocytes. To relate the inflammatory status with glucose tolerance in the cultured myocytes, it was important not only to refer to the subjects from whom cells were isolated (as shown in Table 1) but also to confirm that impaired insulin sensitivity was retained in cultured myocytes. To ascertain this, insulin-stimulated glucose uptake and PKB/Akt phosphorylation of myocytes were measured. Both Non-Ob and Ob-NGT myocytes had comparable levels of basal and insulin-stimulated glucose uptake, with a significant increase in glucose uptake (approximately twofold) in response to insulin (Fig. 3A). It is important that both Ob-IGT and Ob-T2D myocytes had no increase in glucose uptake when challenged with insulin. This lack of insulin sensitivity in these cells was reflected in the loss of insulin-stimulated PKB/Akt Ser 473 phosphorylation (Fig. 3B and C). Hence, the in vivo glucose tolerance status of the donors is reflected in cultured myocytes.

Effect of obesity and A769662 treatment of myocytes on AMPK signaling. Obesity had no effect on the level of AMPK-α1/α2 protein expressed in the cells. However, in the Ob-T2D myocytes, there was a significant increase in the total protein amount of downstream AMPK target, ACC (Fig. 4A and B). It is interesting that obesity was associated with a trend of increased basal AMPK-α2 activity, independent of glucose tolerance of the cells. Figure 4C shows that in obese myocytes, there is a threefold increase in basal AMPK activity compared with that observed in Non-Ob myocytes. Treatment of cultured human myocytes with A769662 for 4 h significantly increased AMPK-α2 activity to equal extents in all cell groups (Fig. 5A). Of interest, AMPK activation in Ob-IGT and Ob-T2D myocytes led to a significantly higher level of ACC phosphorylation compared with Non-Ob and Ob-NGT control subjects. The latter observation cannot be fully accounted for by increased protein expression levels of ACC (Fig. 5C and D). A769662 treatment did not alter the protein expression of AMPK-α1/α2.
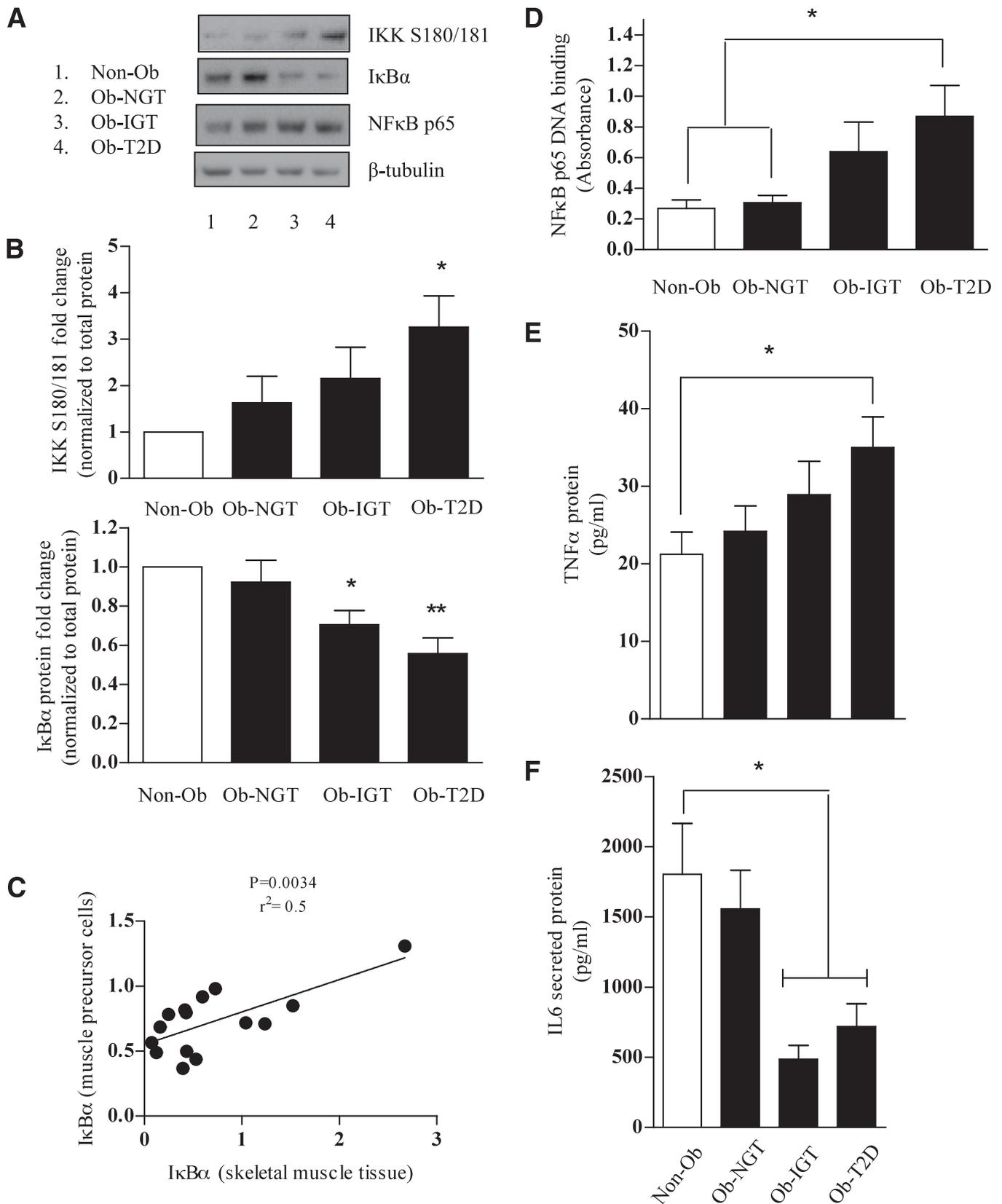
FIG. 2. NF-κB signaling in human myotubes from Non-Ob and obese subjects. Myoblasts isolated from Non-Ob ($n = 5$), Ob-NGT ($n = 5$), Ob-IGT ($n = 5$), and Ob-T2D ($n = 5$) patients were grown in culture until mature myotubes were formed. *A*: Lysates were immunoblotted to assess the phosphorylation status of IKK-α/β and total protein abundance of IκB-α and the p65 subunit of NF-κB. Equal gel loading was ascertained by immunoblotting with an antibody against β-tubulin. *B*: The effect of obesity (filled bars) on IKK-α/β phosphorylation and IκB-α abundance was quantified and expressed as a fold change from Non-Ob cells (open bars). *C*: IκB-α protein abundance in skeletal muscle tissue was compared with abundance in muscle precursor cells from the same individuals. *D*: Lysates were used to measure NF-κB-p65 subunit DNA binding activity in an ELISA assay. *E* and *F*: Cell culture medium was collected 4 h after addition to cells, and concentration of TNF-α and IL-6 was assessed by Meso Scale Discovery ELISA assay. Values shown are mean ± SEM from muscle precursor cells from five individuals for each group. *$P < 0.05$ vs. Non-Ob. **$P < 0.005$ vs. Non-Ob.
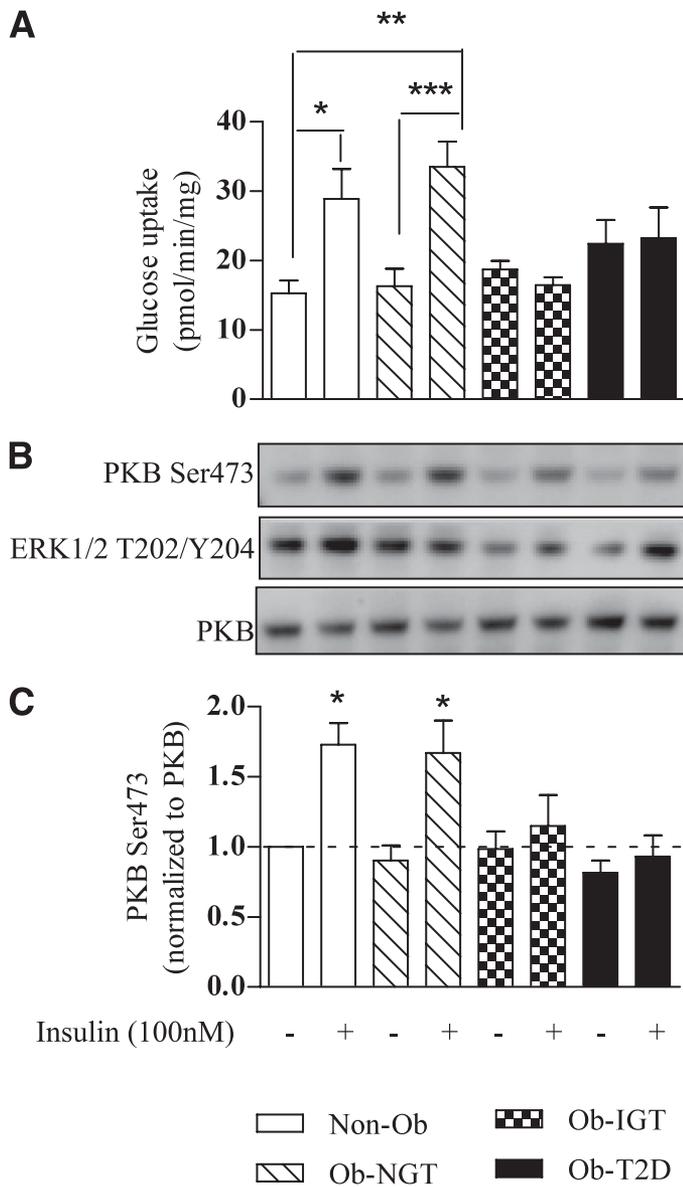
FIG. 3. Phenotypical insulin sensitivity is retained in human myotubes in culture. Myotubes were treated with 100 nmol/L insulin for 30 min before (A) assaying 2-deoxyglucose uptake or for 10 min before (B) immunoblotting to assess the phosphorylation status of PKB/Akt and extracellular signal–related kinase-1/2 and the total protein abundance of PKB/Akt. C: Effect of insulin on PKB/Akt phosphorylation was quantified and expressed as a fold change from untreated Non-Ob cells. Values are mean ± SEM from five separate experiments; glucose uptake values were performed in triplicate. *P < 0.05 vs. Non-Ob. **P < 0.005 vs. Non-Ob; ***P < 0.0005 vs. Non-Ob.

Effect of pharmacological AMPK activation on NF-κB signaling.

It has been reported that AMPK is able to antagonize the effects of a number of proinflammatory markers in a variety of cell lines (12–15). However, its role as a modulator of inflammation has not been well established in human skeletal muscle. Therefore, we assessed the effect of pharmacological activation of AMPK using the specific AMPK activator, A769662, on NF-κB signaling in Non-Ob and obese myocytes. It is important that activation of AMPK using A769662 attenuated IKK phosphorylation to a level that was not significantly different from Non-Ob control subject levels. The loss of IκB-α protein expression was also attenuated in cells from Ob-T2D (Fig. 6A and B). In line with this, A769662 treatment of Ob-T2D cells
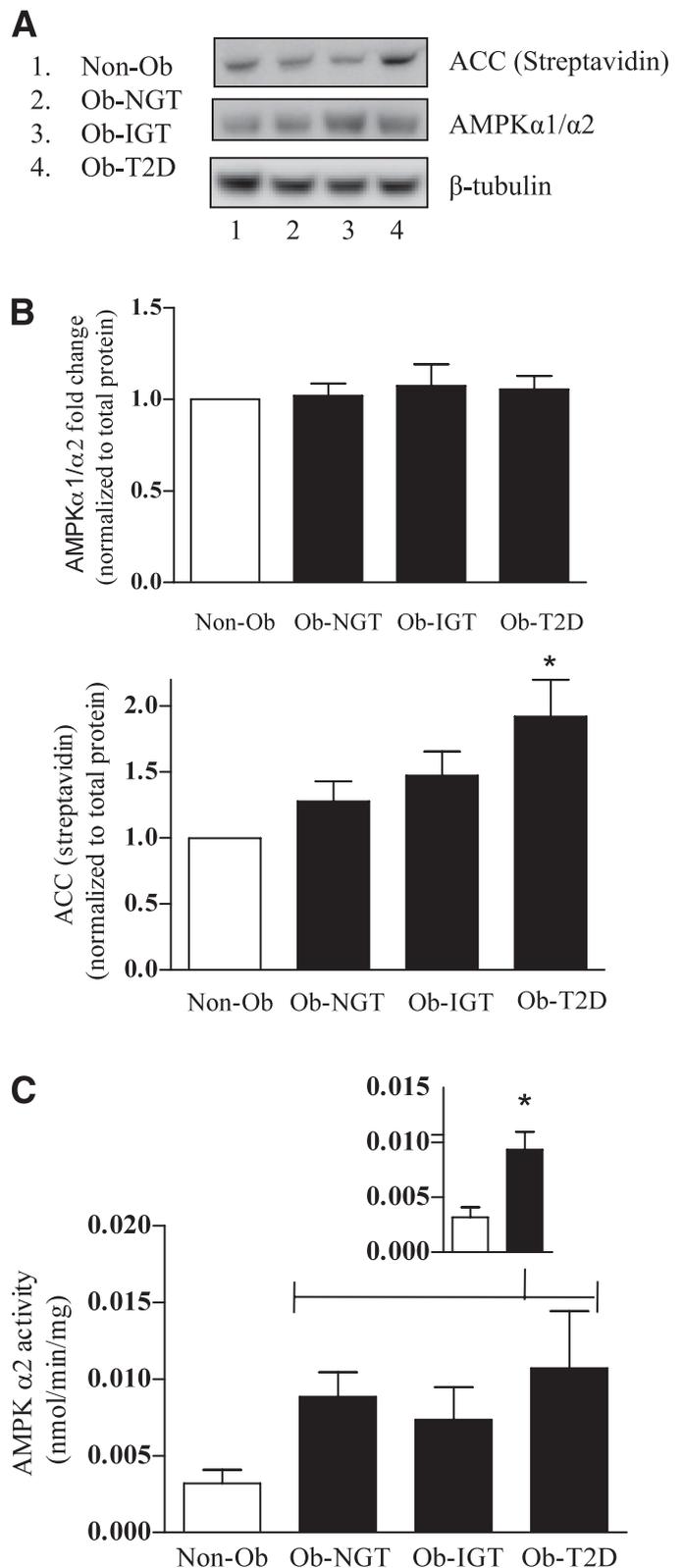
FIG. 4. AMPK signaling in human myotubes from Non-Ob and obese subjects. A: Lysates were immunoblotted to assess the phosphorylation status of ACC and the total protein abundance of AMPK-α1/α2 and ACC (streptavidin). Equal gel loading was ascertained by immunoblotting with an antibody against β-tubulin. B: The effect of obesity (filled bars) on AMPK-α1/α2 and ACC (streptavidin) abundance was quantified and expressed as a fold change from Non-Ob cells (open bars). C: Lysates were immunoprecipitated with a specific antibody against AMPK-α2 before measurement of AMPK activity. The inset graph (C) indicates lean vs. obese (NGT + IGT + T2D). Values are mean ± SEM from five separate experiments. *P < 0.05 vs. Non-Ob.
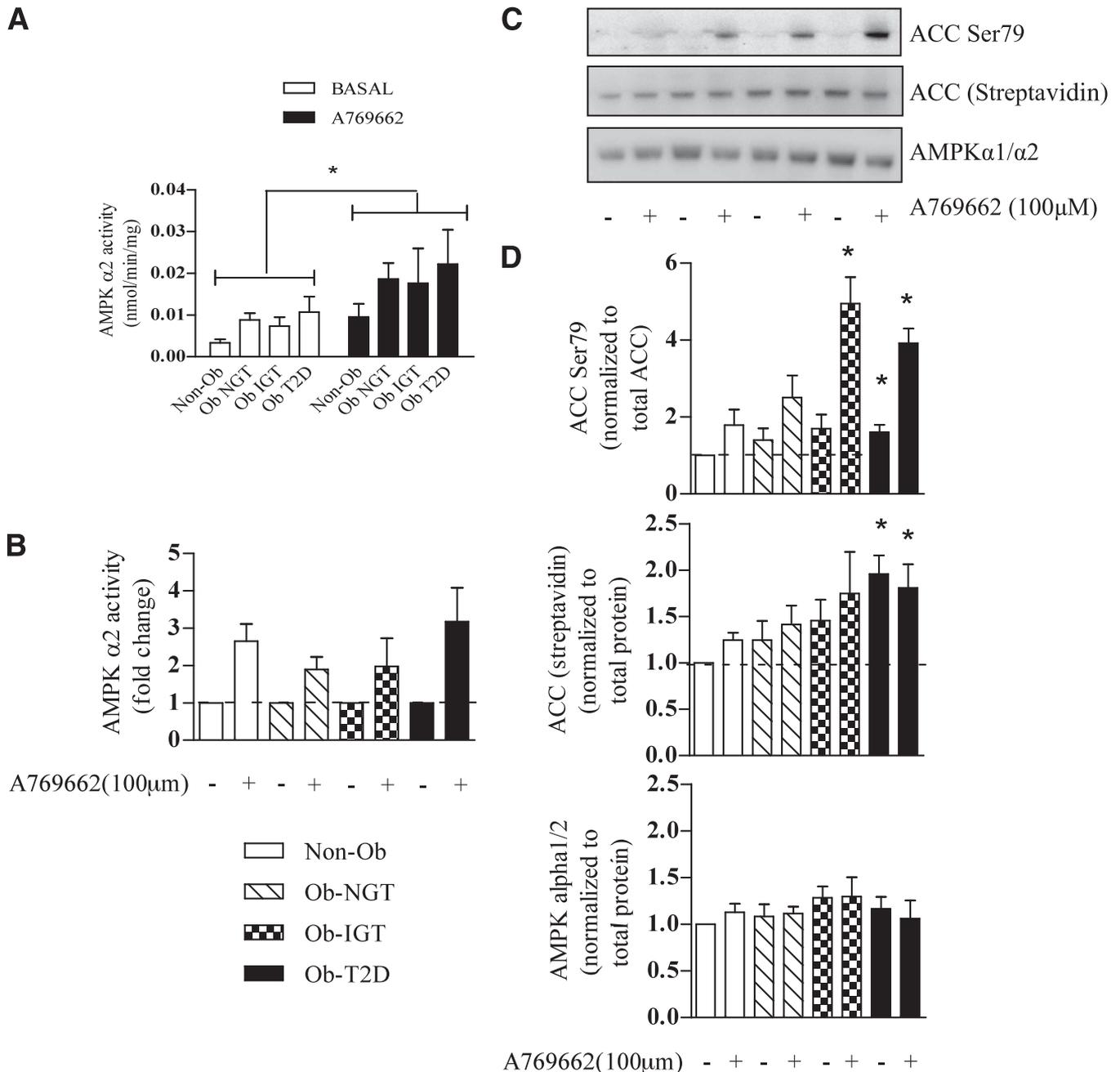
FIG. 5. Effects of pharmacological AMPK activation on AMPK signaling in human myotubes from Non-Ob and obese subjects. Myotubes were treated with 100 μmol/L A769662 for 4 h before (A) immunoprecipitation with a specific antibody against AMPK-α2 before measurement of AMPK activity or (C) immunoblotting to assess the phosphorylation status of ACC and the total protein abundance of AMPK-α1/α2 and ACC (strep-tavidin). B: Effect of A769662 on AMPK-α2 activity was expressed as a fold change from lean controls. D: Effect of A769662 on ACC phosphor-ylation and total protein abundance of ACC (streptavidin) and AMPK-α1/α2 was quantified and expressed as a fold change from untreated, Non-Ob cells. Values are mean ± SEM from five separate experiments. *$P < 0.05$ vs. Non-Ob.

attenuated the level of NF-κB-p65 DNA binding activity to that comparable with Non-Ob myocytes (Fig. 6C). Because levels of TNF-α were robustly increased in the cell culture media from basal Ob-T2D myocytes, we measured concentrations of this cytokine after 4 h of AMPK activation. A769662 suppressed TNF-α in the cell culture media to a level that was not significantly different from that measured in media from Non-Ob cells (Fig. 6D). Although A769662 did not significantly increase IL-6 concentrations in Ob-IGT and Ob-T2D media, there was a trend to increase IL-6 concentrations after A769662 treatment in these groups (Fig. 6E).

Effect of suppression of NF-κB activity using A769662 on insulin sensitivity of Ob-T2D myocytes. A769662 treatment had no additive effect on insulin-stimulated glucose uptake or PKB/Akt phosphorylation in any of the cell groups tested (Fig. 7A and B). As shown in Fig. 2, Ob-IGT and Ob-T2D myocytes show no response to insulin as measured by glucose uptake and PKB/Akt phosphorylation. It should be noted, however, that there was a trend toward increased basal glucose uptake in Ob-T2D myocytes ($P = 0.065$). Of interest, 4 h of A769662 treatment, which was sufficient to attenuate inflammation, did not increase insulin
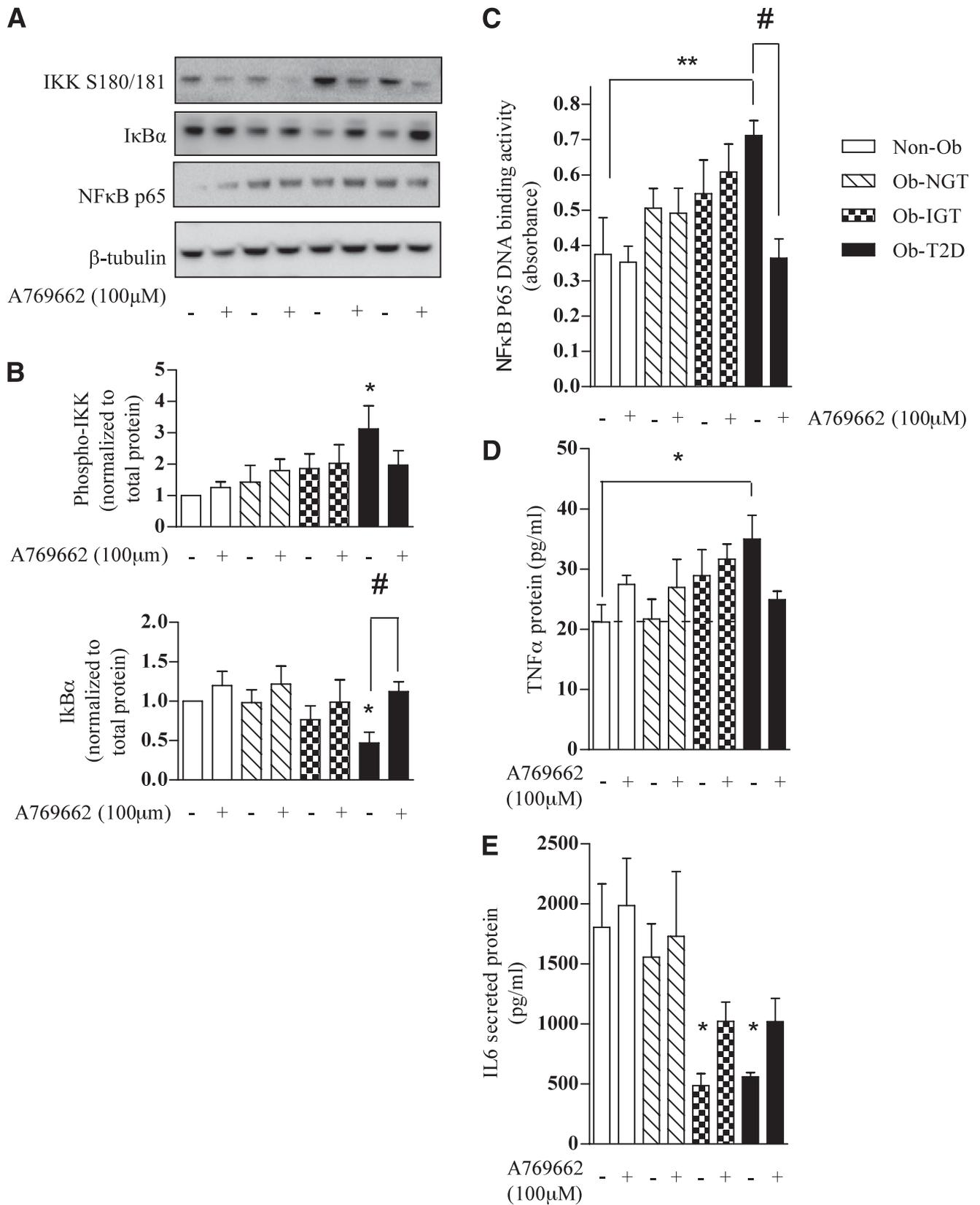
FIG. 6. Effect of pharmacological AMPK activation on NF-kB signaling in human myotubes from Non-Ob and obese subjects. Myotubes were treated with 100 μmol/L A769662 for 4 h before (A) immunoblotting to assess the phosphorylation status of IKK-α/β and total protein abundance of IκB-α and the p65 subunit of NF-κB. Equal loading was ascertained by immunoblotting with an antibody against β-tubulin, (C) measurement of NF-κB-p65 subunit DNA binding activity by ELISA assay, or concentration of (D) TNF-α and (E) IL-6 in culture media by Meso Scale Discovery ELISA assay. B: Effect of A769662 on IKK-α/β phosphorylation and total protein abundance of IκB-α was quantified and expressed as a fold change from untreated Non-Ob cells. Values are mean ± SEM from five separate experiments. *P < 0.05 vs. Non-Ob cells. **P < 0.005 vs. untreated Ob-T2D. #P < 0.05 vs. untreated Ob-T2D.
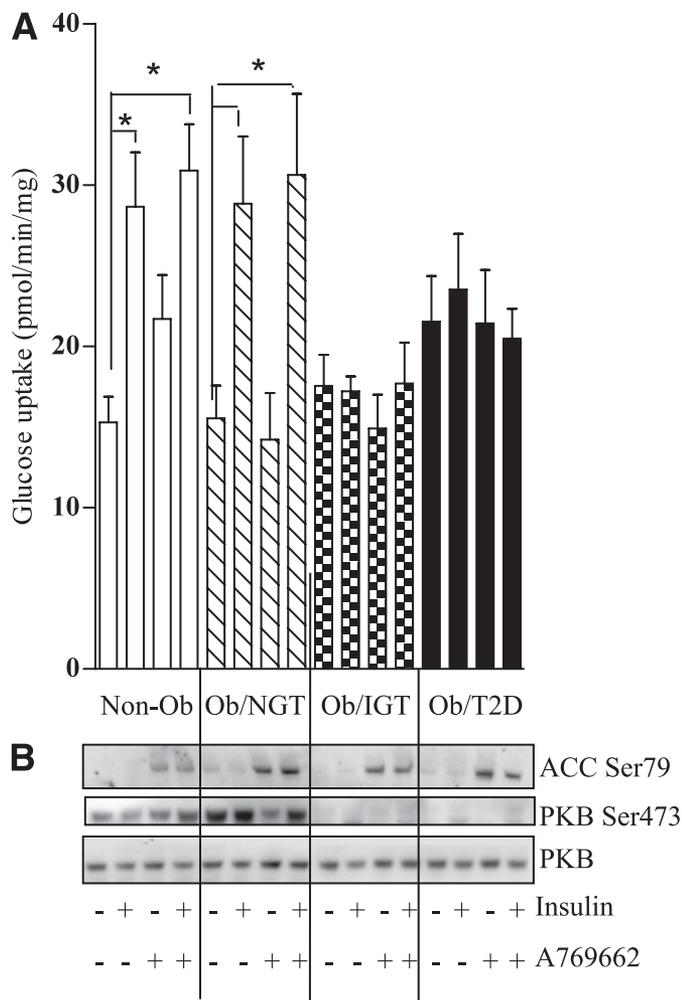
FIG. 7. Effect of pharmacological AMPK activation on insulin signaling in human myotubes. Myotubes were treated with 100 μmol/L A769662 and/or 100 nmol/L insulin for 30 min before (*A*) assaying 2-deoxyglucose uptake or 100 nmol/L insulin for 10 min before (*B*) immunoblotting to assess the phosphorylation status of PKB/Akt and ACC and the total protein abundance of PKB/Akt. Values are mean ± SEM from five separate experiments; glucose uptake values were performed in triplicate. *P < 0.05 vs. control cells.

sensitivity of Ob-IGT or Ob-T2D myotubes (Fig. 6*A*). This finding was also confirmed at the level of PKB/Akt phosphorylation, which remained suppressed in Ob-IGT and Ob-T2D myocytes compared with Non-Ob and Ob-NGT myocytes (Fig. 7*B*).

DISCUSSION

Our findings indicate that skeletal muscle precursor cells that are grown and differentiated into myocytes in culture, despite multiple passages, retain both an obesity/T2D-associated inflammatory phenotype and insulin resistant phenotype seen in vivo. This finding provides evidence for justifying the use of muscle precursor cultures as tools to define the role of increased inflammation in obesity and T2D in humans ex vivo. It is interesting that no increase in inflammation was found in myocytes from Ob-NGT subjects when compared with Non-Ob control subjects. Inflammation was, however, significantly increased in Ob-T2D myocytes when compared with both Non-Ob and Ob-NGT cells (Fig. 1*C*). Similar findings have also been shown in adipose tissue, where adipose tissue from moderately

obese insulin-resistant individuals expressed significantly higher mRNA levels of IL-8, IL-6, and monocyte chemoattractant protein–1 when compared with levels in moderately obese insulin-sensitive tissue (22). One hypothesis that can be drawn from these data is that hyperglycemia (associated with insulin resistance) is a major driving force behind inflammation seen in Ob-IGT and Ob-T2D. In support of this, it has been shown that in healthy individuals, clamping glucose at hyperglycemic levels (20 mmol/L) leads to a significant increase in plasma TNF-α, IL-18, and IL-6 within 2 h (23). A subsequent study shows that a glucose meal (75 g) alone resulted in increased plasma inflammatory and oxidative stress markers, including vascular cell adhesion molecule–1 and intracellular adhesion molecule–1 (24). It is possible that duration of obesity, partitioning of adiposity, or stage of T2D the volunteers were at when biopsies were taken could affect how obesity inflammation and insulin sensitivity interact in these cells. The analysis of these parameters in the human subjects is still ongoing and will be necessary for future understanding of the directionality of the inflammation–insulin resistance relationship.

AMPK is a known target for the treatment of diabetes; however, recently it has been suggested that AMPK confers an anti-inflammatory response (although the mechanisms by which this occurs are currently unclear). Metformin, a widely prescribed insulin-sensitizing drug, and AICAR are both well-established (nonspecific) AMPK activators that have been shown to inhibit NF-κB activity in endothelial cells (13,25). Because of the potential health benefits of anti-inflammatory drugs for both metabolic syndrome and other chronic inflammatory diseases, it is important to establish this role of AMPK in humans. The thienopyridone A769662 directly activates AMPK, mimicking the effects of AMP; however, it has no effect on the binding of AMP to the Bateman domains of the γ-subunit (26). Instead, it has been shown that A769662 activates AMPK by a mechanism involving the β-subunit carbohydrate binding domain in addition to other sites on the γ-subunit, although it directly activates only AMPK-β1–containing complexes (in cell-free assays) (27). It is important, therefore, to note that human skeletal muscle has been shown to express the AMPK-β2 subunit predominantly, with very little β1 expression (28). Despite this, AMPK was significantly activated in all myocytes treated with A769662. We have attributed this to the 50% increased expression of the β1 subunit (with no effect on β2 expression) in cultured myocytes when compared with skeletal muscle tissue (Supplementary Fig. 1).

Having confirmed that A769662 significantly activated AMPK in human skeletal myocytes, we found that the increased inflammation associated with Ob-T2D myocytes could be attenuated by this direct, pharmacological activation of AMPK. More specific, AMPK activation suppressed NF-κB signaling (IKK phosphorylation, IκB-α, and p65 DNA binding) leading to decreased secretion of the inflammatory cytokine TNF-α. However, pharmacological activation of AMPK, although comparable in all cells, failed to attenuate obesity-associated insulin resistance in either Ob-IGT or Ob-T2D myocytes. Similar findings were recently published by Green et al. (16) in rat L6 myotubes, where inflammation induced by palmitate could be attenuated by AMPK activation; however, this failed to increase the insulin sensitivity of these cells. This latter finding would suggest that increased NF-κB signaling and increased TNF-α concentrations in obesity are not the main causative factors in the development of insulin resistance. Yet it is possible that other factors that promote insulin resistance may not be

regulated by AMPK activation, for example, the mitogen-activated protein kinase (MAPK) family. Both JNK and p38 MAPK have been implicated in the pathogenesis of insulin resistance in muscle (29). It has been shown that AMPK activation in L6 muscle cells attenuates palmitate-associated extracellular signal–related kinase phosphorylation but fails to attenuate either palmitate-induced JNK or p38 phosphorylation under the same conditions (16). Further investigation into the causes and potential role of MAPKs in insulin resistance in muscle precursor cells and the affect of AMPK activation on this will be investigated in our future work to fully elucidate the interplay between inflammation and insulin resistance in obese human muscle precursor cells. It is possible that our findings might reflect the complex process by which insulin resistance is developed and that once established in the muscle precursor cells, it probably requires more than just reversing a few contributing factors.

Our findings suggest that obesity, independent of insulin sensitivity, is associated with a significant increase in basal AMPK-α2 activity. This latter finding fits with findings from other laboratories that show a higher level of basal AMPK activity in obese human muscle and in rat muscle cells treated with saturated fatty acids (30,31). Because AMPK activation is important in regulating fatty acid oxidation and energy balance in the cell, increased basal AMPK activity may be a reflection of the cell's attempt to compensate for the obesity-driven oversupply of fatty acids, and accumulation of these in skeletal muscle. In line with this, it has been reported that intracellular accumulation of fatty acids increases mitochondrial oxidative capacity, which leads to the impairment of fuel selection from fat to carbohydrate (32). It is therefore likely that increased basal AMPK activity in obese myocytes reflects increased "impaired" fatty acid oxidation. This impaired fatty acid oxidation could subsequently lead to increased levels of reactive oxygen species (ROS) and the promotion of insulin resistance (33,34). It is important that ROS levels have been shown to be increased in human skeletal muscle from obese insulin-resistant subjects (34). It is also important to note that Ob-T2D myocytes show an increased sensitivity to AMPK activation in terms of ACC phosphorylation. This increase in sensitivity cannot be explained simply by the increased abundance of ACC protein and is not attributable to higher AMPK activity or, indeed, the expression of α-AMPK subunits in these cells. It would be interesting, therefore, to investigate whether this higher level of ACC phosphorylation in response to AMPK activation translates into a higher level of functional fatty acid oxidation or whether this increase further reflects the impaired fatty acid oxidation associated with the oversupply of free fatty acids in obesity.

In conclusion, our findings provide solid evidence that muscle precursor cells that are grown ex vivo in culture are useful tools to investigate the relationship between inflammation and insulin resistance in skeletal muscle. It is also likely that this system retains other disease phenotypes that have not been investigated here. Even though obesity is associated with inflammation in vivo, we demonstrate that obesity is not enough to establish an inflammatory phenotype in muscle precursor cells. In contrast, the increased inflammatory response observed in the muscle precursor cells derived from subjects with T2D possibly reflects the irreversible status of the disease. In addition, we have confirmed the anti-inflammatory role for AMPK in human skeletal muscle, verifying its potential therapeutic

role in inflammatory-associated morbidity and mortality associated with metabolic syndrome. However, it must be noted that attenuation of NF-κB activity by AMPK in muscle may not be sufficient to increase or restore insulin sensitivity of the cells alone, and further investigation into how other inflammatory mediators (including JNK and ROS) may promote insulin resistance under these conditions should be investigated in future research using this primary cell model.

REFERENCES

1. Fève B, Bastard JP. The role of interleukins in insulin resistance and type 2 diabetes mellitus. Nat Rev Endocrinol 2009;5:305–311
2. Funaki M. Saturated fatty acids and insulin resistance. J Med Invest 2009; 56:88–92
3. Barma P, Bhattacharya S, Bhattacharya A, et al. Lipid induced overexpression of NF-kappaB in skeletal muscle cells is linked to insulin resistance. Biochim Biophys Acta 2009;1792:190–200
4. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. Diabetes 1981;30:1000–1007
5. Gaster M, Kristensen SR, Beck-Nielsen H, Schrøder HD. A cellular model system of differentiated human myotubes. APMIS 2001;109:735–744
6. Gaster M, Petersen I, Højlund K, Poulsen P, Beck-Nielsen H. The diabetic phenotype is conserved in myotubes established from diabetic subjects: evidence for primary defects in glucose transport and glycogen synthase activity. Diabetes 2002;51:921–927
7. Bouzakri K, Roques M, Gual P, et al. Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. Diabetes 2003;52:1319–1325
8. Boden G. Fatty acid-induced inflammation and insulin resistance in skeletal muscle and liver. Curr Diab Rep 2006;6:177–181
9. Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes Dev 2004;18:2195–2224
10. Senn JJ. Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. J Biol Chem 2006;281:26865–26875
11. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest 2006;116: 3015–3025
12. Su RY, Chao Y, Chen TY, Huang DY, Lin WW. 5-Aminoimidazole-4-carboxamide riboside sensitizes TRAIL- and TNFalpha-induced cytotoxicity in colon cancer cells through AMP-activated protein kinase signaling. Mol Cancer Ther 2007;6:1562–1571
13. Katerelos M, Mudge SJ, Stapleton D, et al. 5-aminoimidazole-4-carboxamide ribonucleoside and AMP-activated protein kinase inhibit signalling through NF-κB. Immunol Cell Biol 2010;88:754–760
14. Giri S, Nath N, Smith B, Viollet B, Singh AK, Singh I. 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside inhibits proinflammatory response in glial cells: a possible role of AMP-activated protein kinase. J Neurosci 2004;24:479–487

15. Lihn AS, Pedersen SB, Lund S, Richelsen B. The anti-diabetic AMPK activator AICAR reduces IL-6 and IL-8 in human adipose tissue and skeletal muscle cells. Mol Cell Endocrinol 2008;292:36–41
16. Green CJ, Macrae K, Fogarty S, Hardie DG, Sakamoto K, Hundal HS. Counter modulation of fatty acid-induced proinflammatory NFkB signalling in rat skeletal muscle cells by AMPK. Biochem J 2011;435:463–474
17. Scheele C, Larsson O, Timmons JA. Using functional genomics to study PINK1 and metabolic physiology. Methods Enzymol 2009;457:211–229
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254
19. Plomgaard P, Nielsen AR, Fischer CP, et al. Associations between insulin resistance and TNF-alpha in plasma, skeletal muscle and adipose tissue in humans with and without type 2 diabetes. Diabetologia 2007;50:2562–2571
20. Mortensen OH, Nielsen AR, Erikstrup C, et al. Calprotectin—a novel marker of obesity. PLoS ONE 2009;4:e7419
21. Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood 1990;75:40–47
22. McLaughlin T, Deng A, Gonzales O, et al. Insulin resistance is associated with a modest increase in inflammation in subcutaneous adipose tissue of moderately obese women. Diabetologia 2008;51:2303–2308
23. Esposito K, Nappo F, Marfella R, et al. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. Circulation 2002;106:2067–2072
24. Ceriello A, Quagliaro L, Piconi L, et al. Effect of postprandial hypertriglyceridemia and hyperglycemia on circulating adhesion molecules and oxidative stress generation and the possible role of simvastatin treatment. Diabetes 2004;53:701–710
25. Huang NL, Chiang SH, Hsueh CH, Liang YJ, Chen YJ, Lai LP. Metformin inhibits TNF-alpha-induced IkappaB kinase phosphorylation, IkappaB-alpha degradation and IL-6 production in endothelial cells through PI3K-dependent AMPK phosphorylation. Int J Cardiol 2009;134:169–175
26. Goransson O, McBride A, Hawley SA, et al. Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. J Biol Chem 2007;282:32549–32560
27. Scott JW, van Denderen BJ, Jorgensen SB, et al. Thienopyridone drugs are selective activators of AMP-activated protein kinase beta1-containing complexes. Chem Biol 2008;15:1220–1230
28. Thornton C, Snowden MA, Carling D. Identification of a novel AMP-activated protein kinase beta subunit isoform that is highly expressed in skeletal muscle. J Biol Chem 1998;273:12443–12450
29. Tanti JF, Jager J. Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. Curr Opin Pharmacol 2009;9:753–762
30. Watt MJ, Steinberg GR, Chen ZP, Kemp BE, Febbraio MA. Fatty acids stimulate AMP-activated protein kinase and enhance fatty acid oxidation in L6 myotubes. J Physiol 2006;574:139–147
31. Sriwijitkamol A, Coletta DK, Wajcberg E, et al. Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: a time-course and dose-response study. Diabetes 2007;56:836–848
32. Koves TR, Ussher JR, Noland RC, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab 2008;7:45–56
33. Yuzefovych L, Wilson G, Rachek L. Different effects of oleate vs. palmitate on mitochondrial function, apoptosis, and insulin signaling in L6 skeletal muscle cells: role of oxidative stress. Am J Physiol Endocrinol Metab 2010;299:E1096–E1105
34. Lefort N, Glancy B, Bowen B, et al. Increased reactive oxygen species production and lower abundance of complex I subunits and carnitine palmitoyltransferase 1B protein despite normal mitochondrial respiration in insulin-resistant human skeletal muscle. Diabetes 2010;59:2444–2452