RESEARCH ARTICLE

Trans-10, *cis*-12 conjugated linoleic acid inhibits skeletal muscle differentiation and GLUT4 expression independently from NF-κB activation

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Scope: The capacity of skeletal muscle to contribute to glucose homeostasis depends on muscular insulin sensitivity. The expression of glucose transporter (GLUT)-4 is increased during myoblast differentiation, a process essential in maintenance of adult muscle. Therefore, processes that affect muscle differentiation may influence insulin dependent glucose homeostasis. Conjugated linoleic acids, and in particular *trans*-10, *cis*-12 CLA (*t*10, *c*12-CLA), are potent inducers of NF-kB in cultured skeletal myotubes, and NF-kB activation inhibits muscle differentiation. The aims of this study were to evaluate whether CLAs inhibit myogenic differentiation and lower GLUT4 mRNA expression and to address the involvement of NF-kB activation in potential effects of CLA on these processes.

Methods and results: Incubation of C2C12 cells with *t*10, *c*12-CLA blocked the formation of myotubes, which was accompanied by reduced expression of the muscle specific genes creatine kinase, myogenin, myosin heavy chain perinatal and myosin heavy chain IIB, as well as decreased GLUT4 mRNA levels. However, genetic blockade of NF-kB was not sufficient to restore reduced myosin heavy chain protein expression following *t*10, *c*12-CLA treatment. Surprisingly, in contrast to myotubes, *t*10, *c*12-CLA was not able to activate NF-kB transcriptional activity in myoblasts.

Conclusion: In conclusion, *t*10, *c*12-CLA inhibits myogenic differentiation and GLUT4 expression, independently from NF-kB activation.

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1 Introduction

Myogenic differentiation, a process essential in the formation and maintenance of muscle, can be defined as the irreversible transition from the proliferative myoblast stage into fused multinucleated myotubes [1]. In addition to myoblast fusion, myogenic differentiation is characterized by the expression of muscle-specific genes, such as muscle creatine kinase (MCK),

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Abbreviations: CLA, conjugated linoleic acid; **DM,** differentiation medium; **FA,** fatty acids; **FBS,** fetal bovine serum; **GLUT4,** glucose transporter 4; **GM,** growth medium; **IκBα-SR,** IκBα-super repressor; **MCK,** muscle creatine kinase; **MyHC,** myosin heavy chain; **MyHCf,** fast twitch MyHC; **MyHC-peri,** perinatal MyHC; **NF-κB,** nuclear factor kappa B; **PPAR,** peroxisome proliferator-activated receptor; **TLR,** toll-like receptor; **TNF-α,** tumor necrosis factor-alpha

the structural protein myosin heavy chain (MyHC) and muscle specific transcription factors including myogenin [2]. Likewise, the expression of the insulin-sensitive glucose transporter GLUT4 (glucose transporter 4) in skeletal muscle increases during muscle differentiation [3, 4].

The contribution of skeletal muscle to glucose homeostasis is not only determined by insulin-mediated GLUT4 translocation but is also sensitive to differences in GLUT4 expression [5–8]. This indicates that processes affecting muscle differentiation indirectly influence insulin-dependent glucose homeostasis.

It has been reported that cytokines are able to inhibit myogenic differentiation *via* activation of the nuclear factor kappa B (NF-κB) pathway [9, 10]. The intracellular signal transduction of inflammatory cues such as pro-inflammatory cytokines depends to a large extent on the transcriptional regulator NF-κB. NF-κB is considered a key regulator of inflammatory responses and is normally present in a latent form in the cytoplasm, bound to its inhibitory protein IκBα. After stimulation, an intracellular signaling cascade is initiated, resulting in the activation of the serine kinase inhibitor κB kinase. This leads to phosphorylation and subsequent degradation of IκBα. NF-κB is subsequently released from its inhibitory protein and translocates to the nucleus, where it regulates the transcription of many genes involved in inflammation, growth regulation and survival, by binding to its cognate DNA sequence in promoters and enhancers of their corresponding genes [11, 12].

In addition to inflammatory cytokines, fatty acids (FAs) have also been identified as strong inducers of NF-κB in skeletal muscle [13–16]. Previously, we demonstrated that conjugated linoleic acids (CLAs), and in particular the *trans*-10, *cis*-12 isoform of CLA (*t*10, *c*12-CLA), are potent inducers of NF-κB in cultured myotubes [17]. Interestingly, no acute effects on insulin sensitivity were observed in myotubes exposed to CLA [17]. However, although CLAs have been studied extensively for their proposed health benefits, including anti-carcinogenic, anti-inflammatory, anti-obesity and anti-diabetic effects [18], unfavorable effects of especially *t*10, *c*12-CLA have previously been reported, for example, on lipid metabolism, glucose metabolism and insulin sensitivity [19]. Therefore, we hypothesize that CLAs may indirectly affect glucose homeostasis *via* reduced muscle differentiation, provoked by NF-κB activation.

The aims of this study were to evaluate the consequences of CLAs on myogenic differentiation and GLUT4 expression, and address the involvement of NF-κB activation in potential effects of CLA on these processes.

2 Materials and methods

2.1 Cell culture

The C2C12 murine skeletal muscle cell line (ATCC CRL1772. Manassas, VA, USA), stably transfected with the 6κB-TK-luciferase, was used for the assessment of NFκB transcriptional activity. In brief, C2C12 cells were plated (1×10^4 cells/cm^2) on Matrigel (Becton-Dickinson Labware, Bedford, MA, USA)-coated (1:50 in DMEM) dishes, as described previously [20]. C2C12 myoblasts were cultured in growth medium (GM), composed of low glucose DMEM containing antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin; both from Gibco-Invitrogen, Rockville, MD, USA) and 9% v/v fetal bovine serum (FBS; PAA Laboratories). To induce differentiation, GM was replaced by differentiation medium (DM), containing DMEM with 1% v/v heat-inactivated FBS and antibiotics. As a positive control for NF-κB transcriptional activity, murine tumor necrosis factor-alpha (TNF-α) (Calbiochem, San Diego, CA, USA) was added to the dishes.

The L6 rat skeletal muscle cell line, stably transfected with a construct encoding GLUT4 with an exofacial *myc* epitope (L6-GLUT4*myc*), was kindly provided by Dr. Amira Klip from the Hospital for Sick Children (Toronto, ON, Canada). L6 myoblasts were cultured in GM, composed of α-MEM (Gibco-Invitrogen) containing 9% v/v FBS and antibiotics. The plating density used for the experiments was 2×10^4/cm^2. After 24 h of culturing in GM, differentiation was induced by replacing GM with DM, containing α-MEM with 2% v/v heat-inactivated FBS and antibiotics.

Morphology of myotubes was assessed by bright field microscopy after staining of the myotubes with May Grunwald-Giemsa. Cells were washed twice in PBS, fixed in methanol and stained in May-Grunwald Giemsa (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. All experiments described for both cell lines were performed in 5- or 6-day differentiated myotubes unless stated otherwise.

2.2 FA incubations

FA stock solutions of 40 mmol/L were prepared in ethanol (linoleic acid from Sigma; c9, t11-CLA and *t*10, *c*12-CLA from Bio-connect, The Netherlands). Before application to the cells, FAs were conjugated to BSA by diluting the FA solution with DM containing 1% w/v FA-free BSA (Sigma), to obtain final FA concentrations of 5–200 μmol/L. Solutions were filter-sterilized before addition to the cells. Vehicle controls contained 0.125 or 0.5% v/v ethanol and 1% w/v BSA.

2.3 Transfections and plasmids

Stable cell lines were created by transfection with Nanofectin (PAA, UK) according to the manufacturer's recommendations.

To inhibit NF-κB activation, L6 myoblasts (3×10^3 cells/cm^2) were stably transfected with nanofectin in the presence of a plasmid encoding IκBα-super repressor (IκBα-SR)

(3 μg), which was constitutively expressed under control of the SFFV-LTR (pSFFV-NEO IκBα-SR), kindly provided by Dr. Rosa Ten (Mayo Clinic, Rochester, MN, USA). A vehicle cell line was created with the same strategy, using 3 μg plasmid DNA containing the neomycin resistance gene (pSV2-Neo, Stratagene, La Jolla, CA, USA). For selection of positive clones, cells were cultured in GM containing the presence of 800 μg/mL G-418 (Calbiochem).

2.4 NF-κB transcriptional activity

To determine NF-κB transcriptional activity, luciferase activity was measured in the NF-κB sensitive reporter cell line as previously described [20]. After the appropriate incubation time with the various stimuli, cells were washed twice with cold PBS and lysed by adding 100 μL 1 × Reporter Lysis Buffer (Promega, Madison, WI, USA). After incubation on ice for 10 min, the cell lysates were centrifuged (13 000 × *g*, 2 min) 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, Hercules, CA, USA).

2.5 RNA isolation and assessment of mRNA abundance by qPCR

Total RNA from C2C12 cells was isolated using the acid guanidium thiocyanate-phenol-chloroform-isoamylalcohol extraction method (Totally RNA kit, Ambion, Austin, TX, USA). After isolation, RNA was dissolved in RNA storage solution (1 mmol/L Na-citrate, pH 6.4) (Ambion) and stored at −80°C. RNA concentrations were determined using a spectrophotometer (Nanodrop, ND-1000). Four hundred nanogram of RNA was reverse transcribed into cDNA according to manufacturer's instructions (Transcriptor first strand cDNA synthesis kit, Roche Diagnostics GmbH, Mannheim, Germany) with anchored oligo-dT primers. The genome databases of NCBI or Ensembl were used as a source for transcript sequences.

qPCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) and obtained from Sigma Genosys (Haverhill, UK).

Standard curves were made by preparing five serial fivefold dilutions of pooled cDNA aliquots. cDNA samples were diluted 1/25. From the cDNA dilutions, 5 ìL was loaded in a 96-well PCR plate (Thermo Fast plates, semi skirted, transparent, Abgene). Standard dilutions were loaded in duplicate. 96-well plates were covered with Microseals B adhesive seals (Biorad). Real-time PCR reactions were performed in a MyiQ single-color Real-Time thermal cycler (Bio-Rad).

qPCR reactions (20 μL total volume) contained absolute qPCR SyBr Green Fluorescein Mix (Abgene), 300 nM primers (Table 1) and cDNA dilution. The PCR reaction was performed by a two-step PCR using the following cycling conditions: 15″ 95°C, 40 cycles of (15″ 95°C, 45″ 60°C), 30″ 95°C, 30″ 60°C followed by a melting curve (heating from 60°C to 95°C). Ct values were obtained for the standard curve and each sample and the relative DNA starting quantities of the samples were derived from the standard curve by using the MyiQ analysis software (Biorad). The expression of the genes of interest were normalized to three reference genes (β-actin, GAPDH and cyclophilin A) using geNorm software [21].

2.6 Western blotting

The abundance of the fast twitch isotype of MyHC (MyHCf) and GAPDH was evaluated by Western blotting. Cells were washed in PBS, and whole cell lysates were prepared by adding lysis buffer (20 mM Tris, 150 mM NaCl, 1% v/v Nonidet P-40, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and 1%

Table 1. Primer details

Gene	ID[a)]	QPCR primer sequences	
		Forward 5′–3′	Reverse 5′–3′
MCK	NM_007710	AGGTTTTCCGCCGCTTCT	CGGTGCCCAGGTTGGA
Myogenin	ENSMUST00000027730	CCCATGGTGCCCAGTGAA	GCAGATTGTGGGCGTCTGTA
MyHC peri	M12289	GAGTCCCAGGTCAACAAGC	AACCCAGAGAGGCAAGTGAC
MyHC IIB	(Sartorius *et al.*, 1998)	ACAAGCTGCGGGTGAAGAGC	CAGGACAGTGACAAAGAACG
GLUT4	(Jove *et al.*, 2005)	GATGCCGTCGGGTTTCCAGCA	TGAGGGTGCCTTGTGGGATGG
β-Actin	NM_007393	CTGAATGGCCCAGGTCTGA	CCCTCCCAGGGAGACCAA
Cyclophilin A	BC099478	TTCCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG
GAPDH	BC096590	CAACTCACTCAAGATTGTCAGCAA	TGGCAGTGATGGCATGGA

MCK, muscle creatine kinase; MyHC, myosin heavy chain; GLUT4, glucose transporter 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase.
a) ID from http://www.ncbi.nlm.nih.gov/entrez or http://www.ensembl.org.

v/v aprotinin). Lysates were incubated on ice for 30 min, followed by 30 min centrifugation at 14 000 × *g*. A fraction of the supernatant was saved for protein determination, and 4 × Laemmli sample buffer (0.25 M Tris-HCl, pH 6.8, 8% w/v SDS, 40% v/v glycerol, 0.4 M dithiothreitol and 0.04% w/v bromophenol blue) was added, followed by boiling of the samples for 5 min and storage at −20°C. Total protein was assessed by the Bio-Rad DC protein assay kit (Bio-Rad) according to the manufacturer's instructions, and 15 μg of protein was loaded *per* lane and separated using 4–12% Bis-TRIS Criterion XT precast gels (Bio-Rad), followed by electroblot transfer to a 0.45-μm nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked for 1 h at room temperature in 5% w/v nonfat, dried milk. Nitrocellulose blots were washed in PBS-Tween-20 (0.05%), followed by overnight incubation (4°C) with a monoclonal antibody specific for MyHCf (MY-32, Sigma; 1:2000) or GAPDH (Cell Signaling; 1:5000). After three wash steps of 20 min each, the blots were probed with a peroxidase-conjugated secondary antibody, 1:5000 (Vector Laboratories, Burlingame, CA, USA), and visualized by Supersignal® WestPico chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

2.7 Electrophoretic mobility shift analysis

To determine DNA binding activity of NF-κB, complexes binding to an oligonucleotide containing a NF-κB consensus sequence were analyzed by EMSA. Nuclear extracts were isolated to analyze NF-κB DNA binding. To this end, cells were harvested following experimental treatments and lysed on ice in 400 μL buffer containing 20 mmol/L HEPES, pH 7.8, 20 mmol/L KCl, 4 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L DTT, 0.2 mmol/L sodium orthovanadate, 0.4 mmol/L phenylmethyl sulfonylfluoride, 0.3 μg/mL leupeptin and 0.2 mmol/L NaF for 15 min. Subsequently, 25 μL 10% Nonidet P40 was added and samples were vortexed for 15 s followed by centrifugation (14 000 rpm for 30 s). Supernatants were removed and pelleted nuclei were washed with the previously mentioned buffers and resuspended with a buffer containing 100 mmol/L HEPES, pH 7.8, 100 mmol/L KCl, 600 mmol/L NaCl, 0.2 mmol/L EDTA, 20% glycerol, 1 mmol/L DTT, 0.2 mmol/L sodium orthovanadate, 0.667 mmol/L phenylmethyl sulfonylfluoride and 0.2 mmol/L NaF. Nuclei were mixed vigorously for 20 min at 4°C using a rotating platform, centrifuged (14 000 rpm for 5 min) and samples were stored at −20°C (for protein concentration determination) and −80°C (for DNA binding activity measurements). Seven microgram of nuclear cell extracts were used *per* binding reaction, and protein–DNA complexes were resolved on a 5% polyacrylamide gel in 0.25 × Tris borate-EDTA buffer at 160 V for 2 h. Gels were dried and exposed to film (X-Omat Blue XB-1, Kodak, Rochester, NY, USA). Shifted complexes were detected by a phosphoimager (Bio-Rad).

2.8 Statistical analysis

SPSS (version 16.0) was used for statistical analysis. Data were analyzed by one-way ANOVA, and the various treatment groups were compared by using the *post hoc* Bonferroni test in which a $p<0.05$ was considered statistically significant. Data are presented as mean ± SD.

3 Results

3.1 *t*10, *c*12-CLA induces NF-κB transcriptional activation in a dose- and time-dependent manner

The effect of *c*9, *t*11-CLA and *t*10, *c*12-CLA *versus* linoleic acid on NF-κB transcriptional activity was

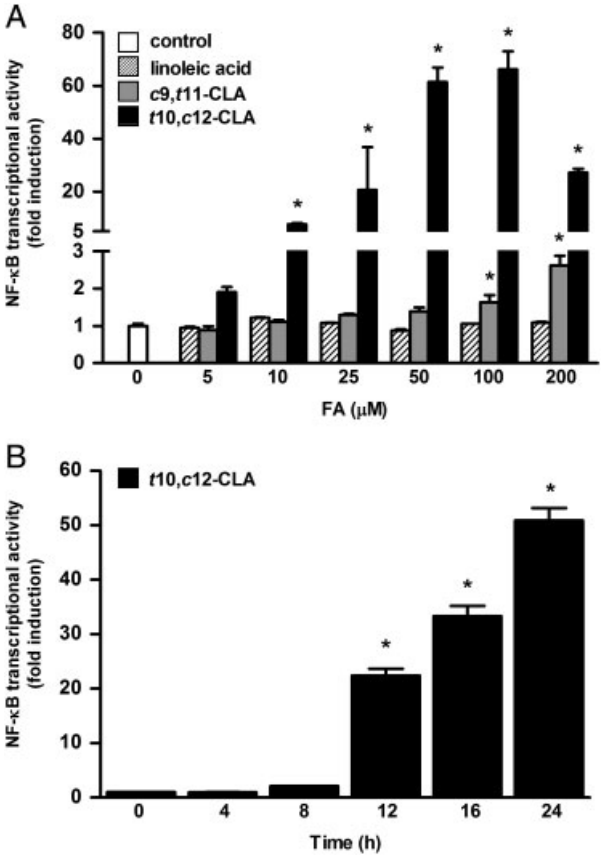

Figure 1. *t*10, *c*12-CLA induces NF-κB transcriptional activation in a dose- and time-dependent manner. C2C12 cells from the NF-κB sensitive reporter cell line were differentiated for 6 days before treatment with linoleic acid, *c*9, *t*11-CLA and *t*10, *c*12-CLA (0–200 μmol/L) for 24 h (A) or treatment with 50 μmol/L of *t*10, *c*12-CLA for 0–24 h (B). Lysates were prepared for assessment of luciferase activity. Values were corrected for protein content and expressed as fold-induction over control. Statistically significant differences between the indicated treatment and corresponding BSA-treated control (*$p<0.05$) were determined by one-way ANOVA.

investigated in C2C12 myotubes, stably transfected with a NF-κB sensitive reporter construct. As shown in Fig. 1A, incubation with linoleic acid did not result in increased NF-κB transcriptional activity, while *c*9, *t*11-CLA incubation resulted in a maximum 2.6-fold increase at 200 μmol/L. Incubation with only 5 μmol/L *t*10, *c*12-CLA already resulted in an increase in NF-κB transcriptional activity, and there was a peak of ~65-fold increase after incubation with 50 and 100 μmol/L *t*10, *c*12-CLA. NF-κB transcriptional activation by *t*10, *c*12-CLA (50 μmol/L) was induced in a time-dependent manner (Fig. 1B). Remarkably, NF-κB activation by t10, c12-CLA required 12 h of incubation, whereas NF-κB activation by TNF-α, which was used as a positive control (data not shown), was clearly detectable after 2–4 h. These data demonstrated that the effects of CLA on NF-κB transcriptional activity are isomer specific with *t*10, *c*12-CLA being the most potent inducer.

3.2 Inhibition of myotube formation by *t*10, *c*12-CLA

Since it is known that activation of NF-κB inhibits myogenic differentiation [9], we evaluated the effects of two CLAs and linoleic acid on myotube formation and muscle specific gene expression during the myogenic differentiation process. Addition of 50 μmol/L *t*10, *c*12-CLA completely inhibited the formation of myotubes (Fig. 2, bottom left). *c*9, *t*11-CLA inhibited myotube formation less potently (Fig. 2, top right), while linoleic acid incubation, although not quantitatively determined, appeared to increase myotube size (Fig. 2, bottom right).

3.3 *t*10, *c*12-CLA reduces muscle specific gene and GLUT4 expression during myogenic differentiation

We next evaluated if the FA-induced changes in morphological differentiation were reflected by changes in the expression of muscle specific genes after induction of differentiation. The expression of MCK, an enzyme expressed in mature muscle, and myogenin, a muscle-specific transcription factor, was reduced by 64 and 59%, respectively, after incubation of *t*10, *c*12-CLA during 3 days after the onset of differentiation (Fig. 3A). The mRNA of the myofibrillar proteins perinatal MyHC (MyHC-peri) and MyHC IIB (MyHC-IIB) genes, which are expressed during later phases of myogenic differentiation, was reduced by 62 and 85%, respectively, after incubation with *t*10, *c*12-CLA in 6 days differentiating myocytes (Fig. 3B). A slight decrease (28%) in MyHC-peri was visible after incubation with *c*9, *t*11-CLA. Remarkably, linoleic acid incubation led to a 70% increase in MCK expression and a doubling of MyHC-IIB expression.

Furthermore, we examined whether the effects of *t*10, *c*12-CLA on myogenic differentiation also affected GLUT4 mRNA levels. As shown in Fig. 3C, GLUT4 mRNA levels were reduced (−50%) after *t*10, *c*12-CLA incubation and induced (+94%) by linoleic acid incubation. No differences on GLUT4 expression were caused by *c*9, *t*11-CLA incubation.

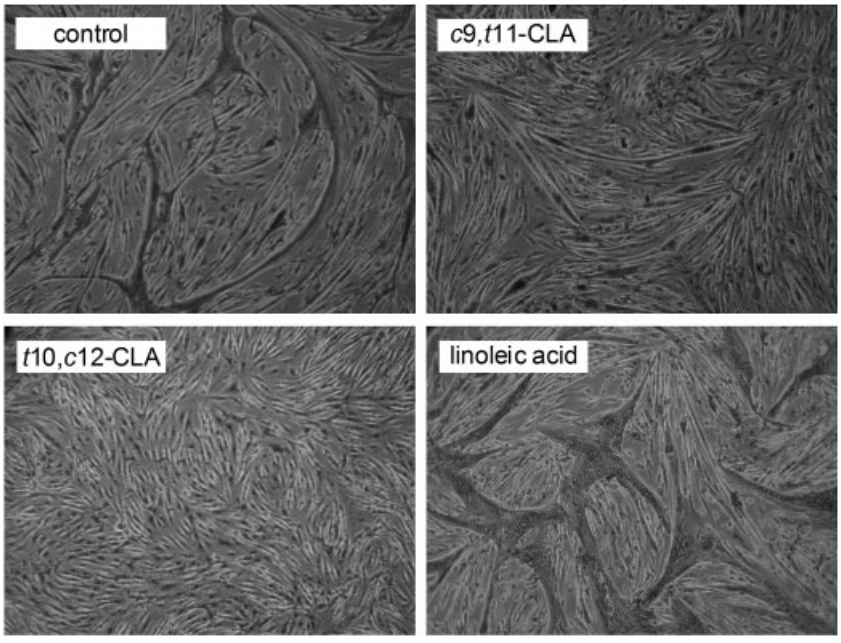

Figure 2. Inhibition of myotube formation by *t*10, *c*12-CLA. C2C12 cells were allowed to differentiate for 5 days in the presence or absence of 50 μmol/L linoleic acid, *c*9, *t*11-CLA or *t*10, *c*12-CLA. Morphology was assessed by bright field microscopy after staining with May-Grunwald Giemsa.

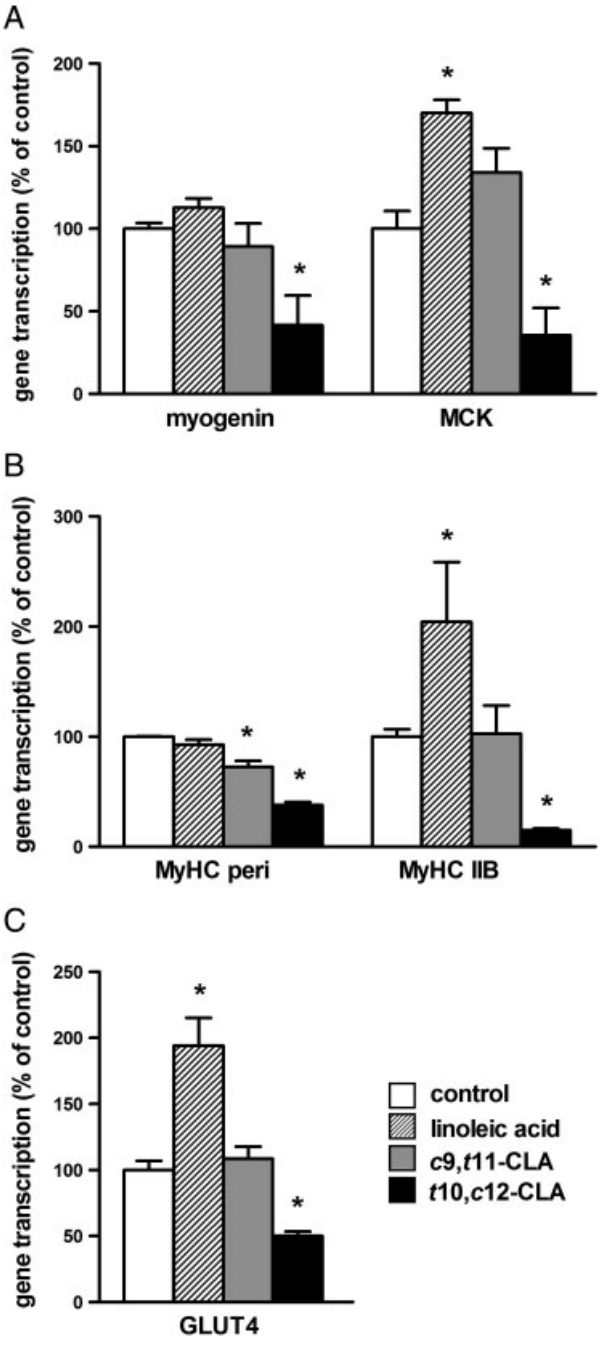

Figure 3. *t*10, *c*12-CLA reduces muscle specific gene- and GLUT4 expression during myogenic differentiation. C2C12 cells were allowed to differentiate for 3 (A) or 6 (B, C) days in the presence of 50 μmol/L of the corresponding fatty acids. Expression levels of myogenin and MCK (A), MyHC-peri and MyHC-IIB (B), and GLUT4 (C) are depicted as percentage of control transcript levels. Statistically significant differences between the indicated treatment and corresponding BSA-treated control ($^*p<0.05$) were determined by one-way ANOVA.

3.4 Inhibition of NF-κB does not prevent the *t*10, *c*12-CLA-induced reduction of differentiation

To test potential causality of NF-κB activation in the reduction of muscle differentiation by *t*10, *c*12-CLA, we measured protein abundance of MyHCf in L6 cells, stably transfected with the IκBα-SR, a non-degradable mutant of IκBα. As expected, induction of maximal NF-κB DNA binding observed in control myotubes was markedly decreased in L6 cells expressing the IκBα-SR (Supporting Information Fig. 1). However, treatment with *t*10, *c*12-CLA during the differentiation process still resulted in a decrease in MyHCf protein expression in the IκBα-SR cell line as well as the vehicle (Fig. 4). Thus, genetic blockade of NF-κB was not sufficient for the restoration of the *t*10, *c*12-CLA-induced disturbed differentiation process.

3.5 Differential NF-κB activation by *t*10, *c*12-CLA in myoblasts and myotubes

We next investigated NF-κB transcriptional activity in C2C12 cells that were continuously exposed to *t*10, *c*12-CLA, *e.g.* for 6 days from the onset of induction of differentiation.

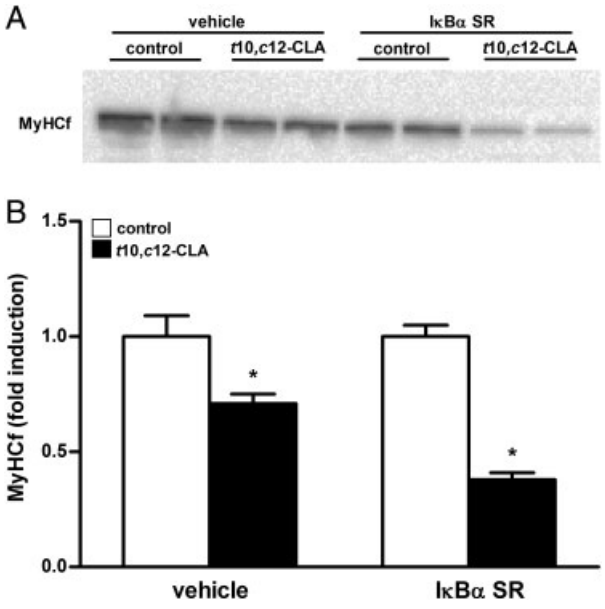

Figure 4. Inhibition of NF-κB does not prevent the *t*10, *c*12-CLA-induced reduction of differentiation. L6 cells were stably transfected with a plasmid encoding a stabilized mutant of the Inhibitor of NF-κB (IκBα-SR) or a vehicle vector (PcDNA 3.1; vehicle). Cells were cultured with differentiation medium for 5 days in the presence of 50 μmol/L *t*10, *c*12-CLA. MyHCf expression was assessed in 15 μg of protein lysate by Western blot analysis. A representative Western blot is shown (A) and values are expression as fold-induction over control (B). Statistically significant differences between the indicated treatment and corresponding BSA-treated control ($^*p<0.05$) were determined by one-way ANOVA.

No cell death was observed after the different treatments with *t*10, *c*12-CLA. Surprisingly, no sustained NF-κB transcriptional activity was observed following *t*10, *c*12-CLA treatment (Fig. 5A). Moreover, in myoblasts, 24-h incubation of *t*10, *c*12-CLA did not induce NF-κB transactivation (Fig. 5B). In contrast, in 3-day differentiated myotubes, NF-κB activation was apparent following 24 h incubation with *t*10, *c*12-CLA. Maximal transactivation of the NF-κB-sensitive promoter construct was observed in myotubes that were first allowed to differentiate for 6 days prior to the addition of *t*10, *c*12-CLA for 24 h (Fig. 5B). TNF-α stimulation resulted in a sevenfold increase in NF-κB transcriptional activity in both myoblasts and myotubes (Fig. 5B), confirming that myoblasts were capable of activating the NF-κB pathway.

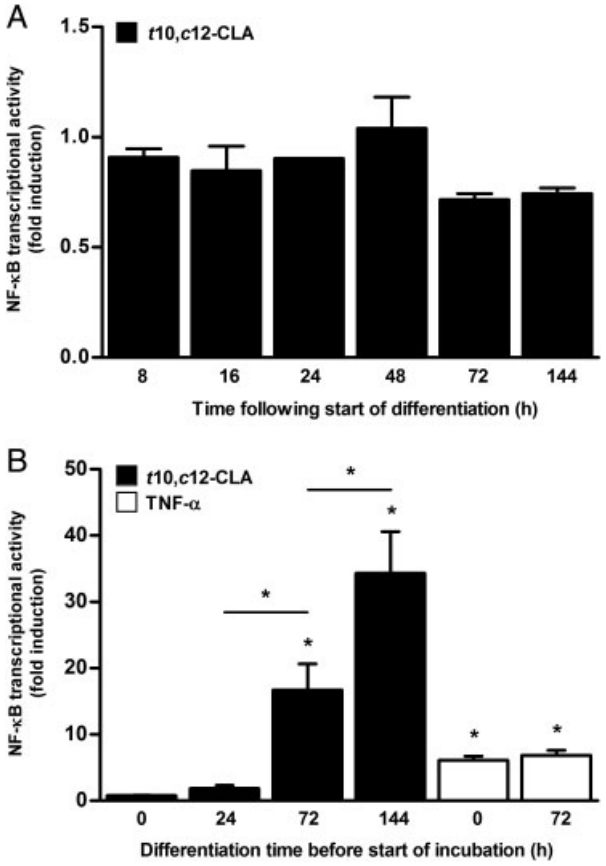

Figure 5. Differential NF-κB activition in myoblasts and myotubes. (A) C2C12 cells were cultured with differentiation medium during 6 days in the presence of 50 μmol/L *t*10, *c*12-CLA or vehicle. NF-κB activity was assessed at several time-points and depicted as fold-induction over the corresponding BSA-treated control. (B) C2C12 cells were differentiated for 0, 1, 3 and 6 days before treatment with 50 μmol/L *t*10, *c*12-CLA for 24 h, or TNF-α (10 ng/mL, 4 h). Statistically significant differences between the indicated treatment and corresponding BSA-treated control and between treatments ($^*p<0.05$) were determined by one-way ANOVA.

4 Discussion

The current study reveals that activation of NF-κB transcriptional activity in skeletal muscle and inhibition of myogenic differentiation by *t*10, *c*12-CLA are not causally related. This is in contrast to what has been reported for blockade of myogenesis by the inflammatory cytokines TNF-α and interleukin-1β [9, 10]. Our findings that myotube formation and expression of muscle specific genes were blocked upon treatment with *t*10, *c*12-CLA in DM are in line with other studies using human primary muscle cells and L6 cells [22, 23]. Conversely, increased formation of multinucleated myotubes upon treatment with linoleic acid, corresponds with studies that have shown stimulatory effects of linoleic acid on the formation of myotubes from primary rat skeletal muscle satellite cells [23–25].

It is remarkable that 50 μmol/L *t*10, *c*12-CLA evoked a >60-fold increase in NF-κB transcriptional activity, which, in comparison, is a much more potent activator of NF-κB in myotubes than the prototypical inducer TNF-α. Furthermore, CLA-isomeric effects were apparent, since there was a noticeable difference in the ability of both CLA isomers in activating NF-κB and inhibiting skeletal muscle differentiation. The same concentration of *c*9, *t*11-CLA had no effect on NF-κB transcriptional activity and muscle differentiation. In a previous report [17], we showed that the isomeric effects on NF-κB transactivation also applied to NF-κB DNA binding.

Surprisingly, in contrast to myotubes, *t*10, *c*12-CLA was not able to activate NF-κB transcriptional activity in myoblasts, and the magnitude of activation appeared to depend on the maturity of the differentiation program. This is remarkable since NF-κB transcriptional activation by TNF-α or any other NF-κB stimuli to our knowledge, is not dependent on the differentiation status of skeletal muscle cells, as identical NF-κB DNA binding and transcriptional activity were observed in response to TNF-α (this study) and interleukin-1β [9] in undifferentiated myoblasts and 5-day differentiated myotubes. Apparently, the signaling constituents required for *t*10, *c*12-CLA-mediated NF-κB activation are not in place in undifferentiated myoblasts. It is known that myoblasts and myotubes display highly differential protein expression patterns [4, 26, 27], but the responsible factor(s) for the observed differences in NF-κB inducibility by *t*10, *c*12-CLA remain elusive. Signaling responses triggered by FAs may result from receptor activation as well as intracellular accumulation of metabolites. For example, palmitate has been shown to induce accumulation of lipid metabolites like ceramides and diacylglycerol, which have been implicated in activation of NF-κB [28]. Furthermore, palmitate can activate toll-like receptor (TLR) signaling in skeletal muscle cells, which can induce NF-κB activity [29], but whether TLRs are required for *t*10, *c*12-CLA-mediated NF-κB activation is currently unknown. In fact, undifferentiated myoblasts were previously reported to express

functional TLRs [30], suggesting that NF-κB activation by *t*10, *c*12-CLA in skeletal muscle cells likely occurred independent of TLR activation, or at least indicating that TLR expression is not sufficient for NF-κB activation in myoblasts by *t*10, *c*12-CLA. In addition, TLR-mediated transcriptional activation of NF-κB by LPS [31], as well as TNF-induced NF-κB transactivation [9] in skeletal muscle were reported to occur within 2–4 h. In contrast to this receptor-mediated signaling, the 8–12 h required for transcriptional activation of NF-κB by 10–12 CLA was more in line with the time frame of palmitate-induced NF-κB transactivation [13]. This suggests NF-κB activation by FA may not be receptor mediated but rather depend on intracellular lipid intermediates.

Besides inhibiting myogenic differentiation, CLAs are also able to inhibit adipocyte differentiation [32, 33]. Interestingly, analogues to our findings in muscle cells, isomer specificity of CLA was also observed in adipocytes, since only *t*10, *c*12-CLA and not *c*9, *t*11-CLA inhibited differentiation and reduced GLUT4 mRNA expression and insulin-induced glucose uptake in differentiating human preadipocytes [34]. The authors speculated on the involvement of peroxisome proliferator-activated receptor (PPAR)-γ, since these isomer-specific effects were paralleled by a reduced expression of PPAR-γ and several of its downstream target genes. Furthermore, *t*10, *c*12-CLA was able to antagonize ligand-dependent PPAR-γ activity [35] and it has been shown that PPAR-γ is required for the differentiation of adipose tissue [36]. Interestingly, decreased PPAR-γ expression in C2C12 cells was also reported to inhibit myogenic differentiation [37]. Nevertheless, whether decreased PPAR-γ expression is responsible for *t*10, *c*12-CLA-induced inhibition of myogenic differentiation remains to be investigated.

Opposing effects of CLA on insulin sensitivity have been described in rodents and humans [38–49]. Many of these studies are performed with mixtures of the two major CLA isomers. In rodent [50, 51] and human [52] studies, the *t*10, *c*12-CLA isomer was associated with decreased body fat. However, most studies that tested isomer specific properties of CLA showed that *t*10, *c*12-CLA induced insulin resistance and hepatic steatosis in mice [42, 53–55]. Also in obese men, treatment with *t*10, *c*12-CLA caused isomer-specific insulin resistance [46]. In human adipocytes, *t*10, *c*12-CLA increased NF-κB activation and reduced GLUT4 expression and insulin-stimulated glucose uptake [56, 57], which is in line with our findings on GLUT4 mRNA expression and NF-κB transactivation in skeletal muscle cells. Despite the association between *t*10, *c*12-CLA inflammation and insulin resistance that emerges from these studies, in previous work we failed to detect acute effects of CLAs on insulin-induced glucose uptake in skeletal muscle myotubes, despite a potent induction of NF-κB [13]. Our data presented in this work suggest that *t*10, *c*12-CLA may affect glucose homeostasis indirectly by inhibiting muscle differentiation and myogenesis-associated GLUT4 expression.

Overall, these data reveal *t*10, *c*12-CLA as a very potent activator of the NF-κB pathway and inhibitor of myogenic differentiation, but demonstrate that these effects of *t*10, *c*12-CLA are not causally related, as genetic blockade of NF-κB signaling failed to restore myogenic differentiation and *t*10, *c*12-CLA did not induce NF-κB activation in undifferentiated myoblasts. The results of numerous studies that described anti-inflammatory [58] effects of mixtures of CLA suggest a potential for CLA as a novel dietary supplement for patients suffering from muscle wasting in chronic inflammatory related diseases. However, the results of this study regarding the isomer-specific inhibition of muscle differentiation and induction of muscle NF-κB signaling by *t*10, *c*12-CLA imply that this CLA isomer may even be detrimental in inflammatory muscle wasting conditions.

In conclusion, we found that the inhibitory effects of *t*10, *c*12-CLA on myogenic differentiation did not require NF-κB activation and coincided with a marked reduction in GLUT4 expression, suggesting that long-term administration of *t*10, *c*12-CLA may reduce insulin sensitivity in skeletal muscle.

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