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CONJUGATED LINOLEIC ACIDS DIMINISH GLYCOGEN SYNTHASE AND GLYCOGEN SYNTHASE KINASE-3 EXPRESSION IN MUSCLE CELLS OF C57BL/6J MICE - *IN VITRO* AND *IN VIVO* STUDY

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Conjugated linoleic acids (CLA) have been extensively advertised as dietary supplements to reduce fat and increase muscle mass. However, the role of CLA in glycogen metabolism is still largely unknown. The aim of this study was to assess the effect of CLA on glycogen synthesis *in vitro* (CCL 136 cell line human) and CLA *in vivo* (C57BL/6J mice). The materials used were the CCL 136 muscle cell line and muscles of female C57BL/6J mice (n = 52), housed at animal laboratory facility and feed with "MURIGRAN", a standard feed prepared for rodents (Agropol, Poland). Chemically pure fatty acids were added to soybean oil. CLA isomers (c9,t11 CLA, t10,c12 CLA, and as a mixture (1:1)) were administered with feed. Supplementation in mice started at week 6 of age and lasted for 4 weeks. Methods used in the study were real time- PCR - quantification of gene expression, Western blot glycogen synthase kinase-3 (GSK3α 9) and glycogen synthase (GS) protein, glycogen staining by PAS. Quantitative determination of glycogen by spectrophotometry and intracellular reactive oxygen species was measured the intracellular oxidation of dichloro-dihydro-fluorescein diacetate (DCFH-DA). *In vitro* data showed that GS and GSK3 expression was lower in cells cultured with different CLAs and a mixture of CLAs. GS gene expression was significantly decreased in cells cultured with c9, t11 CLA ($P < 0.04$) and t10, c12 CLA ($P < 0.05$) as well as the mixture of both isomers. The GSK3α gene expression was reduced in cells cultured with a mixture of CLA ($P < 0.02$), whereas phosphorylation of GSK3α increased in cells cultured with c9, t11 CLA GSK3α ($P < 0.05$). *In vivo* data showed a reduction in the glycogen concentration among mice fed a diet containing t10, c 12 CLA and a mixture of CLA isomers. We conclude that both CLA isomers can affect the synthesis of glycogen in muscle cells through the regulation of GS and GSK3α gene expression.

Key words: *conjugated linoleic acid, glycogen, glycogen synthase, glycogen synthase kinase-3, human skeletal muscle cell line, oxidative stress, reactive oxygen species*

INTRODUCTION

Glycogen synthesis is regulated by several enzymes in humans, including glycogen synthase (GS) and glycogen synthase kinase-3 (GSK3α). GS converts glucose to glycogen, which is regulated by GSK3α. The inhibition of GSK3α activity (by phosphorylation) reduces GS phosphorylation which converts the enzyme to its active form and increases glycogen synthase activity (1-3).

It is also known that insulin-dependent inhibition of GS phosphorylation is regulated by 5' phosphodiesterase (PDE5) and glycogen phosphorylase (4-6). After the influence of insulin, the activity of phosphatidylinositol 3-kinase (PI3K) and two other kinases, phosphatidylinositol-dependent protein kinase 1 (PDK1) and threonine-serine protein kinase (mTORC2), is elevated, leading to the translocation of glucose transporter type 4 (GLUT4) to the cell membrane and then glucose into the cells.

Conjugated linoleic acids (CLAs) are frequently used as dietary supplements generally advertised for their effects on reducing fat content and increasing muscle mass. Moreover, they are said to reduce muscle glycogen breakdown, as well as reduce muscle damage and inflammatory responses (7). Preliminary findings demonstrate that CLA may induce a physiological increase in testosterone synthesis (8). Commercially available CLA supplements mainly consist of two isomers, namely *cis-9*, *trans-11* CLA (c9, t11 CLA) and *trans-10*, *cis-12* CLA (t10, c12 CLA) (9). The role of CLAs in glucose and lipid metabolism has been previously investigated, but with conflicting results (10-12). In a study by Poirier *et al.* CLAs were reported to inhibit glucose, exaggerate insulin resistance in adipocytes, and initiate inflammation and synthesis of acute phase proteins (12). Brown

Table 1. Fatty acid content in the feed distributed to rodent groups (calculeted).

Group	Fat content	Soybean oil	CLA content	LA content	Fat in total
Control	~3%				~3%
SBO	~3%	6,5%			~9.5%
Mix CLA	~3%	5%	1.5%		~9.5%
c9, t11 CLA	~3%	5%	1.5%		~9.5%
t10, c12 CLA	~3%	5%	1.5%		~9.5%
LIN	~3%	5%		1.5%	~9.5%

CLA, conjugated linoleic acid; LA, linoleic acid.
SBO - mice fed a diet containing 6.5% soybean oil;
Mix CLA - mice fed a diet containing 5% soybean oil, 0.75% c9, t11 CLA isomer and 0.75% t10, c12 CLA isomer;
c9, t11 CLA - mice fed a diet containing 5% soybean oil and 1.5% c9, t11 CLA isomer;
t10, c12 CLA - mice fed a diet containing 5% soybean oil and 1.5% t10, c12 CLA isomer; LIN - mice fed a diet containing 5% soybean oil and 1.5% linoleic acid.

et al. (10) conducted that only t10 and c12 CLA were reported to activate the mitogen-activated protein kinase/extracellular signal-related kinase (MEK/ERK) pathway, which alters gene expression and lowers glucose and fatty acid uptake (10). *In vitro* studies showed that CLA reduces liver production of glucose through a decrease in gluconeogenesis (13). At least some of the CLA effects seem to be activated by peroxisome proliferator receptors (PPAR)-γ. There have been reports of the induction of *aP2 gene* expression in the fat tissue of rats exposed to CLA, activating PPAR-γ elements through CLA *in vitro* (14). Some isomers of CLA are capable of improving glucose tolerance *via* insulin-stimulated transportation of glucose to skeletal muscles and through the activity of glycogen synthase (GS) (13). Moreover, Tsao *et al.* (15) showed significantly increased muscle glycogen content with CLA after a single bout of exercise ($P < 0.05$). Muscle glucose transporter type 4 (GLUT4) expression was significantly elevated immediately after exercise, and this elevation continued until 3 h after exercise in the CLA trial. Thus, it was suggested that CLA supplementation can enhance the glycogen re-synthesis rate in exercised human skeletal muscle (15). The aim of this study was to investigate the effects of CLAs on glycogen synthesis both *in vitro* and *in vivo*. As was previously shown, CLAs have negative effects, such as lowering the insulin response capacity by increasing glucose utilization and the prevention of palmitate-induced glucose uptake (16).

MATERIAL AND METHODS

Ethics Statement

The Local Ethics Committee for Experiments on Animals in Szczecin, Poland approved the animal studies (Agreement No 27/09). Test mice were not kept together (2 – 4 mice per group; 3 cages). All cages were with environmental enrichments and separate ventilation that functioned in the specialized animal room of the Pomeranian Medical University in Szczecin, Poland.

Treatment of experimental animals

C57BL/6J female mice (n = 52) were obtained from the animal facility of the Medical University in Bialystok Poland (to perform tests on the unified group of rodents only one and less aggressive female mice were chosen). The material from all animals was collected for analysis after the end of the experiment. Assuming a standard level of test significance and its power: 0.05 and 0.8, respectively, N min. was calculated at the level of 6 animals in the group. In the test groups (c9,t11 CLA, t10,c12 CLA, mixture CLA and LA), a higher value for statistical deviation was secured. Chemically pure fatty acids were added to soybean oil and then mixed with the appropriate amount of ground feed and water, forming a homogeneous mixture. CLA isomers (c9,t11 CLA, t10,c12 CLA, and as a mixture (1:1) (Nu-Chek Prep, USA) were administered with feed. The 1: 1 ratio has allowed us to repeat the layout of our previous experiment (18). Our goal was to observe the effects of individual isomers at the same concentrations, although it is known that in nature, the beneficial cis 9, trans 11 occurs in a significant advantage.

We used "MURIGRAN" (Agropol, Poland), a standard feed prepared for rodents (17, 18). Composition granulate feed: Protein total min. 23%, Fat total min. 3%, Raw ashes max. 7.5%, Fibers max. 5%, Lizin min. 1.50%, Met + Cyst min. 0.8%, Calcium min. 1.1%, Phosphor min. 0.7%, Natrium max. 0.2%, Vit. A 8000 Jm/kg, Vit. D3 1000 Jm/kg, Vit. E 50 Jm/kg.

Supplementation started in 6 weeks old mice which were housed in groups in the animal laboratory of Pomeranian Medical University (*Table 1*):

1) Control - no fatty acid supplementation, n = 6 rodents;
2) Soybean oil (SBO) - mice fed a diet containing 6.5% soybean oil, n = 6 rodents;
3) c9, t11 CLA - mice fed a diet containing 5% soybean oil and 1.5% c9, t11 CLA isomer, n = 10 rodents;
4) t10, c12 CLA - mice fed a diet containing 5% soybean oil and 1.5% t10, c12 CLA isomer, n = 10 rodents;
5) Mix CLA - mice fed a diet containing 5% soybean oil, 0.75% c9, t11 CLA isomer and 0.75% t10, c12 CLA isomer, n = 10 rodents;
6) Linoleic acid (LIN) - mice fed a diet containing 5% soybean oil and 1.5% linoleic acid, n = 10 rodents.

The supplementation in this study was sustained for 4 weeks. The portions prepared for each day of the experiment were ~3 g/mouse/day. In addition, the amount of feed consumed by the animals was recorded on a daily basis. The method used was the counting of the total amount of food leftover in the cages (unconsumed feed was collected and weighed). Each experimental group ate a similar amount of feed, and animals were kept together during the experiment. Feed intake was similar in each group. Also, water was freely available for the mice, and the animals were weighed before and after the experiment. There was no significant weight change observed between the mouse groups, either prior to and or after the study measurements were completed.

The animals were sacrificed *via* exsanguination after intraperitoneal injection of Thiopental (90 mg/kg body weight,

Biochemie GmbH, Austria). Subsequently, muscle tissue from the lower leg (quadriceps muscle of the thigh) was snapped and frozen using liquid nitrogen to be stored for further future analysis.

CCL136 cell culture

The human skeletal muscle cell line CCL136 (LGC Standards, USA) was maintained at 37ºC with 5% CO_2 in Dulbecco's Eagle's modified medium (Sigma-Aldrich, Poland) containing 4500 mg glucose/L (4.5 g/L), 10% fetal bovine serum (FBS; Gibco, USA), and antibiotics (Sigma-Aldrich, Poland). CLA isomers with bovine serum albumin (BSA), linoleic acid + BSA (test samples) (Nu-Chek Prep, USA), and BSA alone, were added to the medium for 48 hours. First the fatty acids were dissolved in ethanol, then sodium hydroxide was added in order to obtain fatty acids salts. The mixtures were dried under nitrogen and the salts were dissolved in double distilled water. To prevent oxidation butylated hydroxytoluene (BHT, Sigma-Aldrich, Germany) was added to each solution. At the end delipidated BSA (Sigma-Aldrich, Germany) was added. The final concentration of the fatty acids was 30 µmol/L. Insulin (Sigma-Aldrich, Poland) was added at a concentration of 0.1 mM/L.

The cells were cultured on 6-well plates 1) no additives, 2) BSA, 3) CLA mix, 4) c9, t11 CLA, 5) t10, c12 CLA, 6) linoleic acid. The medium with BSA (carrier of fatty acids) alone was used as the control, except in one of the experiments, ROS measurement, where the cells cultured in medium with no additives were used as the control. Each experiment was repeated 6 times.

Linoleic acid and its isomers were delivered as complexes with bovine serum albumin (BSA): first the fatty acids were dissolved in ethanol, then sodium hydroxide was added in order to obtain fatty acids salts. The mixtures were dried under nitrogen and the salts were dissolved in double distilled water. To prevent oxidation butylated hydroxytoluene (BHT, Sigma-Aldrich, Germany) was added to each solution. At the end delipidated BSA (Sigma-Aldrich, Germany) was added.

Western blot analysis

1. Lysates of the cells

After incubation with the fatty acids, the cells were washed 3 times with PBS and centrifuged (250 × g, 10 min, 4ºC).Then, 400 µl of RIPA (Radio Immuno Precipitation Assay) lysis buffer with two PhosSTOP tablets (phosphatase inhibitor cocktail tablets) was added to the cells; lysates were then centrifuged. An aliquot of the supernatant was used to measure the protein concentration (Bradford method). The remaining supernatant was used (or frozen at –80ºC) for further analysis.

2. Lysates of the muscle tissue from the animals

Approximately 5 mg of the tissue was homogenized in 300 µl of ice cold lysis buffer with protease inhibitor (5 mM ethylenediaminetetraacetic acid, 1% sodium dichloroisocyanurate, 1% TRITON-X, and 100 mM sodium orthovanadate; Sigma-Aldrich, Poland). The homogenates were then incubated on a shaker for 2 hours at 4ºC at 175 rpm. Subsequent to the incubation, the samples were centrifuged (12000 rpm/20 min/4ºC) and the supernatant was collected (or stored at -80ºC) until further analysis (3 months).

A proper amount (v: v, 1:1) of Bondbreaker Laemmli buffer was added to the samples. The samples were then placed in the thermoblock for 10 minutes (70ºC) and chilled on ice. Then, they were centrifuged and placed back on ice. The samples obtained were electrophoresed in SDS-PAGE and transferred to PVDF membrane at a constant voltage of 100 V for 1 hour. After the transfer, the membrane was blocked with 5% fat-free milk for 1 hour at room temperature and then incubated with the appropriate primary antibody in 4 ml of 5% fat-free milk/TBST (Tris-Buffered Saline with Tween 20) for 2 hours at room temperature. After washing 3 times with TBST, the membrane was incubated with the selected secondary antibodies in 4 mL of 3% BSA in TBST for 1 hour at room temperature. The PVDF membrane was washed in TBST (2 × 5 minutes on the shaker) and in TBS (Tris-Buffered Saline) (2 × 5 minutes on the shaker). Then, the membrane was incubated in the chemiluminescent reagent West Piko (Thermo Scientific, Pierce Biotechnology, USA) for 10 minutes. Next, the films were developed and scanned. Then, densitometric analysis was performed using the GellDOCoIt imaging system (DNR Bioimaging Systems).

The following antibodies used in this study were purchased from Santa Cruz Biotech, USA: glycogen synthase 2 (H-49) - homo sapiens GYS1; mus musculus Gys1; GSK-3α/β (0011-A) - homo sapiens GSK3A; mus musculus Gsk3a; P-GSK-3α (9B8) - homo sapiens GSK3A; mus musculus Gsk3a.

Quantification of gene expression using real-time PCR

RNA from the muscles and CCL136 cells was isolated using an RNeasy Kit (Qiagen). The concentration and purity of the isolated RNA was determined using a Nanodrop Spectrophotometer ND-1000. Reverse transcription was performed using the SuperScript® First Strand Synthesis System for RT-PCR (Invitrogen, USA) with universal primers (random decamers). The cDNA obtained served as a template for further analysis, such as gene expression quantified with the 7500 Fast Real-Time PCR System (Applied Biosystems). PCR reactions were performed in duplicate with a total volume of 20 µL, containing 10 µL of TaqMan® Gene Expression PCR Master Mix (Applied Biosystems, USA), 2 µL of diluted first strand cDNA, and 1 µL of the probe/primer mix (TaqMan® Gene Expression Assays, Applied Biosystems, USA: GYS1 - Hs00157863_m1 and Mm02026726_s1, GSK 3α - Hs00997938_m1 and Mm01719731_g1). The ΔΔCt method (with GAPDH as a house keeping gene for the mouse muscles and cyclophilin for the human cells) was used to compare the expression of the target genes in the experimental versus control conditions, as described in the Applied Biosystems manual.

Quantitative determination of glycogen by spectrophotometry

During this study, 1 g of muscle tissue or 3×10^6 CCL136 cells was placed in a tube containing 2.5 mL of 30% potassium hydroxide solution to isolate glycogen. Quantification of glycogen was measured *via* the Somogyi-Nelson method, as previously described (19). The results were normalized to the total protein content determined with the Bradford method (spectrophotometric method - ASYS UVM340, Biogenet).

Glycogen staining by periodic acid Schiff (PAS)

The approximately 5 µm muscle tissue sections were stained *via* PAS method using Alcian blue-periodic acid-Shiff in order to count the glycogen grains stored in myocytes cells (20). Cells were then incubated for 1 h at room temperature. To estimate the amount of glycogen grains, the stained grains were counted under the microscope (magnification 25 µm), in the 50% randomly selected half view photographs for every group, then the mean was estimated. The quantitative results are expressed as arithmetic means + SD. Statistically significant results were considered those with $P < 0.05$ (Wilcoxon matched-pairs test).

Intracellular reactive oxygen species measurement

Oxidative stress in the cells was determined by measuring the intracellular oxidation of DCFH-DA (dichloro-dihydro-fluorescein diacetate, Sigma-Aldrich, Poland) (21-22). Dichloro-dihydro-fluorescein diacetate (DCFH-DA) becomes fluorescent when it interacts with hydrogen peroxide within the cell. For intracellular ROS detection, cells were pre-loaded (30 min at 37ºC) with 5 µM 2',7'-dichlorofluorescein diacetate (DCFH-DA). Cells were harvested, and the number of cells exhibiting increased oxidized DCF fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm (21). Cells

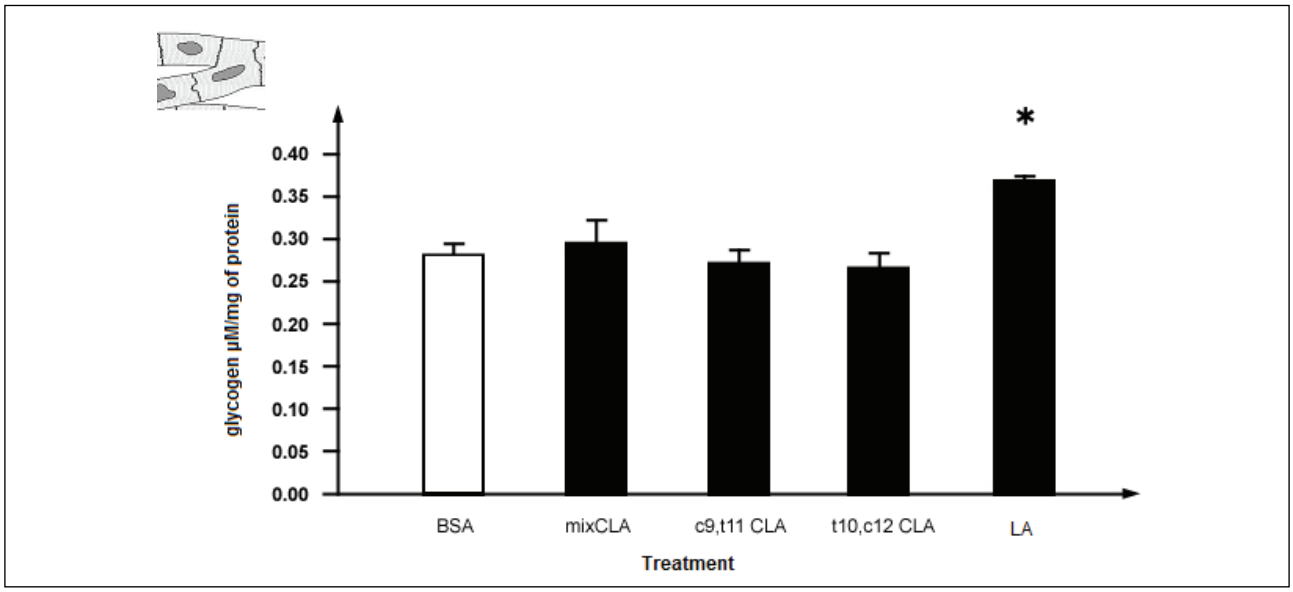

Fig. 1. Effect of conjugated linoleic acid (CLA) on glycogen synthesis in CCL136 muscle cells. Different fatty acids were added to CCL136 cells, as described in the Material and Methods section. After incubation, the glycogen concentration was measured using the Somogyi-Nelson method. *P ≤ 0.05 indicates a significant difference.
BSA - bovine serum albumin; mixCLA - mice fed a diet containing 5% soybean oil, 0.75% c9, t11 CLA isomer and 0.75% t10, c12 CLA isomer; c9, t11 CLA - mice fed a diet containing 5% soybean oil and 1.5% c9, t11 CLA isomer; t10, c12 CLA - mice fed a diet containing 5% soybean oil and 1.5% t10, c12 CLA isomer, LA - linoleic acid.

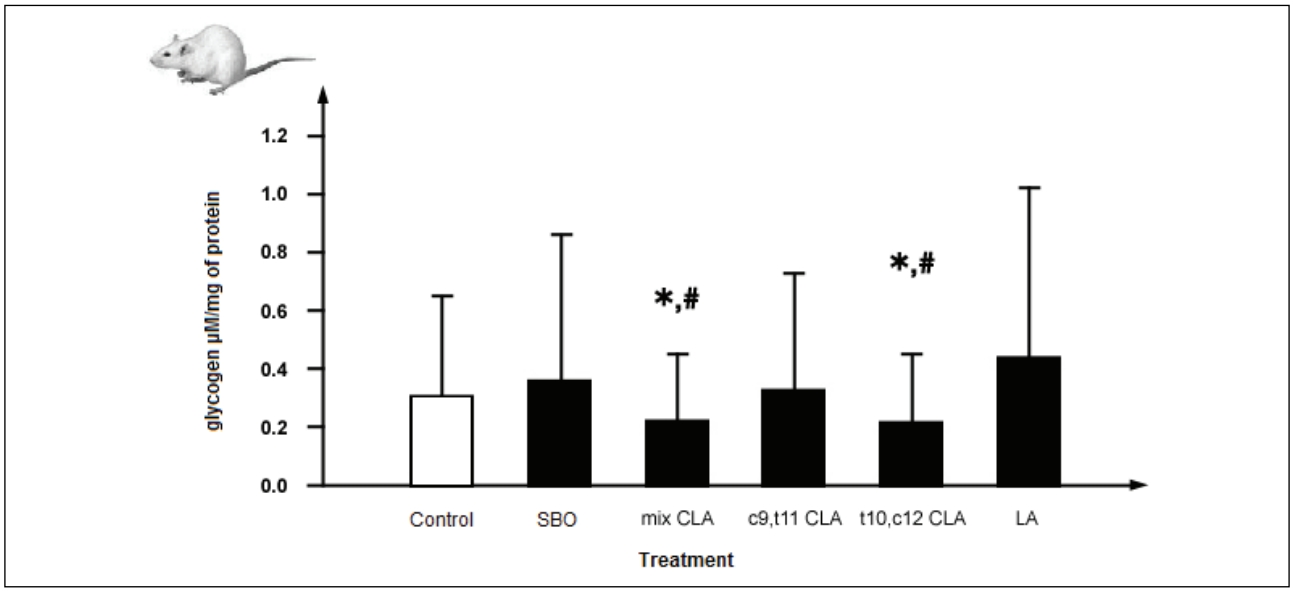

Fig. 2. Effect of conjugated linoleic acid (CLA) on glycogen synthesis in mouse muscle. Mice were fed a standard diet supplemented with CLA as described in the Material and Methods section. After 4 weeks, animals were sacrificed by exsanguination after intraperitoneal injection of thiopental, and the glycogen concentration was measured in the lower leg muscle using the Somogyi-Nelson method. *P ≤ 0.05 indicates statistically significant difference versus Control #P ≤ 0.05 statistically significant difference versus SBO.
SBO - mice fed a diet containing 6.5% soybean oil; mixCLA - mice fed a diet containing 5% soybean oil, 0.75% c9, t11 CLA isomer and 0.75% t10, c12 CLA isomer; c9, t11 CLA - mice fed a diet containing 5% soybean oil and 1.5% c9, t11 CLA isomer; t10, c12 CLA - mice fed a diet containing 5% soybean oil and 1.5% t10, c12 CLA isomer, LA - linoleic acid.

(1.5×10^6 on plate) were cultured on 6-well plates, as described previously. Then, DCFH-DA was added to each well, and the cells were incubated for 30 minutes at 37ºC in the dark. Reactive oxygen species cause intercellular oxidation of DCFH-DA, which results in viewing an enhanced version of the fluorescent dye. For that reason, fluorescence intensity was measured using a FACScan flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 525nm (23).

Statistical analysis

All the results are expressed as the mean ± SD. Because the distribution was not normal in most cases (Shapiro-Wilk test), non-parametric tests were employed. The non-parametric Mann-Whitney test was used to assess the differences between the study groups. The software used for statistical analysis was Statistica 10, Statsoft, Poland. All tests in this study were evaluated at the significance level of $P < 0.05$.

RESULTS

Effect of conjugated linoleic acids on glycogen content in CCL 136 cells

The increase in glycogen content in cells depended on the fatty acid used in the culture. When compared to the control cells (cultured with BSA), the glycogen content in the CCL 136 cells increased by 32% ($P < 0.02$) after incubating with linoleic acid (LA) (*Fig. 1*). When comparing the LA treatment, the glycogen also content was significantly lower ($P < 0.02$) in the CLA incubated cells. The results mirrored no significant differences between control (BSA) and CLA treatment cells.

Effect of conjugated linoleic acids on glycogen content in the muscles of mice

The glycogen concentration was examined *in vivo* in the cells of the posterior lower leg muscles of the mice (gastrocnemius) (*Figs. 2* and *3*). The obtained results were compared with the glycogen concentration in the controls, *i.e.*, in the muscles of mice bred under standard conditions - the group fed with oil supplementation (soybean oil control group).

As shown in *Fig. 2* and *3*, the glycogen concentration of the mouse gastrocnemius muscle was different after it was treated with different CLA isomers. Thus, a lower glycogen content was observed with the supplementation of t10, c12 CLA ($P < 0.04$ when compared to the Control and $P < 0.05$ with the soybean oil group SBO) as well as with CLA mixture ($P < 0.04$ compared to the Control and $P < 0.05$ with the soybean oil group SBO). Furthermore, the results were confirmed by staining the muscle sections with the periodic acid Schiff method, in which glycogen grains are stained red. The supplementation of isomer t10, c12

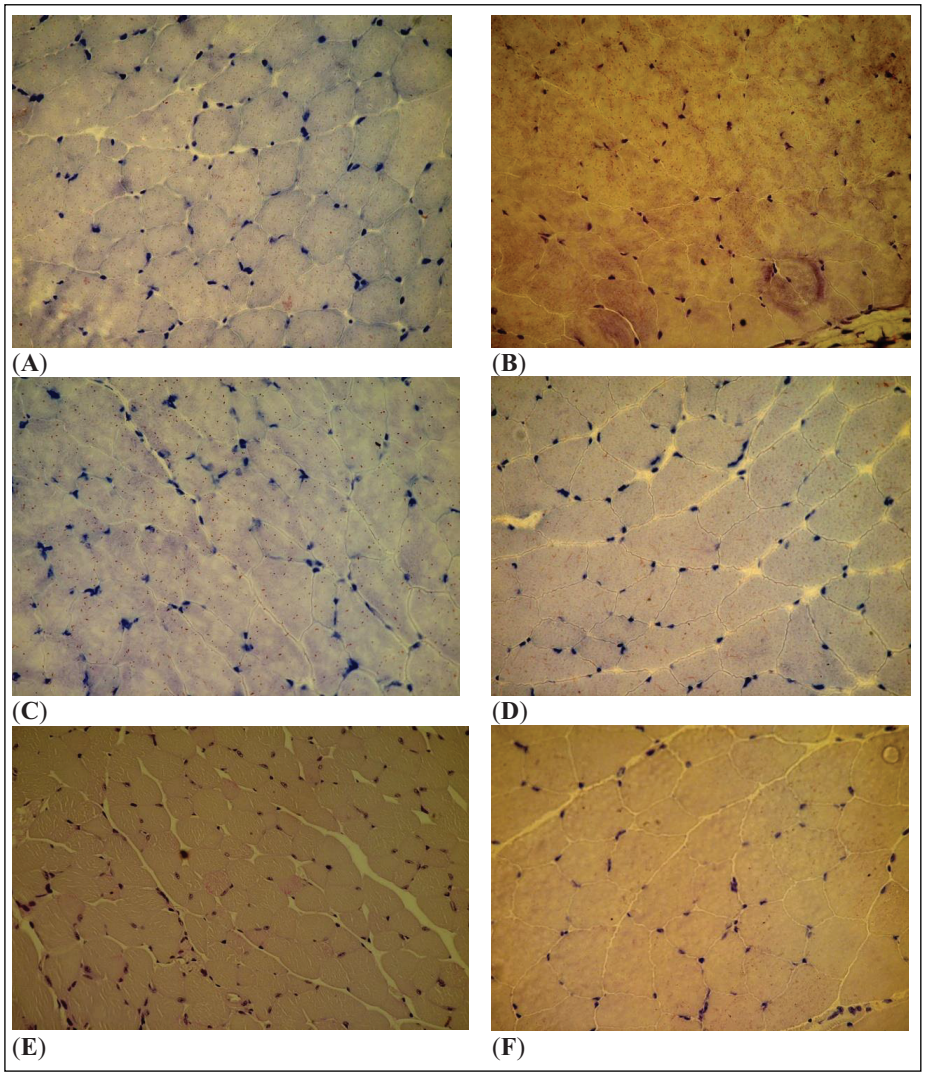

Fig. 3. Effect of conjugated linoleic acid (CLA) on the glycogen content in mouse muscle. Muscle tissue sections were stained with periodic acid Schiff (PAS) to estimate the glycogen content. Glycogen grains in the images are stained red: (A): control; (B): soybean oil; (C): mix CLA; (D): c9,t11 CLA; (E): t10,c12 CLA; (F): linoleic acid. Magnification: 25 μm.

CLA and the CLA mixture led to a visible decrease of glycogen grains when compared to the control and the soybean oil group.

Effect of on GS mRNA and protein expression in CCL136 cells

When comparing cells cultured with BSA, linoleic acid incubation resulted in higher GS gene expression ($P < 0.05$) (*Fig. 4*). In contrast, GS gene expression was significantly decreased in cells cultured with c9, t11 CLA ($P < 0.04$), t10,c12 CLA ($P < 0.05$) and with the mixture of both isomers (*Fig. 4A*). In addition, Western blotting demonstrated a similar trend for the GS protein content, except for the LA incubation, which did not significantly increase GS protein expression (*Fig. 4*).

Similarly, t10, c12 and mixed CLA supplementation resulted in slightly decreased GS mRNA expression, but the results were not significant (results not shown).

The determination of the enzyme protein by Western blotting confirmed a tendency for increased glycogen synthase protein in cells cultured with linoleic acid and a decrease in cells cultured with CLA isomers and their mixture ($P < 0.05$) (*Fig. 4B*).

Effect of conjugated linoleic acids on GSK3α mRNA and protein expression in CCL136 cells

Fig. 5 shows the results for the cells cultured with the mixture of CLA isomers demonstrated a reduction of GSK3α gene expression when compared to the BSA ($p < 0.02$, *Fig. 5A*). There was a noticeable tendency for a reduction in the content of GSK-3α in the muscle of mice fed with the linoleic acid supplement, the mixture of CLA isomers, and the t10,c12 CLA (*Fig. 5A*). Western blot analysis also confirmed the lower expression of GSK3α protein in cells cultured with CLA isomers c9, t11 and t10, c12 and their mixture ($P < 0.05$, *Fig. 5B*). The muscle samples of the mice supplemented with fatty acids (including CLA isomers, their mixture, and linoleic acid) confirmed no significant changes in GSK3α gene expression (results not shown).

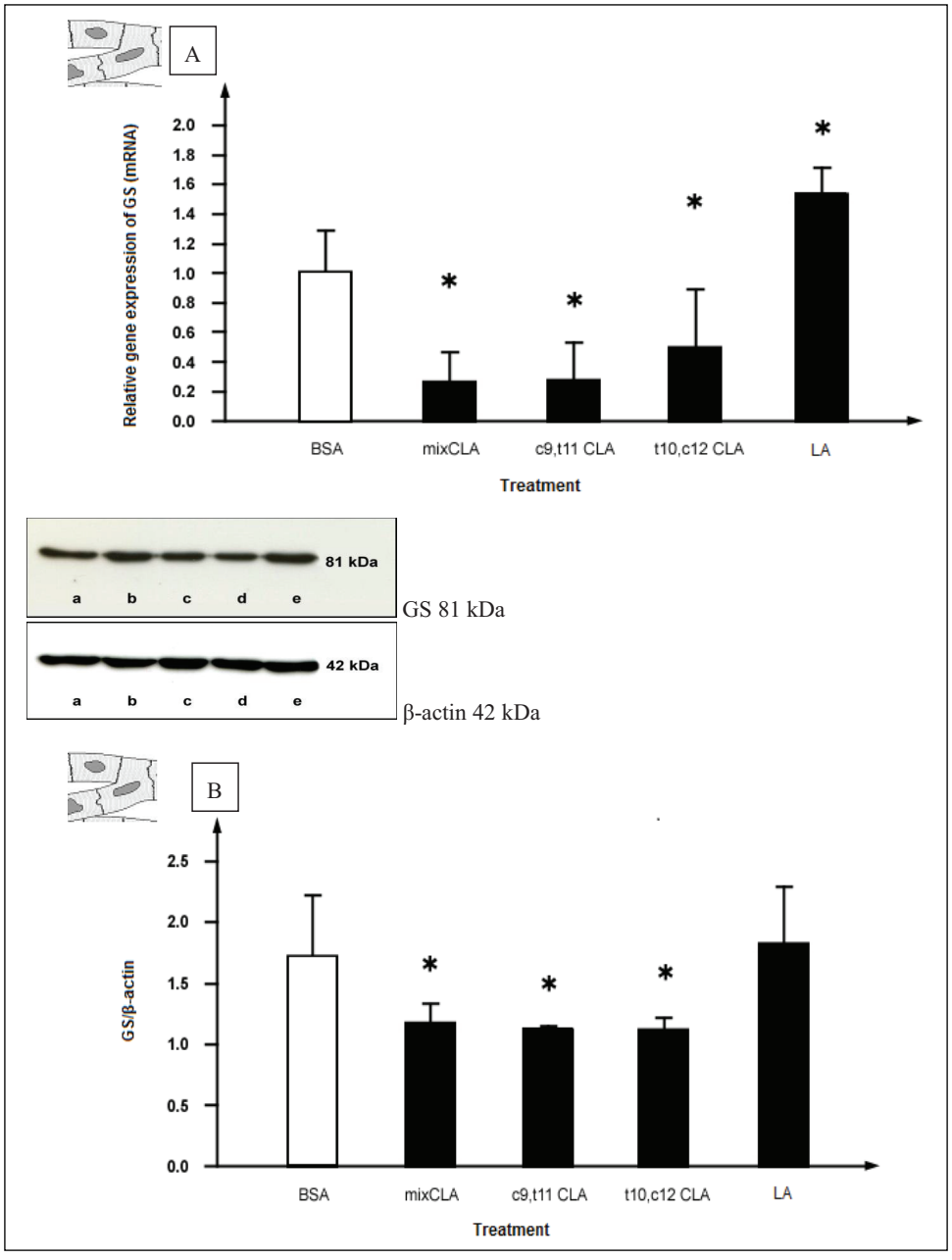

Fig. 4. Effect of conjugated linoleic acid (CLA) on GS mRNA and protein expression in CCL 136 cells. The cells were cultured with CLA for 48 h and then harvested by scraping, (A) mRNA was measured using the real-time PCR method and (B) protein expression was measured using the Western blotting method (densitometric analysis of protein normalized to β-actin; representative Western blot).
*$P \leq 0.05$ indicates statistically significant; **a** - bovine serum albumin (BSA), **b** - mice fed a diet containing 5% soybean oil, 0.75% c9, t11 CLA isomer and 0.75% t10, c12 CLA isomer (mixCLA), **c** - mice fed a diet containing 5% soybean oil and 1.5% c9, t11 CLA isomer (c9, t11CLA); **d** - mice fed a diet containing 5% soybean oil and 1.5% t10, c12 CLA isomer (t10,c12CLA); **e** - linoleic acid (LA).

Effect of conjugated linoleic acids on GSK3α protein phosphorylation in CCL136 cells

GSK3α phosphorylation increased in cells cultured with c9, t11 CLA ($P < 0.05$), and mixed CLA ($P < 0.05$) compared to the BSA-treated cells (*Figs. 6* and *7*).

Effect of conjugated linoleic acids on reactive oxygen species synthesis in CCL 136 cells

When the test results were compared to the BSA control (BSA also increased the production of ROS), there was a statistically significant decrease in the synthesis of ROS in cells cultured with c9,t11 CLA ($P < 0.02$) (*Fig. 8*). There was a statistically significant increased synthesis of reactive oxygen species (ROS) in cells cultured with t10,c12 CLA ($P < 0.05$) compared to the control (Control) but not to the BSA.

DISCUSSION

Conjugated linoleic acids (CLA) are dietary supplements that have been extensively advertised to maintain a low body fat content and normal fat/muscle contents. CLA have been shown to reduce body fat by 43% to 88% (24-27). In mice fed with CLA, the trans-10, cis-12 isomer was associated with lipodystrophy, insulin resistance, and hyperinsulinemia (28). Morover, Schiavon *et al.* have shown that in the adipose tissues, CLA increased the proportions of saturated FAs, 18:0 and 18:2t10c12, and decreased the proportions of monounsaturated FAs (29). In muscles, the effects were the opposite. The results suggest that Δ9 desaturase activity is inhibited by the rpCLA mixture in adipose tissues to a greater extent than in the other tissues (29). The influence of CLA on glycogen metabolism is still largely unknown. Among other molecules, acetylated ghrelin and dexamethasone were shown to affect intracellular signaling of the mass and metabolism of skeletal muscle (*e.g.* affecting Murf-1 an myostatin expression) in C57BL/6 female

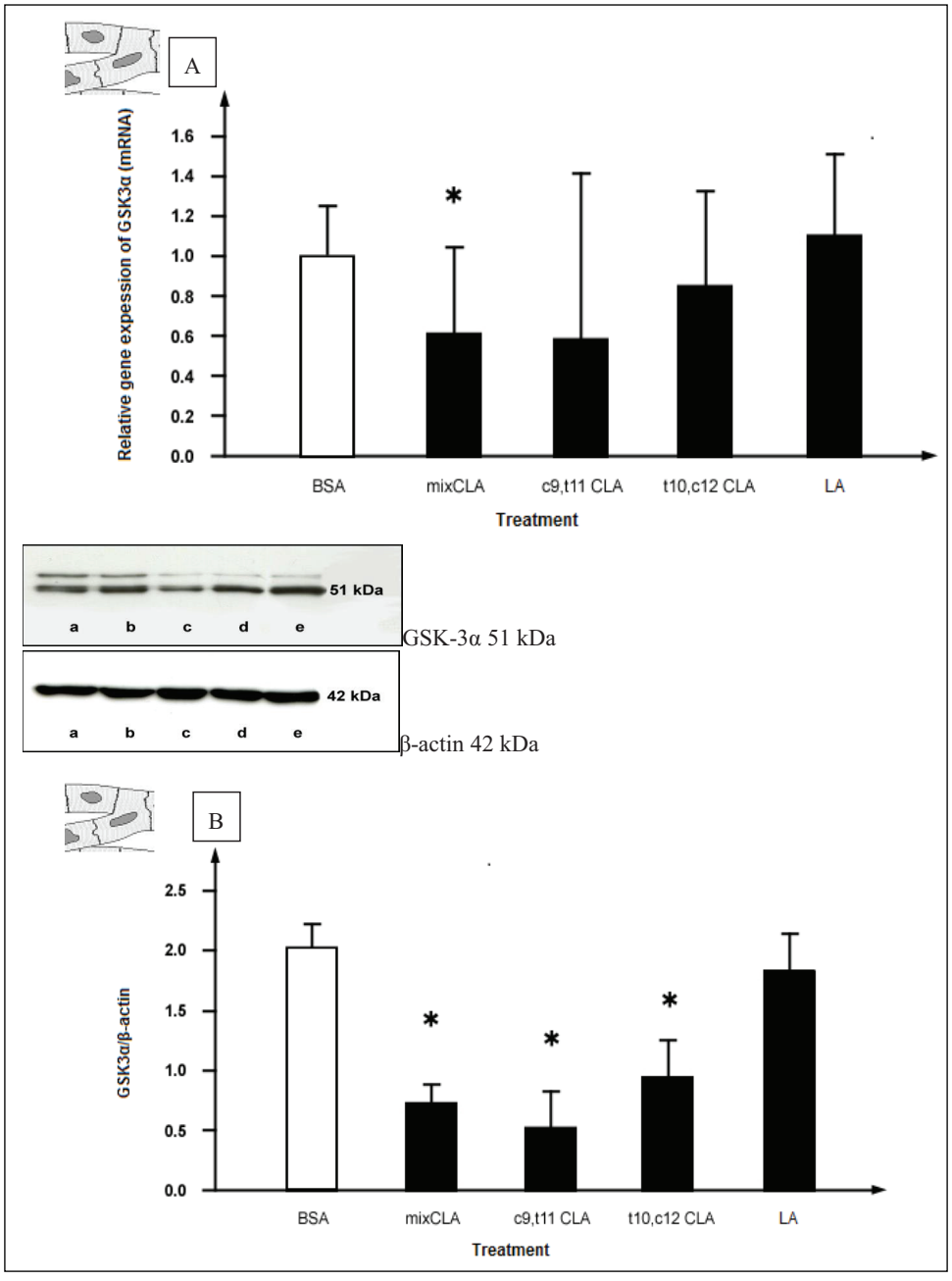

Fig. 5. Effect of conjugated linoleic acid (CLA) on GSK3α mRNA and protein expression in CCL 136 cells. The cells were cultured with CLA for 48 h and then harvested by scraping, (A) mRNA was measured using the real-time PCR method and (B) protein expression using the Western blotting method (densitometric analysis of protein normalized to β-actin; representative Western blot.
*$P \leq 0.05$ indicates statistically significant.
a - bovine serum albumin (BSA), **b** - mice fed a diet containing 5% soybean oil, 0.75% c9, t11 CLA isomer and 0.75% t10, c12 CLA isomer (mixCLA), **c** - mice fed a diet containing 5% soybean oil and 1.5% c9, t11 CLA isomer (c9,t11CLA); **d** - mice fed a diet containing 5% soybean oil and 1.5% t10, c12 CLA isomer (t10,c12CLA); **e** - linoleic acid (LA).

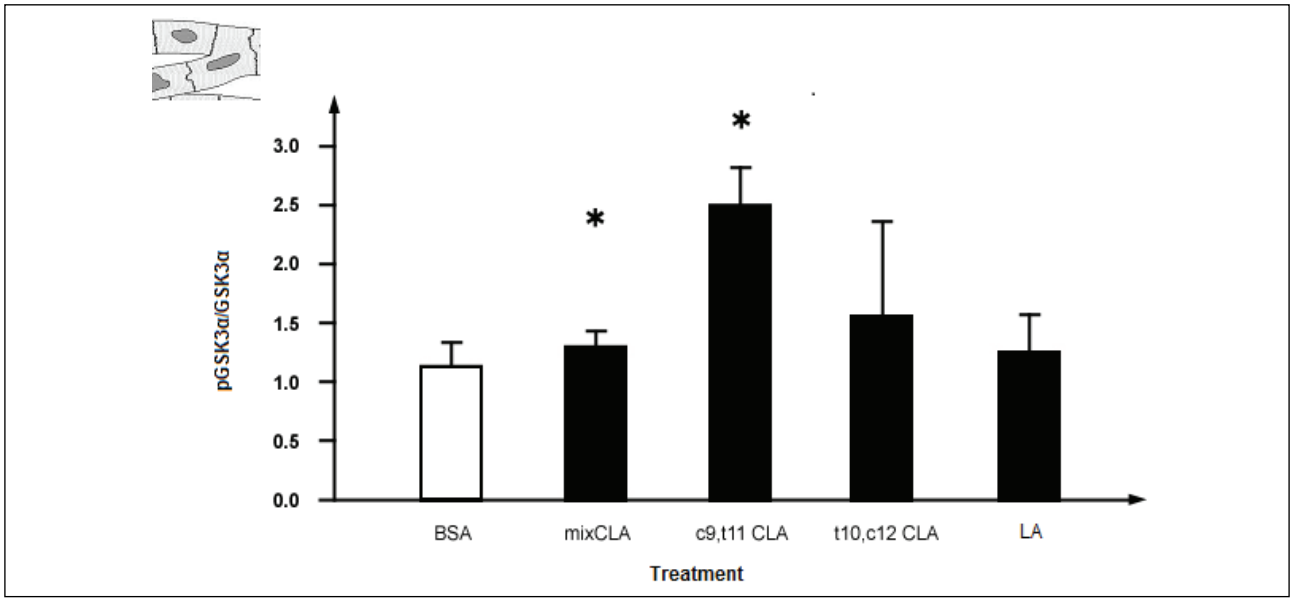

Fig. 6. Effect of conjugated linoleic acid (CLA) on GSK3α protein phosphorylation in CCL136 cells. The cells were cultured with CLA for 48 h and then harvested by scraping, GSK3α protein phosphorylation was measured by the Western blotting method (densitometric analysis of protein normalized to GSK3α; representative Western blot). *P ≤ 0.05 statistically significant.
BSA - bovine serum albumin; mixCLA - mice fed a diet containing 5% soybean oil, 0.75% c9, t11 CLA isomer and 0.75% t10, c12 CLA isomer; c9, t11 CLA - mice fed a diet containing 5% soybean oil and 1.5% c9, t11 CLA isomer; t10, c12 CLA - mice fed a diet containing 5% soybean oil and 1.5% t10, c12 CLA isomer, LA - linoleic acid.

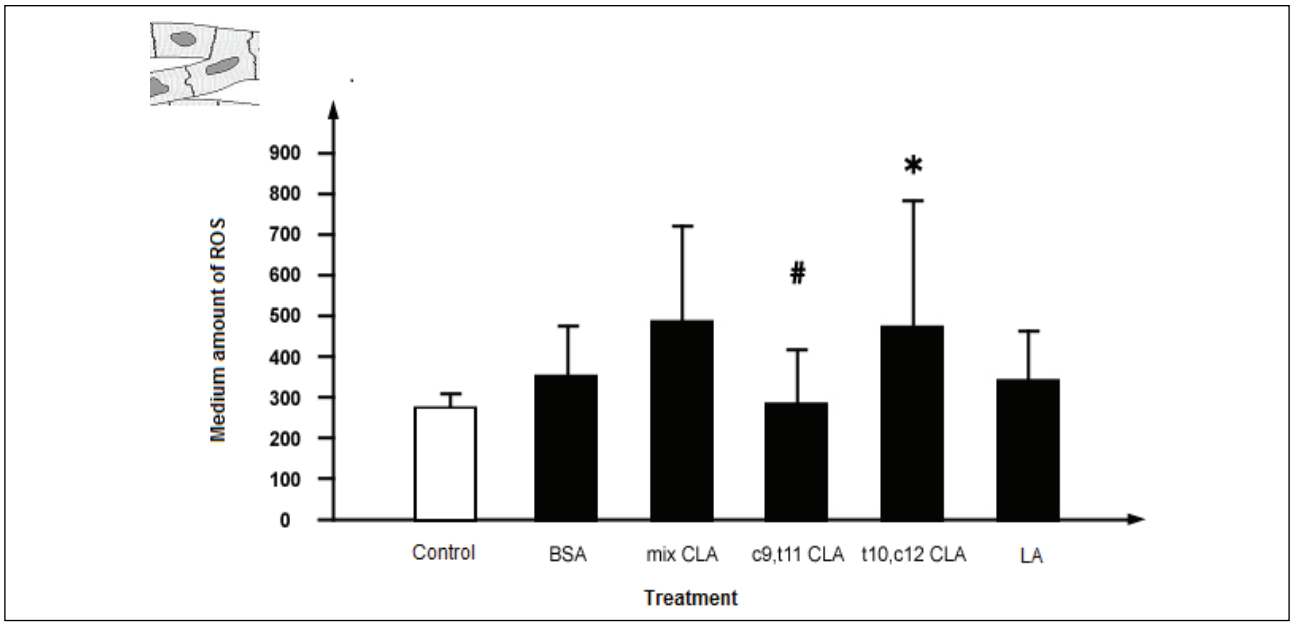

Fig. 8. Reactive oxygen species (ROS) synthesis in CCL 136 muscle cells cultured with conjugated linoleic acid (CLA). The cells were cultured with CLA for 48 h and then harvested by scraping. The ROS concentration was determined by measuring the intracellular oxidation of DCFH-DA. *P < 0.05 significant difference versus control. #P < 0.05 significant difference versus BSA.

mice. Therefore we become interested to elucidate the role of CLA on muscle metabolism in mice animal model (30).

It has been shown that CLA reduces the consumption of stored liver glycogen, which is used for energy metabolism (31). This phenomenon occurs *via* PPARδ dependent mechanisms (32), increasing the endurance capacity of mice (31). CLA supplementation, however, failed to promote the re-establishment of hepatic lipid metabolism in tumor-bearing animals, and therefore is not recommended for cancer-related cachexia (33).

Our results show that CLA changes the expression of GS *in vitro* (*Fig. 4*), without any significant effect on enzyme expression *in vivo*. In CCL 136 cells, CLA led to the reduction in the gene expression of both glycogen synthase and glycogen synthase kinase (GSK) (*Fig. 4* and *6*) without differences in the glycogen content (*Fig. 1*). Analogous effects (CLA did not affect glycogen synthesis and degradation) were previously noticed by other authors (32, 34). We noticed conflicting results between the *in vitro* and *in vivo* models. The explanation here may be insulin resistance, a reduction of free fatty acids by insulin and glucose (31), insulin-

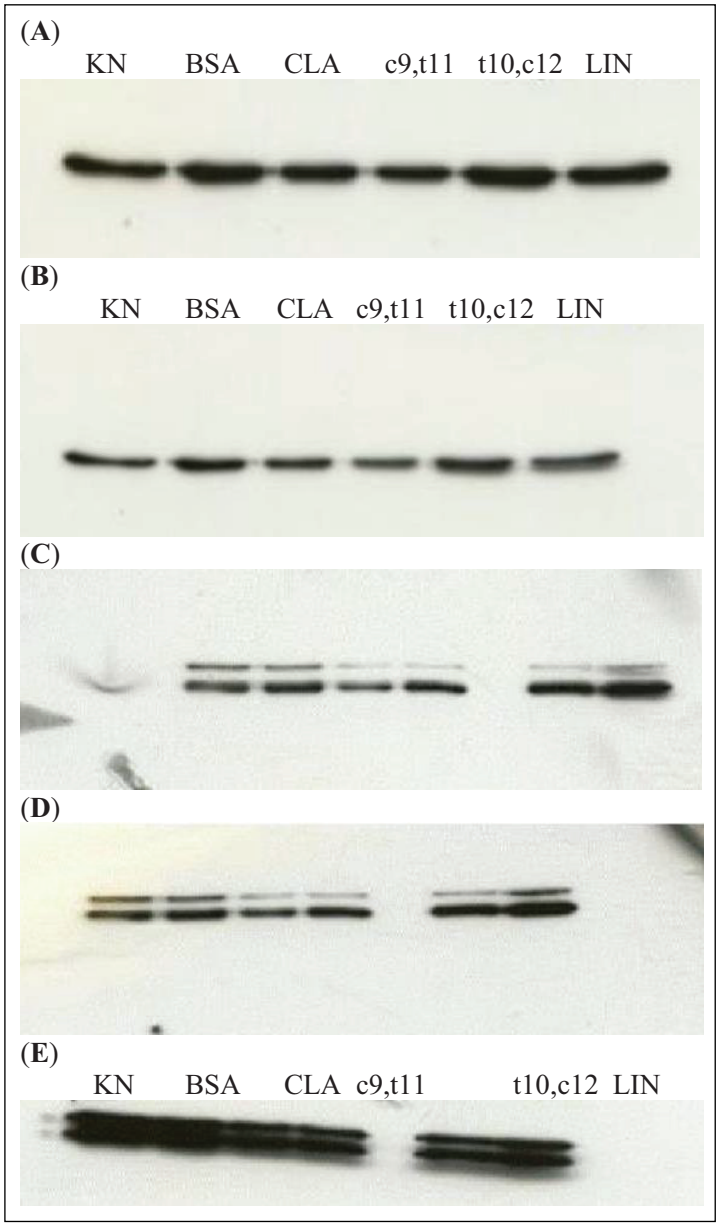

Fig. 7. Examples of the Western blot analysis results of (**A**): pGSK3α gene expression for the human myocytes cells cultured with the mixture of CLA isomers (gel concentration 10%, high glucose medium); time of exposure of the film: 15 min. (**B**): pGSK3α gene expression for the human myocytes cells cultured with the mixture of CLA isomers (gel concentration 10%, high glucose medium); time of exposure of the film: 60 min. (**C**): GSK3α normalization with β-actin (gel concentration 10%, high glucose medium); time of exposure of the film: 3 min. (**D**): GSK3α normalization with β-actin (gel concentration 10%, high glucose medium); time of exposure of the film: 5 min. (**E**): pGSK3α (gel concentration 10%, high glucose medium); time of exposure of the film: 5 min.

mediated glucose transport (36-37) as well as impaired GS activity (37). The t10, c12 CLA isomer has previously been shown to exaggerate insulin resistance (38). Potential mechanisms of insulin resistance induction may be the increase of ROS synthesis or the increase in Ca^{2+} levels (38-40). In our *in vitro* study, t10,c12 CLA significantly increased the ROS content in muscle cells (*Fig. 8*). Similar results were observed in adipocytes where t10, c12 CLA impaired lipid storage. The t10,c12 isomer enhanced fatty acid oxidation and lipolysis, coupled with diminished glucose uptake and utilization (41). On the other hand, c9, t11 CLA did not demonstrate a similar effect (42).

Another important enzyme of the glycogen pathway is kinase glycogen synthase 3α (GSK3α); the phosphorylation of GSK3α inactivates glycogen synthase and decreases glycogen synthesis. We observed higher GSK3α phosphorylation in the presence of c9, t11 CLA as well mixed CLA (*Fig. 7*). The increased phosphorylation of glycogen synthase in the presence of c9, t11 CLA has already been previously observed (41, 43). What is interesting, in contrast to what was seen for c9, t11 CLA CLA, is that the c10, t12 CLA isomer reduced glycogen utilization *in vivo* (in male 129Sv/J mice) (43). Authors showed that after 6 weeks pf treatment with 0.5% t10, c12CLA, the glycogen content was higher in the livers of mice than in control mice (44). Similar results were obtained by Maslak *et al.* (43) although both CLA isomers (c9t11 and t10c12) displayed hepatoprotective activity, the hypolipemic action of the t10c12-CLA isomer proved to be more pronounced than that of c9t11-CLA lipogenic effect. For t10c12-CLA, there were decreased concentrations of serum triacylglycerols and low-density lipoprotein and very-low-density lipoprotein (LDL + VLDL), increased high-density lipoprotein (HDL), and an altered liver lipid content and fatty acid composition due to the down regulation of liver stearoyl-CoA desaturase-1 (SCD-1) and fatty acid synthase expression (44).

The effects of CLA on glucose metabolism may affect the potential role of CLA in skeletal muscle metabolism, and effects are inconsistent in both animal and human studies (45). CLA influences muscle fiber type transformation (45-47), was shown to activate AMPK in murine skeletal muscle cells (48-49). But also CLA supplementation was found to promote endurance capacity in trained mice *via* the upregulation of testosterone biosynthesis (50).

Our *in vitro* data showed that t10, c12 isomer of CLA as well as the mix of isomers can decrease the content of glycogen in the muscle (*Figs. 2* and *3*) by the regulation of the expression of the major enzymes involved in glycogen synthase.

The potential key role of CLAs as inhibitors of glycogen synthesis provides a beneficial aspect for athletes who use CLAs as a supplement. However, more studies are needed to investigate the complex mechanism of CLA's role in inhibiting the process of glycogen synthesis (18).

Fatty acid uptake or content of the cells versus tissues was not measured and this could be a limitation of the study.

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