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Original Paper

Modest Decrease in Pgc1α Results in TAG Accumulation but not in Insulin Resistance in L6 Myotubes

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Key Words
PGC-1α • Lipids • Mitochondria • Skeletal muscle • Insulin resistance

Abstract
Background/Aims: PGC-1α is an important cellular protein (coactivator) regulating myocyte mitochondria number and function, and therefore whole cellular energy status. The aim of this work was to investigate the effects of modest, temporary PGC-1α knock-down on L6 myotubes insulin resistance in a light of cellular lipid metabolism. ***Methods:*** Gas liquid chromatography was applied for assessing FAs content and composition. For the expression of mitochondrial enzymes, as well as FA and glucose transporters, Western Blot technique was adopted. Additionally, radiolabelled glucose and palmitic acid uptake was performed to estimate the nutrients cellular influx. ***Results:*** Modest (-24%) PGC-1α protein ablation resulted in decreased mitochondrial activity in general (reduced Cyt C content) and FAs oxidation in particular (diminished β-HAD expression) without increased FAs cellular influx. The aforementioned intervention led to significantly increased TAG cellular level, but not DAG nor CER. Consequently, no changes in cellular insulin responsiveness were noticed. ***Conclusions:*** Modest (-24%) PGC-1α protein depletion results in lipid accumulation, without causing insulin resistance. Importantly, it seems that this TAG loading is a result of decreased mitochondrial oxidative capacity and/or possibly increased lipid biosynthesis but not fatty acid cellular influx.

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Introduction

Over the last decades of the XX th century, up until now, people's life have undergone great changes. Nowadays, an ordinary person has become increasingly sedentary and lacks of everyday physical activity. According to the WHO (World Health Organization)

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in a year 2010 approximately 290 million people suffered from type 2 diabetes mellitus (T2DM) constituting about 6% of world population [1]. Many authors indicate that systemic lipid imbalances are common in T2DM and it is now clear that lipotoxicity can induce dysregulation of glucose metabolism [2-5]. In particular, striated muscles seem to be vitally important in the development of this pathophysiological phenomenon, since skeletal muscle tissue responses for up to 80% of insulin stimulated postprandial glucose uptake [6]. The classical mechanism by which lipids, especially diacylglycerol (DAG) and ceramide (CER), induce insulin resistance (Fig. 1.) was described, in details, elsewhere [3, 7]. Briefly, in normal physiological conditions insulin induces intramuscular glucose uptake via incorporation of glucose transport proteins (GLUT-4) into plasma membrane. This process consist of several enzymatic reactions, particular steps encompassing: 1) insulin binding to its receptor; 2) subsequent activation of a cascade of intermediate enzymes: IRS-1, PI3K, PKB/Akt; 3) eventually leading to phosphorylation of AS160 and fusion of GLUT-4 transport vesicles with sarcolemma. As an end result facilitated glucose transport rate increases [8]. Increase in muscle diacylgycerol content may interfere and disrupt this pathway, mainly through the activation of protein kinase C (PKCε), which in turn phosphorylates, and therefore deactivates IRS-1 (Fig. 1) [3, 9]. Ceramide over accumulation, on the other hand, activates protein phosphatase 2A (PP2A) that promotes dephosphorylation and inactivation of protein kinase B (PKB/Akt) (Fig. 1). Moreover, CER can also activate c-Jun N-terminal kinases (JNKs) that in turn inhibit IRS-1 [10].

PGC-1α (peroxisome proliferator activated receptor gamma coactivator 1-alpha – PPARγ coactivator 1-α) is a protein that binds to a variety of transcriptional factors (e. g. PPARs') and therefore change the expression of genes involved in cellular metabolism [11]. Although ubiquitously expressed, its prevalence is distinctly and positively correlated with tissue metabolic activity, occurring especially rich in skeletal/heart muscle and liver [11-13]. The main role attributed to PGC-1α in myocyte is a regulation of mitochondria number and function, which in turn effects whole cellular energy status [11, 14]. Additionally to the aforementioned influence on skeletal muscle substrate utilization, some studies also indicate its possible involvement in glucose and LCFAs (long chain fatty acids) facilitated uptake and turnover [15, 16]. However, to best of our knowledge, the relationship between PGC-1α cellular expression in connection to intramyocellular lipid accumulation and insulin resistance hasn't been, so far, directly addressed. Moreover, from a posed question standpoint, some of the previous studies present few serious drawbacks [15, 16], including: 1) application of the knock-out technique (significantly decreasing PGC-1α protein content probably far beyond the state of normal physiological condition [17], long time frame effect enabling activation of cellular/system compensatory mechanisms); 2) usage of mRNA level(s) for determination of appropriate plasma membrane transporter(s) content (changes in mRNA not necessarily reflect changes in protein expression) [18]. Therefore, in an attempt to better understand and address those issues, the following study investigates the effects of modest, temporary PGC-1α knock-down on L6 myotubes insulin resistance in a light of cellular lipid metabolism.

Materials and Methods

Cell cultures

All experiments were performed on commercially available (ATCC - American Type Culture Collection) rat-derived (Rattus norvegicus) L6 skeletal muscle cells according to manufacturer recommendations [19]. Briefly, L6 cells were maintained in myoblast monolayer in high-glucose DMEM (Dulbecco's Modified Eagel's Medium) containing 10% FBS (Fetal Bovine Serum) and 1% antibiotic/antimycotic in a humidified 5% CO2 atmosphere at 37°C. Cells were cultivated to approximately 70% confluence at that time medium was replaced for DMEM containing 2% HS (horse serum) for the induction of spontaneous myoblast fusion. Differentiation medium was changed every 48h-72h. Cells were ready for further experiments after about 6-8 days (as confirmed by visual inspection using phase-contrast microscopy).

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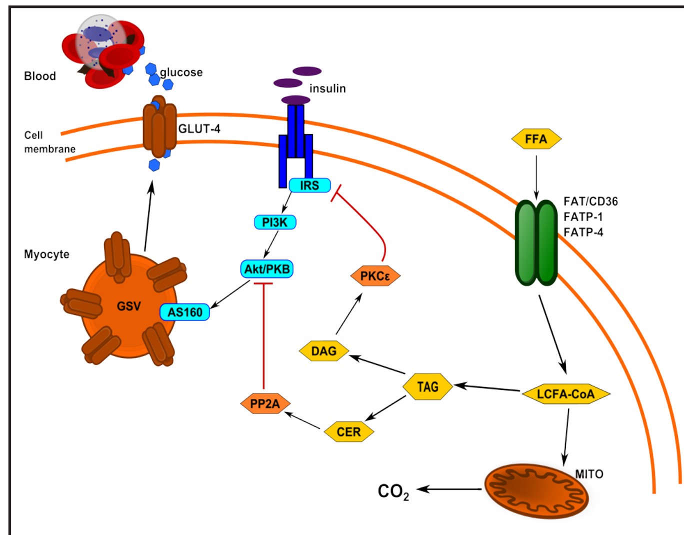


Fig. 1. Bioactive lipid intermediates and their influence on insulin signalling pathway. Akt/PKB – protein kinase B; AS160- Akt substrate of 160 kDa; CER - ceramide; DAG - diacylglycerol; FAT/CD36 – Fatty acid translocase/cluster of differentiation 36; FATP-1 – fatty acid transport protein 1; FATP-4 – fatty acid transport protein 4; FFA – free fatty acids; GSV – GLUT-4 storage vesicle; IRS – insulin receptor substrate; LCFA-CoA – long chain fatty acid acyl-CoA; MITO - mitochondria; PI3K –phosphatidylinositol-4,5-bisphosphate 3-kinase; PKCε – protein kinase C epsilon; PP2A – protein phosphatase 2A; TAG – triacylglycerol.

Gene silencing (siRNA)

Reduction of PGC-1α gene expression was performed using gene silencing method according to manufacturer protocol (Lifetechnologies, former Invitrogen) [20]. The day before transfection cells (L6 myotubes) were transferred to growth medium without antibiotics. Myotubes grown in 6-well culture plates were transfected with 100 nM siRNA (for PGC-1α) or negative-control (noncoding/non-targetting siRNA) with Opti-MEM® I Reduced Serum Medium in DMEM without antibiotics. Lipofectamine mixture was removed after 4-6 h incubation. Then the cells were incubated at 37°C in a 5% CO_2 atmosphere for ~48 hours until assessment of gene knockdown was performed. Three commercially available sequences for PGC-1α were purchased (GGGCAGAUUUGUUCUUCCACAGAUU, ACGAGAGGCUGAAGAGGGAAGAAUA, CCCAUUUGAGAACAAGACUAUUGAA) from which for further experiments one with the highest desired efficiency was chosen.

Real-time PCR

PGC-1α mRNA level was measured with the use of real-time quantitative PCR. The RNA was extracted from cultured myotubes grown in 6-well plates using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Following RNA purification, DNase treatment (Ambion) was performed to ensure that there was no contamination of genomic DNA. Extracted RNA was solubilized in RNase-free water. The quality of RNA was assessed by measuring absorbencies at 260 and 280 nm and verified by running the agarose electrophoresis with ethidium bromide. The RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad). Primers were designed using Beacon Designer Software (Premier Biosoft). Real-time quantitative PCR was performed with SYBR Green JumpStart Taq ReadyMix (Sigma) using a Bio-Rad Chromo4 system. PCR efficiency was examined by serially diluting the template cDNA, and a melt curve was performed at the end of each reaction to verify PCR product specificity. A sample containing no cDNA was used as a negative control to verify the absence of primer dimers. The results were normalized to cyclophilin A expression measured in each sample.

Primer sequences: PGC-1α: 1) F: 5'-ACA GAC ACC GCA CAC ATC GC -3'; R: 5'-GCT TCA TAG CTG TCA TAC CTG GGC -3'; 2) F: 5'-ATG AAG CCA ATG AGC ACG AAA GG -3'; R: 5'-ATC ACA CGG CGC TCT TCA ATT GC -3'; 3) F: 5'-TCT CGA CAC AGG TCG TGT TCC C -3'; R: 5'-TTT CGT GCT CAT TGG CTT CAT AGC -3'. Cyclophilin A: 1) F: 5'-TGT CTC TTT TCG CCG CTT GCT -3'; R: 5'- CAC CAC CCT GGC ACA TGA ATC C -3'; 2) F: 5'-GTC AAC CCC ACC GTG TTC TTC G -3'; R: 5'-TGT GAA GTC ACC ACC CTG GCA C-3'

Western Blot

For the detection of protein content routine Western blotting procedures were applied, as described previously [7, 21]. Briefly, after lysis in ice-cold RIPA buffer (1% NP-40, 0,25% Na-deoxycholate, 150 M NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1μg/ml aprotinin, 1 μg/ml

leupeptin, 1 μg/ml pepstatin 1 mM sodium orthovanadate, 1 mM sodium fluoride) cells were sonicated for 1 min at 4°C. BCA protein assay kit with bovine serum albumin as a standard was used for protein content assessment. Samples were boiled at 95°C for 10 minutes in sample buffer containing 2-mercaptoethanol. The homogenate (60 μg) was subjected to 10% SDS-PAGE and transferred to PVDF membranes, followed by blocking membranes in TTBS buffer (130 mM NaCl, 50 mM Tris-HCl, and 0,05 % Tween-20) containing 5% nonfat dry milk for 1.5 h at room temperature. The membranes were then immunobloted with the corresponding antibodies (Cell Signalling Technology, Novus Biologicals) at a dilution of 1:1000. Primary antibodies included: PGC-1α, Cyt C, β-HAD, HADHSC, CS, COX IV, FASN, pAMPK, AMPK, pACC, ACC, pAkt, Akt, FAT/CD36, FATP-1, 4, GLUT-1, GLUT-4, β-tubulin (for standarization). In the next step membranes were incubated with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:3000; Santa Cruz Biotechnology, USA). Proteins were visualized by using an enhanced chemiluminescence substrate (Thermo Scientific, USA) and quantified by densitometry (Biorad, USA). Ponceau staining technique were used to confirm equal protein loading on the blot membrane. Protein expression (expressed in Optical Density Arbitrary Units) was normalized to β-actin. Control was set at 100 and the experimental group(s) were expressed relative to the control.

2-[3H]-deoxyglucose uptake

The glucose uptake was conducted to assess cellular insulin responsiveness (sensitivity) [7]. Briefly, before determining glucose uptake cells, grown in a 12-well plates, were starved (incubation in DMEM w/o serum) for 3h. Then wells were washed three times with KRB buffer (140 mM NaCl, 20 mM Hepes, 5 mM KCl, 2,5 mM MgSO4, 1 mM CaCl2) and exposed to 100 nM insulin (Novorapid) for 20 min. Subsequently, 10 min incubation with 0,5 mM 2-deoxyglucose containing 1 μCi/ml [3H]-2-deoxyglucose was performed. Nonspecific tracer binding was determined by quantitating cell-associated radioactivity in presence of 10 μM cytochalasin B. Next, after medium removal, cells were rinsed three times with ice-cold PBS buffer. Cells underwent solubilization in 0,05 N NaOH. The resulting fluid was placed in 5 ml vials and taken for liquid scintillation counting. Radioactivity was normalized concerning protein concentration.

[9,10-3H(N)]- palmitic acid uptake

Palmitic acid uptake was performed as described in Chavez [22]. Briefly, L6 myotubes were incubated with incubation medium containing palmitic acid (Sigma-aldrich) bound to bovine serum albumin [fatty-acid free] (Sigma-aldrich) with addition of radiolabelled [9,10-3H] palmitic acid (Perkin Elmer) [1 μCi mL-1]. After 5 min ice-cold PBS was added to terminate palmitate uptake and cells were immediately solubilized in NaOH (0.05 N). The resulting fluid was placed in 5 ml vials and taken for liquid scintillation counting (Beckman). Radioactivity was normalized concerning protein concentration.

Lipid analysis

The lipids were extracted from L6 myotubes using modified Bligh and Dyer method [23]. After extraction, the lipids were separated by thin-layer chromatography (TLC) into specific fractions of ceramid (CER), TAG and DAG [24]. After lipids separation, fatty acids, together with methylpentadecanoic acid (Sigma), used as an internal standard, were transmethylated in 14% methanolic solution of boron trifluoride at 100°C for 90 min. The samples were cooled to room temperature, and 1 mL of pentane and 0.5 mL of water were added. After centrifugation, the upper phase was dried under nitrogen. Individual fatty acid methyl esters, present in each fraction, were quantified according to the retention times of standards by gas liquid chromatography (Hewlett-Packard 5890 Series II gas chromatograph, HP-INNOWax capillary column; Agilent Technologies, Santa Clara, CA, USA). Total amount of CER, DAG and TAG was estimated as the sum of the particular fatty acid species in the assessed fraction and expressed in nanomoles per mg of the protein.

Statistical analysis

All presented data are expressed as mean ± SD. Statistical significance level was set at $\alpha = 0.05$. Normal distribution of data was tested using Shapiro-Wilk normality test. Homogeneity of variance was tested using Fligner-Killeen test. Statistical differences between groups were tested using one-way analysis of variance (ANOVA) approach and an appropriate post hoc test (Tukey HSD). Where appropriate (e.g. lack of normal distribution and/or homogeneity of variance) Kruskall-Wallis test and post-hoc pairwise Wilcoxon test (with Holm-Bonferroni correction) were applied.

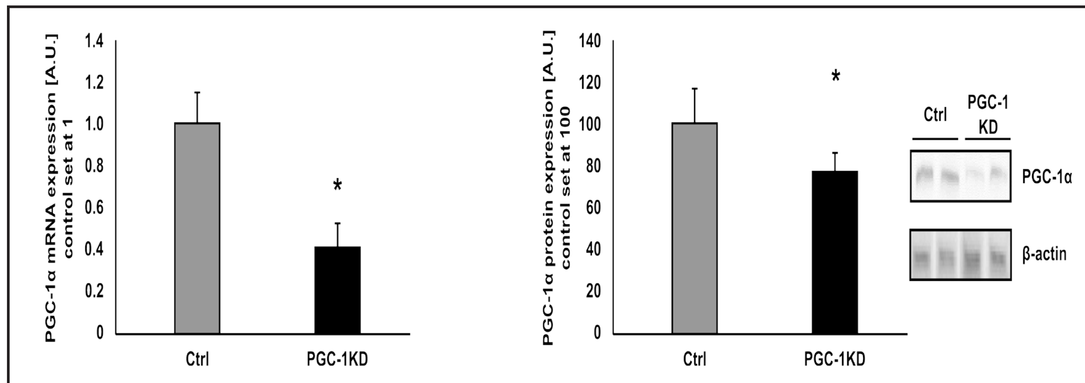


Fig. 2. PGC-1α mRNA and protein expression; n (per group) = 6; * - difference vs. ctrl ($p < 0.05$).

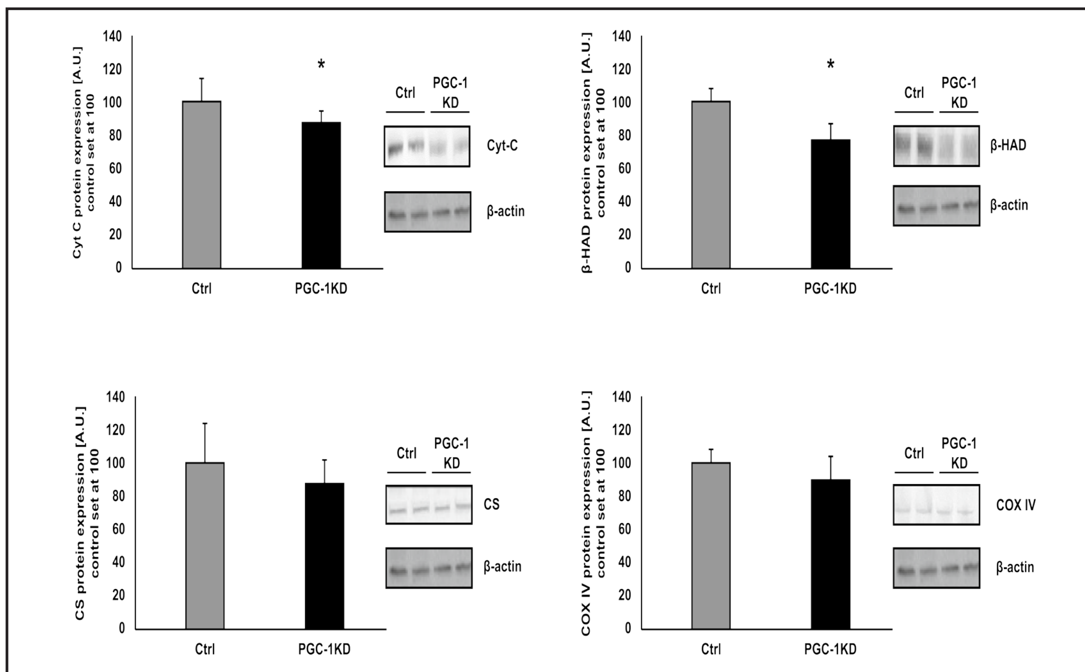


Fig. 3. Total expression of mitochondrial proteins; n (per group) = 6; * - difference vs. ctrl ($p < 0.05$).

Results

Prior to all experimental procedures we evaluated the effectiveness of our mRNA silencing by measuring both mRNA and protein expression of PGC-1α. Forty eight hours after transfection the viability of L6 myotubes was over 80% and PGC-1α mRNA expression was reduced by ~60%, and at the same time protein expression of PGC-1α was decreased by ~24% (Fig 2.). In selected, sets PGC-1α protein expression was checked all over the study period.

Effects of PGC-1α gene silencing on the expression of mitochondrial proteins in L6 myotubes

In the following study we assessed expression level of selected mitochondrial proteins: 1) involved in the function of mitochondrial electron transport chain (Cytochrome C and COX IV); 2) a key, pace-making enzyme of TCA and marker of the intact mitochondria (Citrate synthase); 3) involved in fatty acids β-oxidation (β-HAD and HADHSC). As depicted in Figure 3, modest (-24%) PGC-1α protein down regulation resulted in statistically significant reduction

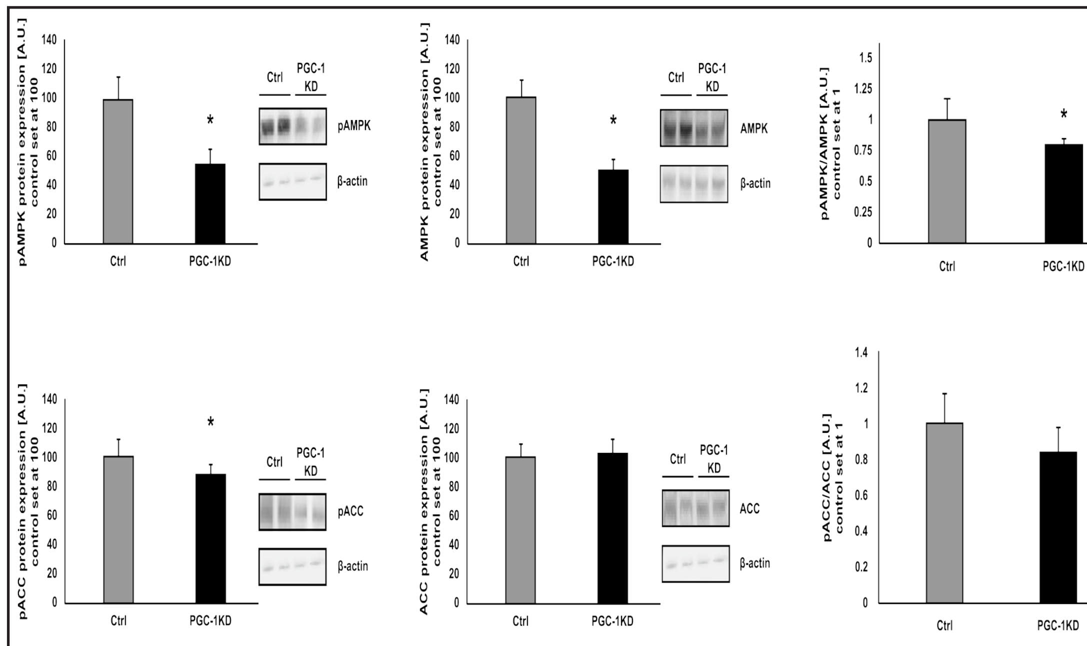


Fig. 4. Total protein expression of: AMPK, pAMPK, pAMPK/AMPK, pACC, ACC, pACC/ACC ratio; n (per group) = 6; * - difference vs. ctrl ($p < 0.05$).

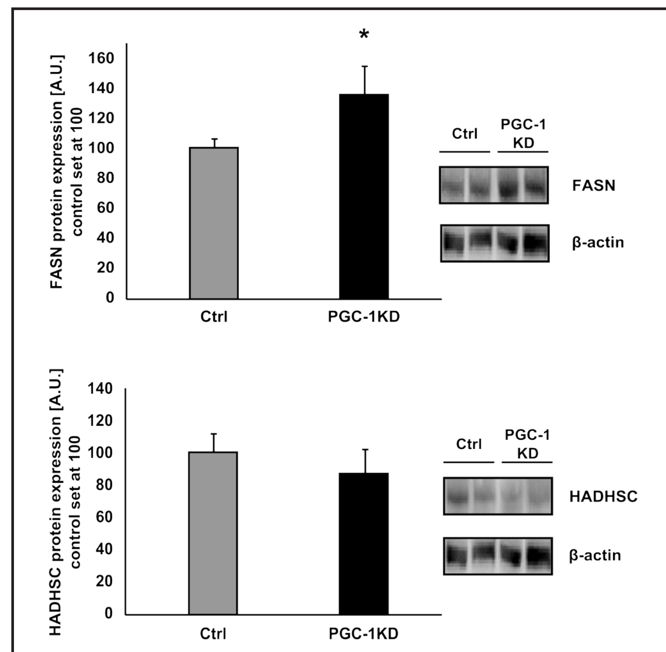


Fig. 5. Protein content of FASN and HADHSC; n (per group) = 6; * - difference vs. ctrl ($p < 0.05$).

of Cyt C and β-HAD expression (-16% and -19% respectively, $p < 0.05$), but not CS (-14%, $p > 0.05$), nor COX IV (-10%, $p > 0.05$) in comparison with control group (Fig. 3). In addition to diminished β-HAD level, also a declining trend (-15%, $p > 0.05$) for HADHSC protein was observed (Fig. 5). Furthermore, we have evaluated the expression of AMPK, pAMPK, ACC and pACC. Both, the cellular AMPK and pAMPK content turned out to be decreased (-50% and -60%, respectively, $p < 0.05$) in PGC-1 KD group. This resulted in a modest (-20%, $p < 0.05$) reduction of AMPK activity (as measured by pAMPK/AMPK ratio, Fig. 4). Consequently, the content of pACC was slightly diminished (-11 %, $p < 0.05$), however pACC/ACC ratio (a measure of ACC enzyme activity) was not changed (+15%, $p > 0.05$) (Fig. 4).

Fig. 6. Intracellular lipid content; n (per group) = 8; * - difference vs. ctrl ($p < 0.05$).

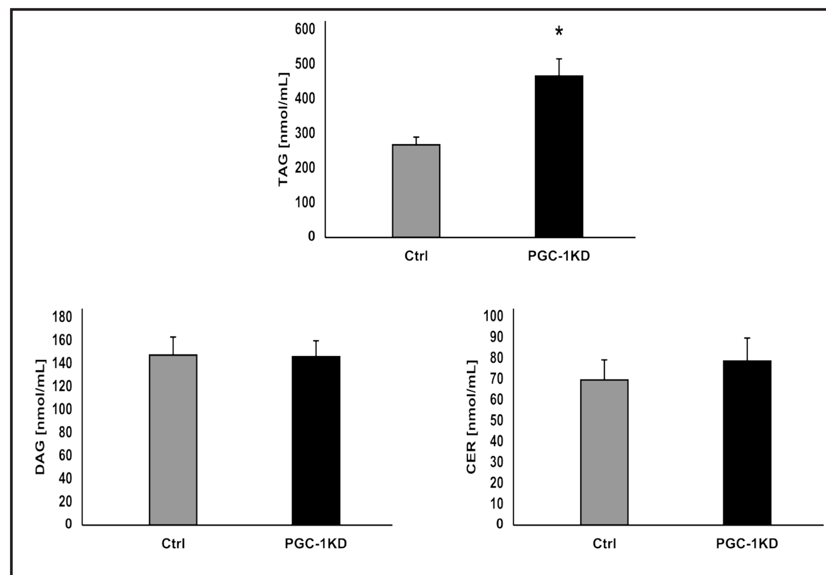


Table 1. TAG composition

TAG composition [nmol/mL]		
Fatty acid	Control	PGC-1KD
Myristic acid ($C_{14}H_{28}O_2$) – C14:0	51.24 ± 5.105	91.05 ± 2.888*
Palmitic acid ($C_{16}H_{32}O_2$) – C16:0	79.46 ± 8.095	153.75 ± 17.891*
Palmitoleic acid ($C_{16}H_{30}O_2$) – C16:1	29.27 ± 4.285	67.22 ± 9.501*
Stearic acid ($C_{18}H_{36}O_2$) – C18:0	34.23 ± 6.477	40.66 ± 4.479
Oleic acid ($C_{18}H_{34}O_2$) – C18:1	38.03 ± 4.911	64.13 ± 9.766*
Linoleic acid ($C_{18}H_{32}O_2$) – C18:2	6.69 ± 1.35	12.75 ± 2.858*
Arachidic acid ($C_{20}H_{40}O_2$) – C20:0	1.59 ± 0.829	1.72 ± 0.294
Linoleic acid ($C_{18}H_{30}O_2$) – C18:3	4.65 ± 0.746	6.92 ± 1.222*
Behenic acid ($C_{22}H_{44}O_2$) – C22:0	0.98 ± 0.318	1.35 ± 0.498
Arachidonic acid ($C_{20}H_{32}O_2$) – C20:4	1.99 ± 0.269	3.14 ± 0.483*
Lignoceric acid ($C_{24}H_{48}O_2$) – C24:0	8 ± 1.605	11.57 ± 2.656*
Eicosapentaenoic acid ($C_{20}H_{30}O_2$) – C20:5	0 ± 0	0 ± 0
Nervonic acid ($C_{24}H_{46}O_2$) – C24:1	0 ± 0	0 ± 0
Docosahexaenoic acid ($C_{22}H_{32}O_2$) – C22:6	12.39 ± 1.403	13.95 ± 2.767
SUM	268.52 ± 20.864	468.21 ± 48.08*

Table 2. CER composition

	CER composition [nmol/mL]	
Fatty acid	Control	PGC-1KD
Myristic acid ($C_{14}H_{28}O_2$) – C14:0	4.29 ± 2.04	5.5 ± 0.317
Palmitic acid ($C_{16}H_{32}O_2$) – C16:0	22.49 ± 1.916	30.92 ± 4.703*
Palmitoleic acid ($C_{16}H_{30}O_2$) – C16:1	1.63 ± 0.251	1.74 ± 0.214
Stearic acid ($C_{18}H_{36}O_2$) – C18:0	21.40 ± 3.577	19.40 ± 3.661
Oleic acid ($C_{18}H_{34}O_2$) – C18:1	5.35 ± 0.342	5.61 ± 0.594
Linoleic acid ($C_{18}H_{32}O_2$) – C18:2	2.99 ± 0.859	3.61 ± 0.868
Arachidic acid ($C_{20}H_{40}O_2$) – C20:0	1.52 ± 0.353	1.31 ± 0.252
Linoleic acid ($C_{18}H_{30}O_2$) – C18:3	0.9 ± 0.08	0.74 ± 0.181
Behenic acid ($C_{22}H_{44}O_2$) – C22:0	1.75 ± 0.246	1.85 ± 0.187
Arachidonic acid ($C_{20}H_{32}O_2$) – C20:4	0.82 ± 0.115	0.73 ± 0.065
Lignoceric acid ($C_{24}H_{48}O_2$) – C24:0	1.68 ± 0.252	1.73 ± 0.336
Eicosapentaenoic acid ($C_{20}H_{30}O_2$) – C20:5	0 ± 0	0 ± 0
Nervonic acid ($C_{24}H_{46}O_2$) – C24:1	3.3 ± 0.201	4.75 ± 0.651*
Docosahexaenoic acid ($C_{22}H_{32}O_2$) – C22:6	1.91 ± 0.522	1.48 ± 0.191
SUM	70.04 ± 9.690	79.37 ± 10.730

Effects of PGC-1gene silencing on 1) the expression of fatty acid transport proteins; 2) palmitate uptake and 3) intramyocellular lipid content

Next, we determined the consequences of modest PGC-1α depletion on cellular lipid content and FAs composition as well as the expression of major fatty acid transport

Table 3. DAG composition

	DAG composition [nmol/mL]	
Fatty acid	Control	PGC-1KD
Myristic acid ($C_{14}H_{28}O_2$) – C14:0	27.45 ± 1.544	23.4 ± 0.557*
Palmitic acid ($C_{16}H_{32}O_2$) – C16:0	44.56 ± 2.484	40.48 ± 5.403
Palmitoleic acid ($C_{16}H_{30}O_2$) – C16:1	7.29 ± 0.364	11.47 ± 0.875*
Stearic acid ($C_{18}H_{36}O_2$) – C18:0	43.37 ± 7.007	45.97 ± 6.73
Oleic acid ($C_{18}H_{34}O_2$) – C18:1	10.4 ± 1.654	10.68 ± 0.445
Linoleic acid ($C_{18}H_{32}O_2$) – C18:2	6.50 ± 0.693	5.87 ± 0.694
Arachidic acid ($C_{20}H_{40}O_2$) – C20:0	1.75 ± 0.519	1.18 ± 0.259
Linoleic acid ($C_{18}H_{30}O_2$) – C18:3	1.00 ± 0.13	1.10 ± 0.096
Behenic acid ($C_{22}H_{44}O_2$) – C22:0	0.74 ± 0.213	0.65 ± 0.382
Arachidonic acid ($C_{20}H_{32}O_2$) – C20:4	2.56 ± 0.243	3.28 ± 0.247
Lignoceric acid ($C_{24}H_{48}O_2$) – C24:0	0.34 ± 0.672	0.30 ± 0.216
Eicosapentaenoic acid ($C_{20}H_{30}O_2$) – C20:5	0.74 ± 0.361	0.94 ± 0.214
Nervonic acid ($C_{24}H_{46}O_2$) – C24:1	0.59 ± 0.181	0.32 ± 0.248
Docosahexaenoic acid ($C_{22}H_{32}O_2$) – C22:6	1.01 ± 0.597	0.75 ± 0.107
SUM	148.36 ± 14.454	146.41 ± 13.634

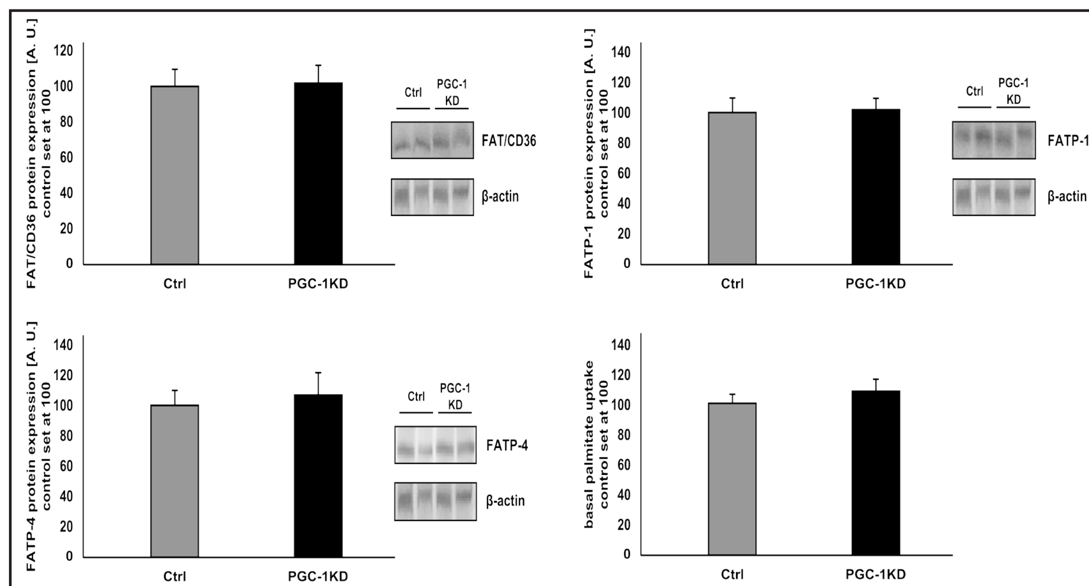


Fig. 7. Total protein expression of fatty acid transporters [n (per group) = 6; * - difference vs. ctrl (p < 0.05)] and basal radiolabelled palmitate uptake [n (per group) = 8; * - difference vs. ctrl (p < 0.05)].

proteins. We noticed that only TAG content was significantly increased in PGC-1α knock-down group (+74%, $p < 0.05$). Interestingly, neither DAG (-2%, $p > 0.05$) nor CER (+13%, $p > 0.05$) amount changed in comparison with control group (Fig. 6). However, we observed significant changes in FAs composition in TAG favouring an increase in unsaturated FAs (e.g. palmitoleic, oleic and linoleic acids) in PGC-1KD group (Table 1). There was also an increase in the amount of palmitic acid in CER and myristic and palmitoleic acids in DAG group (Table 2 and Table 3).

PGC-1α gene silencing was accompanied by virtually no changes in FAT/CD36 (+2 %, $p > 0.05$), nor FATP-1 and FATP-4 (+4% and +7% respectively, $p > 0.05$) protein expression (Fig. 7). Lack of significant changes in FA transport proteins expression was accompanied by constant palmitate transport rate, since we found no differences between studied groups in palmitate uptake assessment (+9%, $p > 0.05$) (Fig. 7).

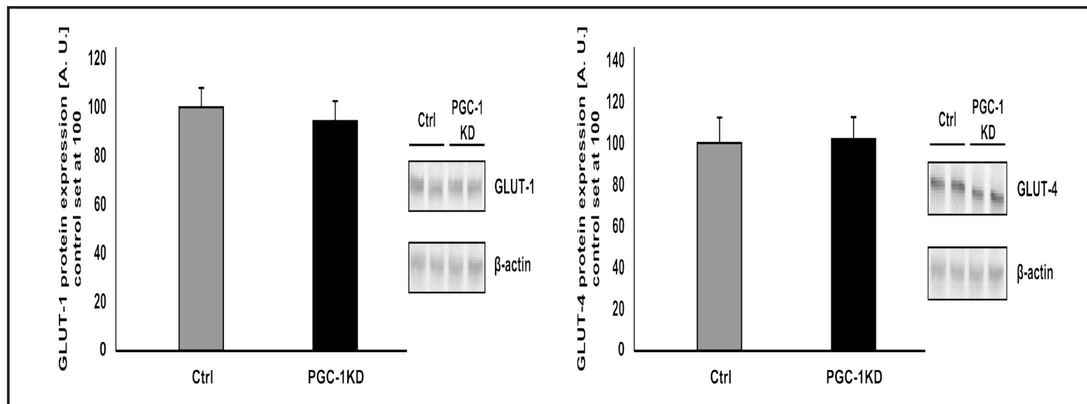
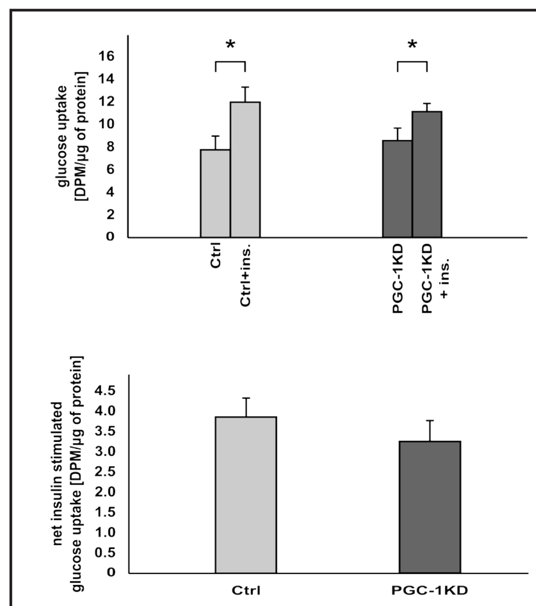


Fig. 8. Total protein expression of glucose transporters; n (per group) = 6; * - difference vs. ctrl ($p < 0.05$).

Fig. 9. Basal, insulin stimulated and net insulin stimulated glucose uptake; n (per group) = 8; * - $p < 0.05$.



Effects of PGC-1α gene silencing on 1) the expression of glucose transport proteins and 2) insulin stimulated glucose uptake

Consequently, we examined L6 myocyte cells insulin sensitivity via insulin stimulated radio-labelled glucose uptake, together with the expression of glucose transporters (GLUT-1, GLUT-4). Modest PGC-1α protein down regulation (-24%) had essentially no effect on GLUT-1 and GLUT-4 protein expression (-4% and +3%, respectively, $p > 0.05$), and only a trend (-15%, $p > 0.05$) toward diminished glucose uptake was observed (Fig. 8, Fig. 9). Moreover, also Akt and pAkt (Ser473) proteins content were evaluated. It seems that PGC-1α ablation has led to, at least partial, inhibition of insulin signalling pathway which was reflected by decrement in pAkt/Akt ratio (-58%, $p < 0.05$; Fig. 10).

Discussion

In the literature elevated intramyocellular lipid content is frequently presented as a marker of skeletal muscle insulin resistance [8, 25]. In the present study we examined the effects of modest (-24% of protein content), transient PGC-1α gene silencing on skeletal muscle insulin resistance. Previously performed gain and loss of function studies indicate

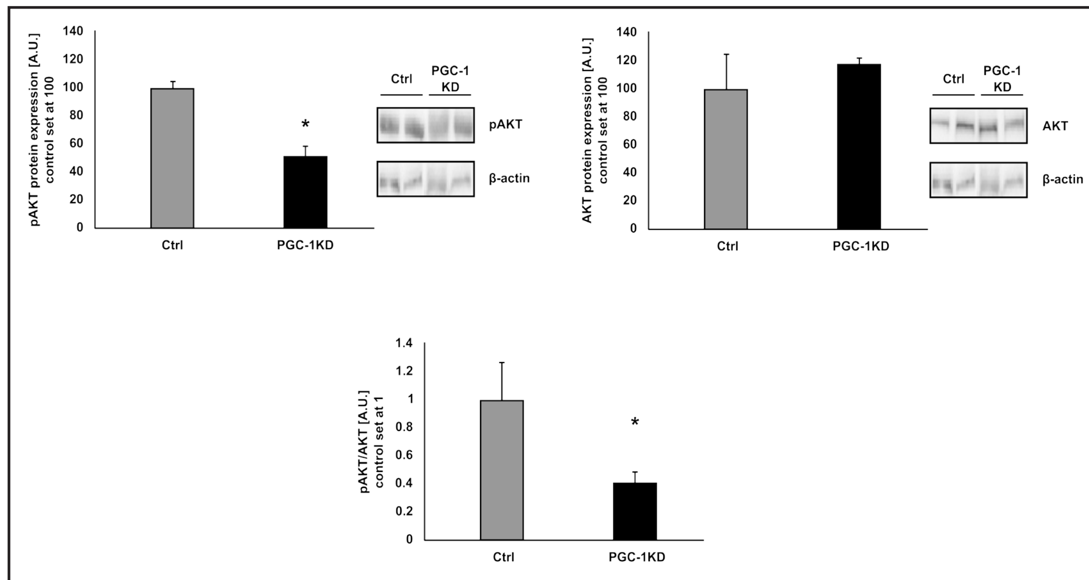


Fig. 10. Protein expression of pAkt, Akt and pAkt/Akt ratio; n = 6 (per group); * - difference vs. ctrl ($p < 0.05$).

that PGC-1α can influence not only the expression of genes regulating lipid oxidation (CPT-1, MCD) [26], but also lipid storage (FAS, DGAT-1) [15]. Nevertheless, it seems that most authors perceive its net effect to be oxidation driving [11, 13, 26]. Therefore, our initial assumption was that, since PGC-1α is considered to be a key regulator of mitochondrial biogenesis and function [11], its depletion will lead to a reduced FAs oxidation rate, resulting in subsequent lipid accumulation and diminished insulin responsiveness.

In accordance with this notion, mitochondrial proteins analysis revealed that in PGC-1KD group, both Cytochrome C (-16%, $p < 0.05$) and β-HAD (-19%, $p < 0.05$) protein expression were significantly decreased, indicating a trend towards reduced mitochondrial oxidation capacity in general, and FAs in particular (β-HAD is a key enzyme of β-oxidation) [27]. Also the expression and activity of cytosol protein AMPK, cellular master switch between anabolic and catabolic processes, was diminished by ~20% (pAMPK/AMPK, $p < 0.05$) after PGC-1α knock down. It is well known that upon its activation, AMPK increases skeletal muscle fatty acid oxidation and glucose uptake [16, 28]. Interestingly, also pAkt/Akt level was significantly decreased (-58%, $p < 0.05$) which could indicate some disturbances in insulin signalling pathway. This change, however, seemed to have only moderate effect on glucose transport, since in our study, we found only a trend (-15%, $p > 0.05$) in 2-[3H]-deoxyglucose uptake between two studied groups. Moreover, further analysis of two main muscular glucose transporter isoforms GLUT-1 and GLUT-4 protein expression, have shown no changes whatsoever. The aforementioned state was quite unexpected, since studies on transgenic mice (whole body PGC-1α overexpression) show rather improved muscular glucose uptake [15, 29] and/or increased GLUT-4 mRNA expression [15]. However, study by Summermatter [30] indicates that this is the case only in physically active transgenic mice and not in their sedentary counterparts.

Additionally, we have evaluated ACC activity. Acetyl-CoA carboxylase (ACC) is an enzyme that in its active unphosphorylated form catalyses conversion of acetyl-CoA to malonyl-CoA - an important substrate for the biosynthesis of fatty acids. Upon ACC phosphorylation by AMPK phospho-acetylCoA (pACC) is created, this in turn results in diminished production of malonyl-CoA which translates to lower FAs formation. Consequently, in accordance with previously mentioned AMPK protein reduction, PGC-1KD group had diminished (-11 %, $p < 0.05$) pACC but not ACC (+5 %, $p > 0.05$) content, and only a trend (-15 %, $p > 0.05$) in pACC/ACC ratio (a measure of ACC activity) was observed. This is another novel finding of our

study, since to best of our knowledge this is the first time when authors examine the effects of PGC-1α gene silencing on ACC activity, although some other articles examining PGC-1α/ACC interrelationship do exist [31]. In conclusion, it seems that, according to our initial assumptions, PGC-1α gene silencing significantly affected cellular catabolic processes as reflected by decreased expression of mitochondrial oxidative enzymes. Moreover, this effect concerns not only general mitochondrial oxidative capacity (Cyt C), but also FAs utilization respectively (β-HAD, HADHCS). Less clear is though PGC-1α influence on fatty acid synthesis. Firstly, we found a trend towards diminished pACC/ACC ratio (15%, $p > 0.05$), however FASN protein expression was significantly enhanced (+35%, $p < 0.05$). Secondly, this modest PGC-1α ablation (-24 %) had no direct (unchanged GLUT-1 and GLUT-4 transporters expression), nor indirect (radiolabelled deoxyglucose uptake) influence on glucose transport. Therefore, we could speculate that more pronounced change in PGC-1α level, would lead to more distinctive changes towards lipid synthesis/accumulation.

To further evaluate the effects of modest PGC-1α depletion we evaluated L6 myotubes intracellular lipid milieu. We narrowed our research to three main lipid fractions, namely TAG, CER and DAG, all implicated in the development of insulin resistance [2, 8, 10]. We found that among evaluated fractions only triacylglycerol (TAG) content was markedly (+74%) increased. This finding is in accordance with previously noticed preserved cellular insulin responsiveness, since so far direct linkage with IR has only been established for diacylglycerol and ceramide [8, 9]. Over accumulation of triacylglycerol is frequently considered to be a marker of skeletal muscle IR present in obesity and T2DM. However, it also occurs in muscles of trained athletes that are highly insulin sensitive and because of this „athlete's paradox", researchers claim that its accumulation is relatively safe physiological phenomenon without direct link to skeletal muscle lipotoxicity [25]. On the other hand deleterious influence of CER and DAG (via their intermediates: PP2A, JNKs and PKCε, respectively) accumulation on insulin signalling pathway has been well documented [9, 10]. Therefore, it seems that modest PGC-1α protein ablation leads to diminished cellular FAs oxidation, which in turn causes their enhanced deposition into a cytosol triacylglycerol fraction. However, since there are no changes in cellular DAG or CER content, we may postulate that this fatty acids utilisation impairment was not sufficiently severe to exceed cellular TAG "buffer system" capacity. Furthermore, we have also observed an increase in unsaturated fatty acids (UNSAT) moieties content in TAG fraction. This seems to be consistent with previously reported reduction of mitochondrial oxidative capacity, since normally these organelles utilize UNSAT more efficiently than their saturated counterparts [22]. Therefore, one would expect that reduced mitochondrial activity, subsequent to PGC-1α protein ablation, will manifest itself in UNSAT accretion.

Additionally to those findings, we measured FA transporters expression (FAT/CD36, FATP-1 and FATP-4) in order to gain some insights concerning fatty acid flux. Interestingly, we observed that total protein content was not changed in any of this fatty acid transporters. This could indicate maintenance of cellular status quo, i.e. normal constant FAs influx into the cell. Although our findings (increased TAG, but not CER nor DAG content, together with unchanged FAT/CD36 expression) still seem to be coherent, then there are some discrepancies with available literature data. Liang et al. [29] showed that in the case of PGC-1α transgenic mice overexpression of this coactivator was associated with marked decrease (~ -50%) in both DAG and CER content, whereas Summermatter [15] reported ~ 2 fold increase in mRNA content for fatty acid transport proteins (FAT/CD6, FABPpm, FATP-4) in transgenic animals. Based on these data one would rather expect an opposite results (increase in CER and DAG accompanied by decrement in FAT/CD36 content) to those observed by us. It is hard to explain those discrepancies, although we must keep in mind the methodological differences between our approach and the one aforementioned. In both of those cases we have whole body PGC-1α overexpression, whereas the word "modest" in their context means ~ +2 fold [29]. Additionally, it is important to notice that mRNA level for FA transporters do not always mirror this of protein content [18], therefore mRNA level may be inadequate indicator of cellular fatty acid transport proteins content. Furthermore, we must remember that LCFAs

cellular uptake, similarly to glucose transport, is a process relying on a translocation of FA transporters to plasma membrane. Interestingly, also in this case total expression of fatty acid transport proteins is not always consistent with its sarcolemmal content [18]. For these reasons, to better assess plasmalemmal number of the aforementioned transporters, we have conducted palmitate uptake experiment. Interestingly, also this time no changes in basal palmitic acid uptake were detected, which gives further support to postulated normal constant FAs influx into the muscle cell. The presented results are, at least partially, convergent with a recent study of Benton and co-workers [32]. In this paper authors examined the effects of modest (+25%) PGC-1α protein overexpression in skeletal muscles of lean and obese Zucker rats. Remarkably, this led to increased mitochondrial biogenesis and increased palmitate oxidation, which is consistent with observations made during the present study. However, in opposite to our findings the authors observed also increased fatty acid and glucose transporters content, paralleled by increased insulin stimulated glucose transport [32]. We, on the other hand, found no evidence for increased fatty acid or glucose uptake. Perhaps, those inconsistencies are caused by not sufficient degeree of PGC-1α protein ablation and/or model specific differences.

In conclusion, we showed that in L6 myotubes modest (-24%) PGC-1α protein depletion results in lipid accumulation, without causing insulin resistance. Importantly, it seems that this TAG loading is a result of decreased mitochondrial oxidative capacity and/or possibly increased lipid biosynthesis but not fatty acid cellular influx. To the best of our knowledge it is the first time when authors examined the effects of temporary, modest physiological PGC-1α ablation on cellular insulin sensitivity and lipid metabolism in skeletal muscle cells.

Abbreviations

PKB/Akt (protein kinase B); AMPK – (5' adenosine monophosphate-activated protein kinase); AS160 (Akt substrate of 160 kDa (also TBC1 domain family member 4); ACC (acetyl-CoA carboxylase); CER (ceramide); COX IV (cytochrome c oxidase, complex IV); CPT-1 (carnitine palmitoyltransferase 1); CS (citrate synthase); Cyt C (cytochrome c); DAG (diacylglycerol); DGAT-1 (diglyceride acyltransferase 1); FAs (fatty acids);FAS (fatty acid synthase); GLUT-1 (glucose transporter type 1); GLUT-4 (glucose transporter type 4); IR (insulin resistance); IRS-1 (insulin receptor substrate 1); JNKs (c-Jun N-terminal kinases); LCFAs (long chain fatty acids); MCD (Malonyl-CoA decarboxylase); pACC (phospho acetyl-CoA carboxylase); PGC-1α – (PPAR-γ coactivator 1-alpha); PGC-1 KD (PGC-1α knock-down); PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase); PKC (protein kinase C); PP2A (protein phosphatase 2A); PPARs' (peroxisome proliferator activated receptors); TAG (triacylglycerol); TCA (tricarboxylic acid cycle (Krebs cycle)); T2DM (Type 2 diabetes mellitus); UNSAT (unsaturated fatty acids); WHO (World Health Organisation); β-HAD (beta-hydroxyacyl-CoA dehydrogenase).

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Disclosure Statement

The authors declare no conflict of interests.

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