Original Paper

The Effects of AS160 Modulation on Fatty Acid Transporters Expression and Lipid Profile in L6 Myotubes

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Key Words

TBC1D4 (AS160) • Fatty acid transporters • Diacylglycerol • Triacylglycerol • Ceramide • Sphingolipids

Abstract

Background/Aims: AS160 is a key intracellular regulator of energy utilization in cells. It was shown to regulate GLUT4 translocation from intracellular depots to the plasma membrane, with subsequent changes in facilitated glucose uptake into the skeletal muscles. Similarly, also free fatty acids (FFAs) transmembrane transport seems to be largely protein-mediated. Therefore, the objective of this study was to examine the effects of moderate AS160 depletion (-82% mRNA, -25% of protein content) on the expression of fatty acid transporters and subsequent changes in lipid profile in L6 myotubes. **Results:** Surprisingly, moderate down regulation of AS160 expression was followed by increased AS160 phosphorylation (~40%). These resulted in a greater expression of fatty acid transporters, namely FABPpm and FAT/CD36, with subsequently increased FAs cellular influx. No changes in the expression of FATP1 and 4 were noticed. Accordingly, we have observed a reduction in total TAG content. This was mainly caused by a significant changes in TAG fatty acids composition favouring a decrease in the amount of palmitic and stearic fatty acid moieties. In contrast, our experimental intervention led to distinctively increased total content of DAG and PL, but concomitantly decreased the content of all measured sphingolipids, e.g. SFA, SA1P, CER, SFO and S1P, in the AS160 knock-down group. **Conclusions:** Modulation of AS160 level and activity led to significant increase in the concentration of DAG and PL, which was associated with changes in FAs composition and expression of fatty acid transporters. Interestingly, the intervention also simultaneously decreased the content of sphingolipids.

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Introduction

Skeletal muscles play a dominant role as a key metabolic tissue regulating glucose and lipid homeostasis [1]. It is well established that glucose uptake requires an intact insulin

signaling cascade. Briefly, binding of insulin to its receptor initiates a signaling process that results in the translocation of glucose transport proteins (GLUT-4) into a plasma membrane and the facilitated diffusion of glucose into the cell [2, 3]. Studies in cultured adipocytes and myocytes identified the role of Rab GTPase-activating protein (Rab GAP) and Akt substrate of 160 kDa (AS160, also known as TBC1D4) in GLUT4 retention and release [4, 5]. In its basal state, GAP domain of AS160 is active and consequently maintains Rab proteins in their inactive form thus sustaining GLUT4 intracellular retention [3, 6]. In response to insulin AS160 becomes phosphorylated by Akt, and AS160 Rab GAP activity is suppressed. The latter eventually facilitates GLUT4 vesicle movement, docking and fusion with the plasma membrane (Fig. 1) [4]. It is well known that insulin stimulated AS160 phosphorylation is impaired in insulin resistance and type 2 diabetes, whereas total expression of this protein does not change [7]. Recent studies shown that site specific impairments in AS160 phosphorylation (Ser318, Ser588, Ser751) have great implications for insulin resistance [8, 9]. Insulin also regulates facilitated diffusion of fatty acids, which is essential for maintaining balance between fatty acids (FAs) uptake and their utilization by myocytes. In myocytes FAs serve an important role as an energy fuel. Moreover, they are also precursors of signaling molecules or mediators influencing the expression of various genes encoding proteins involved in lipid metabolism [10, 11]. Fatty acids uptake is facilitated by proteins, which are co-expressed in the same tissue. Protein-mediated uptake mechanisms involve a number of fatty acid transporters, namely: scavenger receptor cluster of differentiation 36 (CD36), plasma membrane-associated FA binding protein (FABPpm) and FA transport proteins (FATPs) [12-15]. FATPs are expressed in a tissue-specific manner, with FATP1, 4 and 6 isoform expressed in skeletal muscle tissue. It seems that acute regulation of fatty acid transport can occur *via* insulin stimulated translocation of FAT/CD36 and FABPpm from intracellular depots to the plasma membrane [16-18]. Furthermore, permanent relocation of FAT/CD36 to the plasma membrane is often observed in type 2 diabetes and diet-induced insulin resistance which strongly correlates with intracellular retention of the glucose transporter 4 (GLUT4) [13, 15]. Recent studies in cardiomyocytes indicate that trafficking of CD36, a most important facilitator of FA uptake, is regulated by AS160 in a similar way to that of GLUT4 [11]. Besides these observations, it is not known whether deficiency of AS160 might modify protein-mediated fatty acid transport in muscle tissue.

It is well established that dysregulation of skeletal muscle lipid metabolism is of primary importance in the development of skeletal muscle insulin resistance [19, 20]. The latter is usually perceived as an excess of intramuscular accumulation of bioactive lipids [diacylglycerol (DAG), ceramide (CER)] that interfere with insulin signaling pathway (Fig. 1). In turn, intramuscular lipid accumulation has been linked with enhanced rates of fatty acid transport, which exceeds the rates of β -oxidation in this tissue [20-23]. Ceramide, the central molecule in sphingolipids structure and metabolism, is composed of sphingosine and long chain fatty acid [24, 25]. Interestingly, despite the close structural homology of ceramide and other sphingolipid metabolites including sphingosine and sphingosine-1-phosphate, the biological role of these lipids is different and in most cases even opposite [26, 27]. However, it is unknown whether down regulation of AS160 might change FAs transporters expression with related changes in lipids profile in muscle cells. Therefore, in the present study we have examined the effects of moderate AS160 downregulation (-25%, 48 h) on the expression of fatty acid transporters, i.e. FAT/CD36, FABPpm, FATP 1, 4 and intramuscular lipids (TAG, DAG, PL) as well as sphingolipids [sphinganine (SFA), sphinganine-1-phosphate (SA1P), ceramide (CER), sphingosine (SFO) and sphingosine-1-phosphate (S1P)] profile and FAs composition in L6 myotubes.

Materials and Methods

Cell culture

All experiments were performed on commercially available L6 (rat-derived) myoblasts purchased from the American Type Culture Collection. L6 skeletal muscle cells were grown according to the method

Fig. 1. Bioactive lipid intermediates *versus* insulin signalling pathway. Akt/PKB – protein kinase B; AS160 – Akt substrate of 160 kDa (also known as TBC1D4); CER – ceramide; DAG – diacylglycerol; FAT/CD36 – fatty acid translocase/cluster of differentiation 36; FATP-1,4 – fatty acid transport proteins 1 and 4; FFA – free fatty acids; GLUT-4 – glucose transporter type 4; GSV – glucose transporters (GLUT-4) storage vesicles; IKK – I κ B kinase; IRS – insulin receptor substrate; JNK – c-Jun N-terminal kinase; LCFA-CoA – long chain fatty acid acyl-CoA; MITO – mitochondria; PI3K – phosphatidylinositol-4,5-bis-phosphate 3-kinase; PKC ϵ – protein kinase C epsilon; PP2A – protein phosphatase 2A; TAG – triacylglycerol. Adopted from [31].

described previously [28]. Briefly, L6 cells were cultured in high-glucose DMEM (Dulbecco's Modified Eagle's Medium) containing 10% FBS (Fetal Bovine Serum) and 1% antibiotic/antimycotic in a humidified 5% CO₂ atmosphere at 37°C. Since the amount of myoblast fusion into myotubes declines with increasing passage, for the following experiments we used only cells that were at low passages (between 2 and 5). Cells were cultivated to approximately 60% confluence, at that time medium was replaced for DMEM containing 2% horse serum for the induction of differentiation into myotubes. Differentiating medium was changed every 48h, during that time, myoblasts fused to form elongated, multi-nucleated myotubes. Experiments were initiated when visual inspection confirmed that cells are differentiated (usually after about 7 days). Myotubes were used for experiments 7 days following differentiation (as confirmed by visual inspection using phase-contrast microscopy).

Small interfering RNA gene silencing (siRNA)

Reduction of AS160 gene expression was done using gene silencing method according to the manufacturer recommendations (Lifetechnologies, former Invitrogen) [29]. The day before transfection L6 myotubes were transferred to growth medium without antibiotics such that they were ~60% confluent at the time of transfection. The myotubes grown in 6-well culture plates were transfected with 100 nM siRNA (for AS160) or negative-control (noncoding/non-targetting siRNA) with Opti-MEM® I Reduced Serum Medium in DMEM without antibiotics. The lipofectamine mixture was removed after 6 h of incubation. At that time the cells were incubated at 37°C in a 5% CO₂ atmosphere for ~48 hours until the estimation of gene knock-down effectiveness was performed. Three commercially available sequences for AS160 were tested, whereas for further experiments we chose the sequence with the highest efficiency (~80% reduction of mRNA, and 25% of protein content).

Sequence siRNA for AS160:

- 1) GAG UCG CAG AUG GCC UGC CAC GUUU
- 2) GCA CAA AGA GAA AGC UGA AUU UGCA
- 3) CCA GAG CCU GGA CUU AGC CAG UAUA

Real-time PCR

AS160 mRNA content was measured with the use of real-time quantitative PCR. The RNA was extracted from L6 myotubes using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Extracted RNA was solubilized in RNase-free water. After RNA purification, DNase treatment (Ambion) was made to ensure that there was no contamination of genomic DNA. The quality of RNA was verified by running the agarose electrophoresis with ethidium bromide and assessed by measuring absorbances at 260 and 280 nm. Then the RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad), while primers were designed using Beacon Designer Software (Premier Biosoft). Real-time PCR was made using SYBR Green JumpStart Taq ReadyMix (Sigma) via a Bio-Rad Chromo4 system. PCR efficiency was examined by serially diluting the template cDNA, and a melt curve was done at the end of each reaction to verify PCR product specificity. A sample having 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. For further studies sequence with the highest efficiency of expressions (AS160, cyclophilin A) was used.

Primer sequences:

AS160:

1) F: 5'-AGA AGG GGT CCC TAA AAG TCG GC-3'

R: 5'-GTT GGG CAA TCT GTG TCT CAG GC-3'

2) F: 5'-AGA TTG CCC AAC AAA CAC CAG CC-3'

R: 5'-TGA GTG GGA AAA GTC CTC CCC AA-3'

3) F: 5'-CTC TGG AGA AGC TGG AGA GAG CG-3'

R: 5'-GGC CTG AAT TTT AGC GTG AGC CA-3'

Cyclophilin A:

1) F: 5'-TGT CTC TTT TCG CCG CTT GCT G-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'

3) F: 5'-AGC ACT GGG GAG AAA GGA TT-3'

R: 5'-AGA TGC CAG GAC CTG TAT GC-3'

Protein extraction and Western Blot

Routine Western blotting procedures were used to detect protein content as described previously [30, 31]. Briefly, the cells were lysed in ice-cold RIPA (radioimmunoprecipitation assay) buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride) and sonicated for 1 min at 4°C. Protein concentration was then determined using BCA protein assay kit with bovine serum albumin as a standard. Next, samples were boiled at 95°C for 10 minutes in sample buffer containing 2-mercaptoethanol. The protein (60 µg) was subjected to SDS-PAGE and transferred to PVDF membranes, followed by blocking membranes in TTBS buffer (50 mM Tris-HCl, 130 mM NaCl and 0.05 % Tween-20) containing 5% nonfat dry milk for 90 min at room temperature. Then membranes were incubated with the corresponding antibodies at a dilution of 1:1000. Primary antibodies were purchased from Santa Cruz Biotechnology (AS160, FAT/CD36, FABPpm, FATP1,4), Cell Signaling (pAS160_{Thr642}) and Novus Biologicals (β-tubulin). Thereafter the membranes were incubated with anti-rabbit or anti-goat IgG horseradish peroxidase-conjugated secondary antibody (1:3000; Santa Cruz Biotechnology, USA). Protein bands were visualized by using an enhanced chemiluminescence substrate (Thermo Scientific, USA) and quantified by densitometry (Biorad, USA). Equal protein concentrations were loaded in each lane as confirmed by Ponceau staining technique on the blot membrane. Protein expression was then normalized to β-tubulin and reported as arbitrary units. As a final point, the control was set to 100% and the experimental groups were expressed relative to the control.

Palmitate uptake

Palmitate uptake was performed according to Chavez & Summers [21]. Briefly, the cells were incubated with medium supplemented with palmitic acid (Sigma-Aldrich) bound to fatty acid-free bovine serum albumin (Sigma-aldrich) and a trace of radiolabelled [9,10-³H] palmitic acid (Perkin Elmer) at the

specific activity of 1 $\mu\text{Ci mL}^{-1}$. Palmitate uptake was then terminated (after 5 min) by adding an ice-cold PBS and cells were immediately solubilized in 0.05 N NaOH. Thereafter, the fluid was placed in 5 ml vials and taken for liquid scintillation counting (Beckman, Brea, CA, USA). Finally, the radioactivity was normalized relating to the protein concentration.

Sphingolipid analysis

Concentration of sphingoid bases. Concentration of sphingosine, sphinganine, S1P and SA1P was measured simultaneously using the method of Min et al. [32]. Internal standards (10 pmol of C17-sphingosine and 30 pmol of C17-S1P, Avanti Polar Lipids, USA) were added to the samples before ultrasonication. The dried lipid residues were re-dissolved in ethanol, converted to their o-phthalaldehyde derivatives and analyzed using HPLC system (ProStar, Varian, USA) equipped with a fluorescence detector and C18 reversed-phase column (Varian, OmniSpher 5, 4.6 x 150 mm). The isocratic eluent composition of acetonitrile (Merck, Darmstadt, Germany): water (9 : 1, v/v) and a flow rate of 1 mL/min were used. The column temperature was maintained at 30°C.

Concentration of ceramide. An aliquot of the chloroform phase containing lipids extracted as described above was transferred to a fresh tube containing 40 pmol of N-palmitoyl-D-erythro-sphingosine (C17 base) as an internal standard. Samples were then evaporated under a nitrogen stream, re-dissolved in 1M KOH in 90% methanol and heated at 90°C for 60 min to convert ceramide into sphingosine. This procedure does not convert all sphingolipids, such as sphingomyelin, galactosylceramide or glucosylceramide, into free sphingoid bases [33]. Next, the samples were partitioned by the addition of chloroform and water. The upper phase was rejected, but the lower phase was evaporated under nitrogen and then re-dissolved in ethanol. The level of free sphingosine liberated from ceramide was next analyzed using HPLC as described above. The calibration curve was prepared using N-palmitoylsphingosine (Avanti Polar Lipids) as a standard. The chloroform extract used for the assay of ceramide contains small amounts of free sphingoid bases. Therefore, the concentration of ceramide was corrected for the content of free sphingosine determined in the same sample.

Concentration of other lipid species. The intracellular lipids (TAG, DAG, PL and FFA) were analyzed by gas-liquid chromatography (GLC). Studied lipids were extracted using the Folch method of extraction [34] changed according to van der Vusse et al. [10]. Briefly, L6 myotubes were extracted in chloroform-methanol (2:1, vol/vol) containing butylated hydroxytoluene (0.01%) as an antioxidant and heptadecanoic acid as an internal standard. Lipid samples were then separated by thin-layer chromatography silica plates (Kieselgel 60, 0.22 mm, Merck, Darmstadt, Germany) with a heptane: isopropyl ether: acetic acid (60:40:3, vol/vol/vol) resolving solution. Lipid bands were made visible by spraying plates with a 0.2% solution of 3',3'-dichlorofluorescein in methanol and recognized under ultraviolet light using standards on the plates. Then the gel bands were scraped off the plate, transferred into screw cap tubes and transmethylated with BF₃/methanol. The fatty acid methyl esters (FAMES) were dissolved in hexane and analyzed by GLC. A Hewlett-Packard 5890 Series II gas chromatograph with Varian CP-SIL capillary column (100 m, internal diameter of 0.25 mm) and flame-ionization detector were used. In accordance with the retention times of standards, the individual long-chain fatty acids quantification was performed. Next the content of lipids was estimated as the sum of the particular fatty acid species and expressed in nanomoles per milligram of protein.

Statistical analysis

All analysis were run using R statistical software (version 3.1.2). One way ANOVA followed by Tukey HSD test was applied to test the differences between studied groups. Whenever their assumptions did not hold, i.e. lack of normal distribution and/or homogeneity of variance, Kruskal-Wallis rank test with the subsequent pairwise Wilcoxon test was conducted. Differences were considered to be statistically significant at the level of 0.05. All displayed data are expressed as mean $\pm$ SD. As stated in the Materials and Methods section in order to avoid any unspecific effects of our technique (siRNA silencing with lipofectamine reagent) we have employed negative control (NC) group (containing non-targeting siRNA fragment). All results with respect to AS160 knock-down (AS160 KD) were compared with the abovementioned group. For the sake of clarity only two groups are presented: AS160 KD and the appropriate control (NC) group.

Results

Based on our previous pilot studies, we have assessed that the highest desired silencing effect occurs 48 h after transfection - with the post-transfectional L6 myotubes viability at the level of over 80% (data not shown). As a first step we determined the effectiveness of our mRNA silencing by measuring both mRNA and protein expression of AS160. Forty eight hours of transfection caused significant decreases in the levels of mRNA (-82%, $p < 0.05$, Fig. 2A) and the protein expression of AS160 (-25%, $p < 0.05$, Fig. 2B). The abovementioned intervention contributed to the increased AS160 phosphorylation at Thr⁴⁶² residue ($\sim +40\%$, $p < 0.05$, Fig. 2C).

Effects of AS160 modulation on fatty acid transporters expression and palmitate uptake in L6 myotubes

Subsequently, we investigated whether the expression of fatty acid transporters was changed after AS160 gene silencing. Moderate AS160 down regulation (followed by enhanced AS160 phosphorylation) increased the expression of FAT/CD36 and FABPpm (+28% and +40%, respectively, $p < 0.05$, Fig. 3A, B) with subsequent increase in PA cellular influx (+14%, $p < 0.05$, Fig. 4). However, the expressions of FATP-1 and FATP-4 did not differ between the studied groups (-13% and -18%, respectively, $p > 0.05$, Fig. 3C, D).

Fig. 2. AS160 mRNA and protein expression. N (per group) = 6; * - difference vs. ctrl ($p < 0.05$); A. U. - arbitrary units; control set as 100.

Fig. 3. Total expression of fatty acid transport proteins: fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm), fatty acid transport proteins (FATP-1, 4); N (per group) = 6; * - difference vs. ctrl ($p < 0.05$); A. U. - arbitrary units; control set as 100. For samples from the one batch, the same β -tubulin bands were used for normalization.

Fig. 4. Basal palmitate uptake; N (per group) = 8; * - difference vs. ctrl ($p < 0.05$).

Fig. 5. Intracellular lipid content [nmol mg of protein]; N (per group) = 8; * - difference vs. ctrl ($p < 0.05$). FFA – free fatty acids; DAG – diacylglycerol; TAG – triacylglycerol; PL – phospholipids.

Effects of AS160 modulation on lipids content and composition in L6 myotubes

We have also examined the consequences of the AS160 modulation on cellular lipid content as well as fatty acids composition. AS160 silencing reduced the total content of triacylglycerol-fatty acids by 22%, but this change was not significant ($p > 0.05$, Fig 5C). This trend towards reduction was caused mostly by a significant changes in FAs composition in TAG - favouring a decrease in palmitic and stearic acids moieties (-34% and -49%, $p < 0.05$, Table 3) in AS160-KD group. In contrast AS160 protein modulation distinctly increased the total content of DAG (+40%, $p < 0.05$) as compared to control myotubes (Fig. 5B). This alteration was mainly due to the elevation in the content of diacylglycerols containing saturated fatty acids (+52% palmitic, +29% stearic and +362% lignoceric, $p < 0.05$, Table 2) as well as unsaturated fatty acids (+95% palmitoleic, linoleic +56%, linolenic +100%, +66% eicosapentaenoic and +100% arachidonic, $p < 0.05$, Table 2). Interestingly, AS160 silencing has also significantly augmented total phospholipid content in L6 myotubes (+30%, $p < 0.05$, Fig. 5D). The most profound increase was observed for PL containing palmitic and stearic fatty acids (+46% and +43%, respectively, $p < 0.05$, Table 4). On the contrary, our experimental intervention had no significant effects on the FFAs level, although a trend towards an increase was noticed (+20%, $p > 0.05$, Fig. 5A). There were, however, some significant changes in the content of individual fatty acids affecting an increase in stearic acid (+41%, $p < 0.05$, Table 1), but a decrease in myristic acid (-67%, $p < 0.05$, Table 1). The content of other FFAs remained quite stable (Table 1).

Effects of AS160 modulation on the sphingolipids content in L6 myotubes

Consequently, we assessed intracellular sphingolipids content in transfected cells. Examination of the effects of AS160 protein modulation in L6 myotubes revealed that there is a significant decrease in the formation of sphingolipids. Indeed, we have noticed that SFA content (the most important substrate for *de novo* sphingolipid synthesis) was significantly

Table 1. FA – FFA; N (per group) = 8; * - difference vs. Ctrl (p < 0.05)

FA - FFA [nmol/mg of protein]	Ctrl	AS160 KD
Myristic acid (C ₁₄ H ₂₈ O ₂) – C14:0	0.88 ± 0.154	0.29 ± 0.103*
Palmitic acid (C ₁₆ H ₃₂ O ₂) – C16:0	6.94 ± 0.902	7.58 ± 1.343
Palmitoleic acid (C ₁₆ H ₃₀ O ₂) – C16:1	0.12 ± 0.034	0.15 ± 0.019
Stearic acid (C ₁₈ H ₃₆ O ₂) – C18:0	7.35 ± 0.655	10.36 ± 1.614*
Oleic acid (C ₁₈ H ₃₄ O ₂) – C18:1	0.7 ± 0.227	0.66 ± 0.06
Linoleic acid (C ₁₈ H ₃₂ O ₂) – C18:2	0.26 ± 0.036	0.32 ± 0.044
Arachidic acid (C ₂₀ H ₄₀ O ₂) – C20:0	0.22 ± 0.161	0.22 ± 0.05
Linoleic acid (C ₁₈ H ₃₀ O ₂) – C18:3	0.05 ± 0.025	0.05 ± 0.01
Behenic acid (C ₂₂ H ₄₄ O ₂) – C22:0	0.04 ± 0.008	0.05 ± 0.011
Arachidonic acid (C ₂₀ H ₃₂ O ₂) – C20:4	0.11 ± 0.025	0.11 ± 0.015
Lignoceric acid (C ₂₄ H ₄₈ O ₂) – C24:0	0.02 ± 0.01	0.02 ± 0.01
Eicosapentaenoic acid (C ₂₀ H ₃₀ O ₂) – C20:5	0.02 ± 0.008	0.03 ± 0.01
Nervonic acid (C ₂₄ H ₄₆ O ₂) – C24:1	0.03 ± 0.01	0.04 ± 0.015
Docosahexaenoic acid (C ₂₂ H ₃₂ O ₂) – C22:6	nd.	nd.

Table 2. FA – DAG; N (per group) = 8; * - difference vs. Ctrl (p < 0.05)

FA - DAG [nmol/mg of protein]	Ctrl	AS160 KD
Myristic acid (C ₁₄ H ₂₈ O ₂) – C14:0	1.4 ± 0.955	1.58 ± 0.085
Palmitic acid (C ₁₆ H ₃₂ O ₂) – C16:0	10.66 ± 1.568	16.16 ± 2.084*
Palmitoleic acid (C ₁₆ H ₃₀ O ₂) – C16:1	0.21 ± 0.12	0.41 ± 0.035*
Stearic acid (C ₁₈ H ₃₆ O ₂) – C18:0	8.93 ± 0.767	11.56 ± 0.998*
Oleic acid (C ₁₈ H ₃₄ O ₂) – C18:1	0.73 ± 0.483	0.85 ± 0.107
Linoleic acid (C ₁₈ H ₃₂ O ₂) – C18:2	0.23 ± 0.118	0.36 ± 0.054*
Arachidic acid (C ₂₀ H ₄₀ O ₂) – C20:0	0.12 ± 0.066	0.17 ± 0.015
Linoleic acid (C ₁₈ H ₃₀ O ₂) – C18:3	0.03 ± 0.014	0.06 ± 0.03*
Behenic acid (C ₂₂ H ₄₄ O ₂) – C22:0	0.04 ± 0.024	0.04 ± 0.006
Arachidonic acid (C ₂₀ H ₃₂ O ₂) – C20:4	0.1 ± 0.057	0.2 ± 0.025*
Lignoceric acid (C ₂₄ H ₄₈ O ₂) – C24:0	0.08 ± 0.039	0.37 ± 0.043*
Eicosapentaenoic acid (C ₂₀ H ₃₀ O ₂) – C20:5	0.03 ± 0.009	0.05 ± 0.011*
Nervonic acid (C ₂₄ H ₄₆ O ₂) – C24:1	0.05 ± 0.032	0.04 ± 0.005
Docosahexaenoic acid (C ₂₂ H ₃₂ O ₂) – C22:6	nd.	nd.

decreased in AS160 knock-down group (-15%, p < 0.05, Fig. 6A). In addition, SA1P level was also diminished after moderate down regulation of AS160 (-31%, p < 0.05, Fig. 6B). Similarly, the intervention resulted in statistically significant reduction of ceramide (-35%, p < 0.05, Fig. 6C) and SFO, as well as its phosphorylated form S1P (-42% and -40% p < 0.05, respectively, Fig. 6D, E).

Discussion

In the current study we examined, for the first time, the effects of moderate (-25% of protein content) AS160 down regulation on the expression of fatty acid transporters and

Table 3. FA – TAG; N (per group) = 8; * - difference vs. Ctrl (p < 0.05)

FA - TAG [nmol/mg of protein]	Ctrl	AS160 KD
Myristic acid (C ₁₄ H ₂₈ O ₂) – C14:0	0.85 ± 0.217	0.72 ± 0.111
Palmitic acid (C ₁₆ H ₃₂ O ₂) – C16:0	3.58 ± 0.992	2.36 ± 0.333*
Palmitoleic acid (C ₁₆ H ₃₀ O ₂) – C16:1	0.46 ± 0.058	0.47 ± 0.066
Stearic acid (C ₁₈ H ₃₆ O ₂) – C18:0	1.27 ± 0.516	0.65 ± 0.166*
Oleic acid (C ₁₈ H ₃₄ O ₂) – C18:1	1.16 ± 0.385	1.08 ± 0.105
Linoleic acid (C ₁₈ H ₃₂ O ₂) – C18:2	0.08 ± 0.071	0.03 ± 0.034
Arachidic acid (C ₂₀ H ₄₀ O ₂) – C20:0	0.06 ± 0.022	0.06 ± 0.032
Linoleic acid (C ₁₈ H ₃₀ O ₂) – C18:3	0.09 ± 0.045	0.09 ± 0.054
Behenic acid (C ₂₂ H ₄₄ O ₂) – C22:0	nd.	nd.
Arachidonic acid (C ₂₀ H ₃₂ O ₂) – C20:4	0.12 ± 0.016	0.13 ± 0.076
Lignoceric acid (C ₂₄ H ₄₈ O ₂) – C24:0	0.39 ± 0.189	0.72 ± 0.282*
Eicosapentaenoic acid (C ₂₀ H ₃₀ O ₂) – C20:5	0.34 ± 0.042	0.28 ± 0.071
Nervonic acid (C ₂₄ H ₄₆ O ₂) – C24:1	0.01 ± 0.027	0.02 ± 0.021
Docosahexaenoic acid (C ₂₂ H ₃₂ O ₂) – C22:6	nd.	nd.

Table 4. FA - PL. N (per group) = 8; * - difference vs. Ctrl (p < 0.05)

FA - PL [nmol/mg of protein]	Ctrl	AS160 KD
Myristic acid (C ₁₄ H ₂₈ O ₂) – C14:0	10.67 ± 1.75	10.98 ± 1.788
Palmitic acid (C ₁₆ H ₃₂ O ₂) – C16:0	40.9 ± 7.442	59.59 ± 14.809*
Palmitoleic acid (C ₁₆ H ₃₀ O ₂) – C16:1	9.78 ± 0.845	12.71 ± 1.574*
Stearic acid (C ₁₈ H ₃₆ O ₂) – C18:0	22.16 ± 2.861	31.68 ± 7.042*
Oleic acid (C ₁₈ H ₃₄ O ₂) – C18:1	15.59 ± 1.641	18.22 ± 2.396*
Linoleic acid (C ₁₈ H ₃₂ O ₂) – C18:2	8.82 ± 1.063	10.1 ± 1.348
Arachidic acid (C ₂₀ H ₄₀ O ₂) – C20:0	0.4 ± 0.102	0.31 ± 0.119
Linoleic acid (C ₁₈ H ₃₀ O ₂) – C18:3	0.23 ± 0.451	nd.
Behenic acid (C ₂₂ H ₄₄ O ₂) – C22:0	nd.	nd.
Arachidonic acid (C ₂₀ H ₃₂ O ₂) – C20:4	10.39 ± 1.175	12.43 ± 1.524*
Lignoceric acid (C ₂₄ H ₄₈ O ₂) – C24:0	1.26 ± 1.051	1.1 ± 0.193
Eicosapentaenoic acid (C ₂₀ H ₃₀ O ₂) – C20:5	1.91 ± 0.301	1.95 ± 0.28
Nervonic acid (C ₂₄ H ₄₆ O ₂) – C24:1	nd.	nd.
Docosahexaenoic acid (C ₂₂ H ₃₂ O ₂) – C22:6	1.49 ± 0.312	1.68 ± 0.215

lipid profile in L6 myotubes. In general, the application of the knock-down technique in cell cultures allows for observing direct AS160 actions due to the short time frame and transient protein downregulation. The latter should prevent (or at least significantly reduce) cellular/system compensatory mechanisms obscuring the picture and hindering further analysis [31]. Moreover, moderate AS160 downregulation may be of great value itself. Some of the previous works [35, 36] have shown that, with respect to hormones or regulatory proteins, there may be a significant difference in the observed response between biological systems with severe- and mild-type therapeutic interventions. It has been postulated, that at least in some cases, experimentally evoked changes of moderate extent are closer to the one observed in the normal (patho)physiological conditions (like type 2 diabetes mellitus), and therefore constitute a better model of the processes [35]. Surprisingly, our transient, short time-frame intervention evoked a substantial increment of AS160 phosphorylation (~ +40%) (Fig. 2 C). The latter, although unexpected, seems to be of particular significance since previous studies of AS160 protein revealed that in insulin resistance and type 2 diabetes insulin stimulated

Fig. 6. Intracellular lipid content [nmol/mg of protein]; N (per group) = 8; * - difference vs. ctrl ($p < 0.05$). SFA – sphinganine; SA1P – sphinganine 1-phosphate; CER – ceramide; SFO – sphingosine; S1P – sphingosine 1 phosphate.

AS160 phosphorylation is impaired, whereas its total expression remains unchanged [7, 8]. This would indicate that the level of pAS160, rather than the total AS160 itself, is responsible for the biological actions of this protein. However, the reason(s) for this miscellaneous shift in the both parameters remains elusive.

Our data demonstrate that transient AS160 gene silencing increased the expression of FAT/CD36 and FABPpm which seems to have contributed to the concurrent increase in the fatty acid cellular influx. Accordingly, the changes in fatty acid transporters expression were also accompanied by a significant rise in the concentration of DAG and PL lipid fractions. However, our AS160 experimental intervention was associated with a reduction in the content of all measured sphingolipids in L6 myotubes.

Long chain fatty acids (LCFAs) are an efficient energy source for skeletal muscle and therefore the uptake of LCFAs into cells is an important step in energy metabolism [37]. Since it is well known that lipid metabolism in skeletal muscle is dysregulated in obesity and Type 2 Diabetes, most attention has been given towards examining a role of fatty acid transporters in this pathological phenomenon. In animal models of IR [22, 38] and in humans with IR and T2D [39] it was observed that intramuscular lipid accumulation is connected with the increased plasmalemmal FAT/CD36 content, whereas the total expression of that protein was not altered. Indeed, its permanent relocation to the plasma membrane augments the rate of fatty acids uptake and contributes to the accumulation of intramuscular lipids, which

in turn can cause insulin resistance by interfering with insulin signaling pathway (Fig. 1). Moreover, recent studies have shown that blocking either FAT/CD36 or FABPpm is sufficient to markedly inhibit fatty acid transport and therefore rates of fatty acid esterification and oxidation in heart and skeletal muscle, suggesting a close collaboration between these two transporters at the plasma membrane level [13, 15, 20, 40]. The data presented herein showed that moderate AS160 down regulation followed by the increment in pAS160 level has upregulated total expression of the fatty acid transporters, namely FABPpm and FAT/CD36 in L6 myotubes. To the best of our knowledge only one article on this specific topic exists where FAT/CD36 expression after knock-down of AS160 was measured [11]. The authors of these data have found that in HL-1 cardiomyocytes the trafficking of FAT/CD36, a major facilitator of myocardial FA uptake, is regulated by AS160 [11]. In that experiment, the level of total FAT/CD36 was not altered, but surface FAT/CD36 expression in basal (unstimulated) cells was increased about 2.5-fold. These results suggested that surface recruitment of FAT/CD36 in cardiomyocytes is regulated by AS160 and that the protein mediates the effects exerted by insulin and/or AMPK on FAT/CD36 translocation [11]. Moreover, also other results reported by Samovski et al. seem to resemble some of our findings. As mentioned before the authors noticed that AS160 KD had caused an increased FAT/CD36 translocation. Secondly, they reported that in cell lines with AS160 4P overexpression (blocked AS160 phosphorylation places, and therefore its reduced activity) FAT/CD36 translocation was abrogated. Thirdly, the stimulation of GFP-Rab8a (downstream target of AS160 activity) abolished the effects of AS160 4P overexpression. These results indicate that AS160 downregulation may exert stimulating effect with respect to FA transport (similarly to our study), whereas inhibition of its phosphorylation/activity abrogated the abovementioned effect. This indirectly suggests that the increase of AS160 phosphorylation (as observed in our study) would have caused a stimulation of FAs transport (presented here increase in basal palmitate acid uptake). On the other hand, recent study of Maher et al. has shown that overexpression of TBC1D1, which is also a paralogue of Rab-GTPase-activating proteins, did not alter the total content of selected fatty acid transporters, namely: FAT/CD36, FABPpm and FATP4 in skeletal muscle. Moreover, in that study fatty acid oxidation was reduced as a result of inhibition of β -HAD enzymatic activity, but not reductions in fatty acid transporter proteins level [1]. Additionally, there are some studies revealing that FABPpm overexpression in skeletal muscle did not alter triacylglycerol formation, but resulted in a significant increase of fatty acid oxidation [12, 41]. This was attributed to enlarged fatty acids transport into the skeletal muscle cells, as the simultaneous overexpression of FABPpm/mAspAt in mitochondria did not modify fatty oxidation in mitochondria [42]. However, we did not observe any significant changes in FATP1 and 4 expression after AS160 gene silencing. Perhaps, the most plausible explanation of these findings is that FAT/CD36 and FABPpm, but not FATPs, play a major role in the protein mediated fatty acid transport into myocytes [12, 42]. Supporting this conclusion is the study presenting that overexpression of FATP1 and 4 in rat skeletal muscle increases the rate of long-chain fatty acids transport into giant sarcolemmal vesicles, however, this increase was far more modest than an increase in FABPpm [18]. Furthermore, FATPs and FAT/CD36 are present within distinct plasma membrane compartments, which excludes a possibility of direct interaction between FATP and FAT/CD36 proteins in mediating fatty acid transport [14, 17]. As we have indicated above, the total expression of fatty acid transport proteins not always reflects their sarcolemmal content. Therefore, to better investigate the effect of AS160 depletion on the expression of FA transporters we have performed palmitate uptake experiment. Interestingly, also this time an increase in unstimulated palmitic acid uptake was observed, which is consistent with intensified FAs influx into muscle cells (as postulated above). Remarkably, just recently an interesting paper of Jain et al. has been published [43]. In the article the authors have examined the influence of Akt2 knock-out on fatty acids transport and their transporters. Akt2 is an important insulin signaling pathway chain link and an upstream enzyme for AS160 phosphorylation (Fig. 1). It turned out that in the case of Akt2 KO mice, both insulin-induced and contraction-induced fatty transporters translocation (FAT/CD36 and FATP-1) were significantly downregulated. Moreover, the aforementioned

changes were accompanied by a reduced phosphorylation of AS160 (Thr⁴⁶²), but not total AS160 level [43]. Those findings converge substantially with the results presented here. Moreover, they suggest the involvement of AS160 in insulin-dependent (and perhaps also contraction dependent) fatty acids transporters translocation and FAs uptake.

In general, the increased entry of FAs into myocytes, without corresponding increase in lipid oxidation, contributes to the accumulation of intramuscular lipids [23]. Moreover, strong correlation exists between lipid accumulation and insulin sensitivity in non-adipose tissues - such as skeletal muscle [20, 39, 44]. However, in some cases, like in the “athlete’s paradox”, intramyocellular triacylglycerol (IMTG) content is increased, suggesting that the accumulation of IMTG provides a protective effect within the cells by limiting the accumulation of other lipid intermediates such as diacylglycerols and ceramides, which are known to have an inhibitory effect on insulin signaling and glucose metabolism [20, 23, 45]. Herein, we have also characterized the effect of moderate AS160 down regulation (followed by its increased phosphorylation) on lipid pools and fatty acids composition in skeletal muscle cells. The present study showed that AS160 gene silencing significantly increased DAG and PL content, and also tended towards a rise of FFAs concentration. On the other hand, in our study, we observed a decrease in the TAG amount. This diminished TAG content correlates with a reduction in the palmitate and stearate acids levels. Interestingly, depending on the degree of saturation, FFAs may exert different effects on insulin signaling and cell metabolism. Previous studies have demonstrated that unsaturated fatty acids may be targeted towards triglyceride synthesis and storage, whilst saturated moieties may be channeled into other pathways, such as DAG synthesis [46]. In line with this statements, we observed enhanced accumulation of intramuscular DAG containing more saturated fatty acids than unsaturated. Furthermore, there are also studies indicating that channeling of long-chain fatty acids by FAT/CD36 to triacylglycerols, as observed in C2C12 myotubes, does not occur in heart and skeletal muscles [47]. However, in muscles, it is well known that FAT/CD36 contributes to regulation of fatty acid oxidation, but not their esterification [18]. Similarly, Chadt et al. have shown an elevated fatty acid oxidation in isolated mouse skeletal muscle with the depletion of both Rab-GTPase activating proteins (TBC1D1 and TBC1D4) [48]. Taken altogether, it appears therefore, that the metabolic effects of AS160 on lipids content and composition are attributable to the alterations in fatty acids transport.

Herein, we show that modulation of AS160 level and activity changes sphingolipid metabolism as well. Ceramide, the central molecule in sphingolipid structure and metabolism can accumulate in cells *via* three main routes: the hydrolysis of the membrane sphingomyelin, *de novo* synthesis from long chain fatty acids or salvage pathway [24, 49-51]. We paid our attention, mostly, to ceramide generated from *de novo* and salvage pathway. In our study, concentrations of SFA, SA1P and CER were markedly reduced in AS160-KD group. This suggests that *de novo* synthesis (in which sphinganine is a key intermediate) of ceramide is reduced in L6 myotubes with AS160 gene silencing. Further, ceramide could also be degraded by the enzyme ceramidase, therefore yielding fatty acid and sphingosine [26]. Similarly, we have shown that the concentrations of both sphingosine and its phosphorylated form S1P are diminished in these conditions. As a result, decreased amount of sphingosine, reflecting reduced ceramide metabolism, has been observed in the present study. We postulate that, a fatty acyl-CoA, usually palmitoyl-CoA, might not have been used for *de novo* ceramide synthesis, but instead might have been directed into DAG synthesis. This hypothesis seems more likely, because of the increased DAG concentration which was observed in our study. However, so far there are no data connecting AS160 expression with sphingolipids concentration in skeletal muscle or other tissue.

As for further perspectives, it might be interesting to identify the mechanism(s) responsible for the observed AS160 phosphorylation increment. It would be also advisable to repeat and/or elaborate our findings with ‘constitutively active’ AS160 (as described by Amira Klip’s group and others) [11, 52]. The aforementioned model and scientific methodology would help to determine AS160 downstream targets with respect to FAs transporter vesicles translocation.

In conclusion, this study demonstrates that AS160 silencing effects lipids and/or sphingolipids profile and their fatty acids composition in L6 myotubes. Surprisingly, the reduction in AS160 expression resulted in a substantially increased phosphorylation level of the protein. The latter, in turn, increased the level of some fatty acid transporters, indicating enhanced FAs entry into the muscle cells. In addition, we have shown that different lipid fractions, namely DAG, PL and also FFA were increased in our study. Finally, the intervention with respect to AS160 level and activity has led to a decrease of free sphingoid bases and their 1-phosphates in L6 myotubes.

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Disclosure Statement

The authors declare no conflict of interests.

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