FEBS Letters

FEBS PR
science publishing b

Investigation of the putative rate-limiting role of electron transfer in fatty acid desaturation using transfected HEK293T cells

Journal:	*FEBS Letters*
Manuscript ID	Draft
Wiley - Manuscript type:	Research Letter
Date Submitted by the Author:	n/a
Complete List of Authors:	Zámbó, Veronika; Semmelweis University, Medical Chemistry, Molecular Biology and Pathobiochemistry Simon-Szabó, Laura; Pathobiochemistry Research Group, Hungarian Academy of Sciences, Semmelweis University (MTA-SE) Sarnyai, Farkas; Semmelweis University, Medical Chemistry, Molecular Biology and Pathobiochemistry Mátyási, Judit; B&B Analytics Ltd. Gór-Nagy, Zsófia; Budapest University of Technology and Economics, Department of Inorganic and Analytical Chemistry Somogyi, Anna; Semmelweis University, Medical Chemistry, Molecular Biology and Pathobiochemistry Szelényi, Péter; Semmelweis University, Medical Chemistry, Molecular Biology and Pathobiochemistry Kereszturi, Éva; Semmelweis University, Medical Chemistry, Molecular Biology and Pathobiochemistry Tóth, Blanka; Budapest University of Technology and Economics, Department of Inorganic and Analytical Chemistry Csala, Miklos; Semmelweis University, Medical Chemistry, Molecular Biology and Pathobiochemistry
Keywords:	lipotoxicity, fatty acid desaturation, endoplasmic reticulum, electron transfer chain
Abstract:	Elevated fatty acid (FA) levels contribute to severe metabolic diseases. Unbalanced oversupply of saturated FAs is particularly damaging, which renders stearoyl-CoA desaturase (Scd1) activity an important factor of resistance. An Scd1-related oxidoreductase protects cells against palmitate toxicity, so we aimed to test whether desaturase activity is limited by Scd1 itself or by the associated electron supply. Unsaturated/saturated FA ratio was markedly elevated by Scd1 overexpression while it remained unaffected by the overexpression of Scd1-related electron transfer proteins in HEK293T cells. Electron supply was not rate-limiting either in palmitate-treated cells or in cells of enhanced Scd1 expression. Our findings indicate the rate-limiting role of Scd1 itself, and that FA desaturation cannot be facilitated by reinforcing the electron supply of the enzyme.

Investigation of the putative rate-limiting role of electron transfer in fatty acid desaturation using transfected HEK293T cells

Veronika Zámbó[1]*, Laura Simon-Szabó[2], Farkas Sarnyai[1], Judit Mátyási[3], Zsófia Gór-Nagy[4], Anna Somogyi[1], Péter Szelényi[1], Éva Kereszturi[1], Blanka Tóth[4]*, Miklós Csala[1]

[1]Semmelweis University, Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Budapest, Hungary
[2]Pathobiochemistry Research Group, Hungarian Academy of Sciences, Semmelweis University (MTA-SE), Budapest, Hungary
[3]B&B Analytics Ltd., Érd, Hungary
[4]Budapest University of Technology and Economics, Department of Inorganic and Analytical Chemistry, Budapest, Hungary

*Corresponding authors:

Veronika Zámbó Ph.D.; Semmelweis University, Dept. of Medical Chemistry, Molecular Biology and Pathobiochemistry, H-1428, Budapest, POB: 2, Hungary; Email: zambo.veronika@med.semmelweis-univ.hu; Tel: + 36 1 2662615

Blanka Tóth Ph.D.; Budapest University of Technology and Economics, Dept. of Inorganic and Analytical Chemistry, H-1111, Budapest, Szt. Gellért tér 4., Hungary; Email: tblanka@mail.bme.hu; Tel: + 36 1 4631415

Abbreviations: Cyb5: Cytochrome b5; Cyb5R: Cytochrome b5 reductase; FFA: Free fatty acid; Scd1: Stearoyl-CoA desaturase 1; Ncb5or: NADH cytochrome b5 oxidoreductase; SFA: Saturated fatty acid; UFA: Unsaturated fatty acid; ER: Endoplasmic reticulum; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GC-FID: Gas chromatography-flame ionization detector; $NAD(P)^+$: nicotinamide adenine dinucleotide phosphate (oxidized); NAD(P)H: nicotinamide adenine dinucleotide phosphate (reduced)

Abstract

Elevated fatty acid (FA) levels contribute to severe metabolic diseases. Unbalanced oversupply of saturated FAs is particularly damaging, which renders stearoyl-CoA desaturase (Scd1) activity an important factor of resistance. An Scd1-related oxidoreductase protects cells against palmitate toxicity, so we aimed to test whether desaturase activity is limited by Scd1 itself or by the associated electron supply. Unsaturated/saturated FA ratio was markedly elevated by Scd1 overexpression while it remained unaffected by the overexpression of Scd1-related electron transfer proteins in HEK293T cells. Electron supply was not rate-limiting either in palmitate-treated cells or in cells of enhanced Scd1 expression. Our findings indicate the rate-limiting role of Scd1 itself, and that FA desaturation cannot be facilitated by reinforcing the electron supply of the enzyme.

INTRODUCTION

Excessive fatty acid (FA) supply causes disturbance in the cellular signaling and metabolic functions, which can culminate in severe cell damage or even cell death [1]. It has been repeatedly demonstrated that saturated FAs (e.g. palmitate) are far more deleterious than the endogenous cis-unsaturated FAs (e.g. oleate) or even the dietary trans-unsaturated FAs (e.g. elaidate and vaccenate) [2]. Moreover, the damage caused by palmitate is often attenuated by simultaneously administered unsaturated FAs [3, 4]. These observations highlight the importance of a balanced availability of saturated and unsaturated FAs in the cells. The intrinsic ability of the cells to maintain such a balance by inserting double bonds into saturated FAs is dependent on the activity of stearoyl-CoA desaturase (Scd1), which creates the first double bond of cis configuration in stearoyl-CoA (C18:0) or palmitoyl-CoA (C16:0) at position 9 yielding oleoyl-CoA (C18:1 cis Δ9) or palmitoleoyl-CoA (C16:1 cis Δ9), respectively. The key role of Scd1 in maintaining a balanced FA supply is supported by several *in vitro* studies demonstrating that Scd1 overexpression reduces saturated FA-induced toxicity and apoptosis similarly to co-administration of unsaturated FAs [5-7]. The iron-containing Scd1 enzyme is localized in the endoplasmic reticulum (ER) membrane and acts through monooxygenation and subsequent dehydration of the FA chains. The two electrons required for monooxygenation derive from cytosolic NADH or NADPH carriers, and are delivered to the active center by the assistance of flavoprotein and hemoprotein oxidoreductases. The classical electron transfer chain is composed of two integral membrane proteins of the ER, the flavoprotein cytochrome b_5 reductase (Cyb5R) and the hemoprotein cytochrome b_5 (Cyb5) [8]. In addition, NADH cytochrome b5 oxidoreductase (Ncb5or), a soluble cytosolic protein containing both Cyb5R-like and Cyb5-like domains [9-11] can serve as an alternative route for the electrons to Scd1 (Fig. 1).

Cells might attempt to adapt to a surplus of saturated FAs by increasing the expression and hence the capacity of either Scd1 enzyme itself, or the associated electron transfer proteins or both. The existence of alternative electron transfer components and the protective role of Ncb5or against palmitate toxicity suggest that some enhancement of the proximal conductivity of the chain is required. The present work aimed to reveal how and at what extent the overall desaturating activity of the cell depends on the expression level of each protein component involved in the process. To this end, we overexpressed Scd1, Cyb5R, Cyb5, Ncb5or or their combinations in human HEK293T cells and assessed the whole cellular FA content for alterations in the unsaturated/saturated ratio. The results indicate that it is the level of the desaturase enzyme itself rather than that of the associated electron transfer chain members that defines the capacity to desaturate fatty acids in our cellular model.

MATERIALS AND METHODS

Materials used

pcDNA3.1- plasmid was purchased from Clontech Laboratories, Inc., Mountain View, CA. Lipofectamine 2000, SuperScript III First-Strand Synthesis System for RT-PCR Kit, Dulbecco's Modified Eagle's Medium (DMEM), Opti-MEM, fetal bovine serum (FBS), 1% antibiotic-antimycotic solution and trypsin-EDTA (0.25%) were obtained from Thermo Fisher Scientific, Waltham, MA. RNeasy Plus Mini Kit was from Qiagen, Germantown, MD. All other reagents and solvents were of analytical grade.

Expression plasmid constructions

The coding sequence of human *CYB5*, *CYB5R*, *SCD1* and *NCB5OR* were PCR amplified by using CYB5-XhoI sense (5' – AAA TTT **CTC GAG** ATG GCA GAG CAG TCG GAC - 3') CYB5- HindIII antisense (5' – TTT AAA **AAG CTT** TTC AGT CCT CTG CCA TGT A - 3'); CYB5R-BamHI sense (5' – AAA TTT **GGA TCC** ATG GGG GCC CAG CTC AGC ACG T - 3') CYB5R- HindIII antisense (5' - AAA TTT **AAG CTT** TCA GAA GAC GAA GCA GCG CTC- 3'); SCD1-XhoI sense (5' –AAA TTT **CTC GAG** CTC AGC CCC CTG GAA AGT GAT- 3') SCD1-HindIII antisense (5' –AAA TTT **AAG CTT** GTT TGG GGT CCC TCA GGT TCC- 3'); NCB5OR-XhoI sense (5' - AAA TTT **CTC GAG** GGG TTT GAA GAT GCT GAA C - 3') and NCB5OR-HindIII antisense (5' - AAA TTT **AAG CTT** GTT GAA TAA AGG ACA ATG ACA G - 3') primers. The template cDNA had been produced by reverse transcription of mRNA isolated from HepG2 cells. The purified PCR products were cloned into pcDNA3.1- plasmids between their appropriate restriction sites. All constructs were verified by sequencing.

Cell culture and transient transfection

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution at 37 °C in a humidified atmosphere containing 5% CO_2.

For transient transfection, 6 × 10^5 HEK293T cells were seeded per well in 6-well plates. Transfection with pcDNA3.1-_CYB5, pcDNA3.1-_CYB5R, pcDNA3.1-_SCD1, pcDNA3.1-_NCB5OR or empty pcDNA3.1- plasmid (2 µg each) was performed using Lipofectamine 2000 and Opti-MEM according to the manufacturer's instructions. Cells were harvested and processed 24h after transfection.

Cell treatment with BSA-conjugated fatty acid

Palmitate were dissolved in isopropanol (Molar Chemicals) to a concentration of 50 mM and were diluted to a concentration 5 mM, conjugated with 4.16 mM fatty acid free BSA (Sigma) in 1:4 ratio on 37˚ C for 1 hour. The working solution for palmitate treatment was in FBS-free and antibiotic-free medium at 50 µM final concentration. Incubation time was 15 hours.

Preparation of cell lysates

Cell lysates were made for Western blot analysis by removing the medium, washing the cells with PBS twice. 100 µL RIPA lysis buffer (0.1% SDS, 5mM EDTA, 150 mM NaCl, 50 mM Tris, 1% Tween 20, 1mM Na_3VO_4, 1mM PMSF, 10 mM benzamidine, 20 mM NaF, 1mM pNPP and protease inhibitor cocktail) was added to each well and the cells were scraped and briefly vortexed. After 15 min incubation at room temperature the lysates were centrifuged for 5 min at max speed in a benchtop centrifuge at 4 °C to remove cell debris. Protein concentration of the supernatant was measured with Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific) and the samples were stored at -20 °C until use.

Western blot analysis

Cell lysates (20 µg protein) were electrophoresed in 12-15% SDS polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 5% non-fat milk powder in phosphate buffered saline in 0.1% Tween-20 (PBST) solution for 1h. Primary antibodies were applied overnight at 4 °C, and secondary antibodies for 1 h at room temperature. Primary antibodies: anti-CYB5R3 (Santa Cruz, sc-398043), anti-CYB5 (Santa Cruz, sc-130311), anti-NCB5OR (Santa Cruz, sc-100529), anti-GAPDH (Santa Cruz, sc-32233), anti-SCD1 (#2438S) from Cell Signaling. Horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG (#7074) and horse anti-mouse IgG (#7076) were purchased from Cell Signaling. HRP was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

RT-PCR

Total RNA was purified from transfected HEK293T cells by using RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instruction and RNA samples were DNase I (Thermo Fisher Scientific) treated. cDNA samples were produced by reverse transcription of 0.5 µg DNA-free RNA using SuperScript III First-Strand Synthesis System for RT-PCR Kit (Thermo Fisher Scientific). Semi-quantitative measurement of *CYB5*, *CYB5R*, *SCD1* and *NCB5OR* expression was performed by PCR using the following primer pairs: CYB5 sense primer (5' – GCA CCA CAA GGT GTA CGA TT - 3') and CYB5 antisense primer (5' – TGA TAA GAG TTT CCG GAG GCT TG - 3'), CYB5R sense primer (5' – CCA GCT CAG CAC GTT GGG - 3') and CYB5R antisense primer (5' – GAG CCG AGA GGT AGA TGT GC - 3'), SCD1 sense primer (5' –GGC TTG CTG ATG ATG TGC TTC- 3') and SCD1 antisense primer (5' –AGG AGT GGT GGT AGT TGT GGA- 3'), NCB5OR sense primer (5' - ATG AAC TAA TGA GAG CAG CAG - 3') and NCB5OR antisense primer (5' - TGG CAA TGG

TGA CTA AAG AG - 3'). The PCR was elaborated by iProof High-Fidelity DNA Polymerase Kit (BioRad) with the following thermocycle conditions: 98 °C 1 min, then 25 cycles of 98 °C 10 sec, SCD1, NCB5OR: 55°C; CYB5, CYB5R: 65 °C 30 sec and 72 °C 30 sec. The final extension was 72 °C 10 min. As a reference control, a 261 nt fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was also amplified using GAPDH sense primer (5' - GTC CAC TGG CGT CTT CAC CA - 3') and antisense primer (5'- GTG GCA GTG ATG GCA TGG AC -3') The PCR thermocycle conditions were 98 °C 1 min, then 25 cycles of 98 °C 10 sec, 65 °C 30 sec and 72 °C 30 sec, completed by a final extension of 72 °C 10 min.

GC-FID analysis of fatty acid profiles

Measuring the saturated, unsaturated fatty acid content, cells were washed once with PBS, then harvested in 100 µl PBS by scraping. Samples were centrifuged for 5 min at 1,500 rpm in a benchtop centrifuge at room temperature and supernatants were discarded. Cell pellets were resuspended in 150 µl PBS. 100 µl of each suspension was transferred to a RNase-free microcentrifuge tube for total RNA isolation. 50 µl cell suspension was transferred to a clear crimp vial for GC-FID measurement. 150 µl of methanol containing 2 W/V% NaOH was added to the 50 µl cell suspension in the crimp vials, the samples were incubated at 90 °C for 30 min, and then cooled to room temperature. 400 µl of methanol containing 13-15% of boron trifluoride was added to the samples, and the vials were incubated at 90 °C for 30 min. After cooling to room temperature, 200 µl of saturated NaCl solution and 300 µl of n-hexane were added. Fatty acid methyl esters were extracted to the upper phase containing n-hexane, and this phase was transferred to a vial for GC analysis. GC analysis was carried out in a Shimadzu GC-2014 gas chromatograph equipped with a Zebron ZB-88 capillary column (60 m x 0.25 mm i.d., 0.20 µm film thickness) with an 88% (propyl-

nitrile)-aryl-polysiloxane stationary phase and a flame ionization detector (FID). For chromatographic separation of fatty acids, the following oven time-temperature program was used: the flow velocity was 35 ml/min, the temperature started at 100 °C and reached 210 °C with an increase of 5 °C/min. 1 µl of extracted sample was injected into the GC.

Statistics

Data are presented in the diagrams as mean values ± S.D. and were compared by ANOVA with Tukey's multiple comparison post hoc test using GraphPad Prism6 software. Differences of P value below 0.01 were considered to be statistically significant.

RESULTS

Effect of Scd1 or Ncb5or overexpression and/or palmitate administration on the fatty acid profile of HEK293T cells

Human SCD1 and NCB5OR cDNAs were inserted into pcDNA3.1- expression vector, and HEK293T cells were transiently transfected with the constructs of verified sequences. The efficiency of transfections and the expression level of the two genes were assessed by detecting the mRNAs and proteins in cell samples 24 h after transfection. Semi-quantitative RT-PCR analysis showed a well-detectable endogenous expression of both investigated genes in the control (i.e., empty vector-transfected) cells, and it also revealed a large increase in the appropriate mRNA expression levels upon transfection (Fig. 2A). Western blot analysis revealed mall amounts of endogenous Scd1 protein in control cells, and the amount of endogenous Ncb5or protein was hardly detectable. Transfection with an expression construct resulted in a pronounced elevation of the corresponding protein level (Fig. 2B).

The FA content of the cells was analyzed by GC-FID after saponification of complex lipids and a subsequent methyl esterification. The extent of FA desaturation was characterized by the calculated ratio of the two major monounsaturated over the two major saturated FAs, i.e. the Scd1-products palmitoleate (C16:1 cis Δ9) and oleate (C18:1 cis Δ9) over the Scd1-substrates palmitate (C16:0) and stearate (C18:0). Transfection with an empty vector did not cause any evident change in the cellular FA composition (Table 1). The unsaturated/saturated ratio was 1.25 ± 0.07 v.s. 1.25 ± 0.06 in the untransfected v.s. mock transfected cells, respectively, and the latter were used as control in the following experiments. Overexpression of Scd1 in the cells remarkably affected the FA profile, which is well reflected by a marked elevation in the palmitoleate and oleate contents of the cells (Table 1), the former of which was statistically significant, and also by an obvious increase in the unsaturated/saturated ratio (Fig. 2C.) Such a shift towards the unsaturated FAs, however, was not seen in the Ncb5or-

transfected cells as the unsaturated FA levels did not change, and the calculated ratio did not increase significantly (Fig. 2C and Table 1).

Since the protective role of Scd1 and Ncb5or was demonstrated in palmitate-treated cells, the potential effect of Scd1 and Ncb5or overexpression on the desaturating activity was also tested under the metabolic stress of palmitate administration, i.e. a saturated FA overload. Palmitate was administered as a BSA conjugate, thus a BSA only control was also implemented. BSA did not cause any obvious change in the FA profile while palmitate-treatment remarkably increased the palmitate and stearate contents of the cell (Table 1) and slightly lowered the unsaturated/saturated ratio of cellular FAs (Fig. 2D), which indicates that the treatment did challenge the desaturating capacity of the cells. Overexpression of Scd1 caused a marked increase in the amount of palmitoleate and oleate again, and hence affected the level of desaturation as the calculated ratio raised above the palmitate-treated and even above the palmitate-untreated controls but overexpression of Ncb5or still remained ineffective (Fig. 2D and Table 1).

Fatty acid desaturation in the cells overexpressing various components of the related electron transfer chains

The failure of Ncb5or overexpression to increase the desaturating activity of the cells suggests that either Ncb5or is unable to improve the electron supply of Scd1 or Scd1 does not need such an enhancement because the electron transfer chain is not saturated. To test these hypotheses, the level of desaturation was also studied at increased levels of the protein members of the classic chain, i.e. Cyb5R and Cyb5 or both.

Human CYB5 and CYB5R cDNAs were inserted into pcDNA3.1- expression vector, and HEK293T cells were transiently transfected with the constructs of verified sequences. The

efficiency of transfections and the expression level of the two genes were assessed by detecting the mRNAs and proteins in cell samples 24 h after transfection.

The endogenous mRNA expression of both genes was evident in the mock-transfected cells, and the RT-PCR analysis also revealed the expected elevation in the mRNA levels upon transfection (Fig. 3A). Little endogenous Cyb5R and even less Cyb5 proteins could be visualized in the Western blot images while these proteins were abundant in the cells transfected with the appropriate expression construct (Fig. 3B). Overexpression of Cyb5R or Cyb5 or their combination did not affect the FA profile of the cells remarkably; the unsaturated/saturated ratio remained below that of the control samples (Fig. 3C and Table 1).

Since none of the electron chain components could facilitate the activity of Scd1, we concluded that the strengthening of the electron transfer chain remains ineffective probably because its potential is not maximized. Therefore, the effect of Cyb5R, Cyb5, Cyb5R+Cyb5 and Ncb5or overexpressions were also investigated at the conditions of accelerated desaturation in Scd1-transfected cells. Although double and triple transfections did not elevate the individual protein levels as effectively as the corresponding single transfections, a remarkable increase in the quantity of each gene product was also seen in the co-transfected cell samples compared to control (Fig. 3B). It is probably due to relatively lower Scd1 protein levels that co-transfection of Cyb5R, Cyb5, Cyb5R+Cyb5 or Ncb5or with Scd1 slightly decreased the unsaturated/saturated ratio compared to single Scd1 overexpression. Nevertheless, the expected synergistic elevation in the desaturation level was not seen in any case (Fig. 3D and Table 1).

DISCUSSION

FA metabolism plays a critical role in energy homeostasis, and it has a major impact on membrane fluidity and intracellular signaling too. Due to their amphipathic character and limited water-solubility, non-esterified also known as free FAs (FFAs) are adsorbed to albumin in the blood plasma, and they easily penetrate the cell membranes for intracellular utilization. It is also due to this amphipathic nature that FA processing (i.e., elongation and desaturation) is carried out by membrane-associated enzyme systems, which are usually localized in the ER. Human cells are also capable of creating double bonds of cis configuration in CoA-conjugated FA chains. The first double bond in a saturated chain is formed invariably between the 9^{th} and 10^{th} carbon atoms by Scd1 [12], and additional double bonds can be inserted subsequently by other desaturase enzymes into the already mono-unsaturated acyl-CoAs [13]. Scd1, therefore, catalyzes the committed and rate-determining step in the generation of endogenous unsaturated acyl-CoAs. Maintenance of an unsaturated acyl-CoA pool for a balanced supply of saturated and unsaturated acyl-CoAs is essential for an efficient triglyceride biosynthesis [8, 14] and for the preservation of appropriate membrane fluidity [15, 16].

Mounting evidence supports contribution of elevated FFA levels to the development of various severe pathologies (e.g., cardiovascular diseases, non-alcoholic fatty liver disease, the metabolic syndrome, diabetes and cancer). Excessive FA supply promotes cellular dysfunction through a combination of metabolic and signaling stress, referred to as lipotoxicity and it can even lead to a programmed cell death, called lipoapoptosis [17]. The ER is a primary target of lipotoxicity. An FFA-induced and receptor-mediated activation of phospholipase C (PLC) [18], as well as the membrane permeabilizing effect of the accumulating cytoplasmic fatty acyl-CoAs [19] cause a Ca^{2+} depletion in the ER [20], and hence interfere with luminal protein folding in the organelle. The lipotoxic ER stress plays an

important role in the above-mentioned diseases through enhancing insulin resistance, inflammation, autophagy and apoptosis.

The mechanisms underlying FA toxicity have been extensively studied in cultured cells, and it became evident that saturated FAs (e.g. palmitate) cause a much greater damage than either cis or trans mono unsaturated FAs (e.g. oleate) at the same concentration. Moreover, a simultaneous addition of oleate often ameliorates the toxicity of palmitate. It is a putative explanation that saturated acyl-CoAs on their own cannot be readily channeled into TG synthesis due to the inefficient acylation of fully saturated DG intermediates, which is supported by the accumulation of DGs in palmitate-treated cells [21]. Another possible explanation is based on the finding that increasing saturation of the ER membrane can trigger the ER stress response independently of the sensing of unfolded proteins in the organelle [22]. Regardless of the mechanisms that make the saturated FA overload highly toxic, the phenomenon itself highlights the importance of local acyl-CoA desaturation in the cells, and the potential protective role of Scd1 against saturated FA-induced toxicity.

This protective role of desaturation has been indeed demonstrated in some in vitro studies [7, 23-27], and further supported by the findings in SCD1 muscle transgenic mice [28]. It is to be noted that SCD1 knock-out mice are resistant to diet-induced weight gain and obesity-related metabolic disease [29] but it is due to a defective synthesis of TGs and cholesteryl esters in the lean animals, and does not contradict the importance of acyl-CoA desaturation in the cells under the pressure of high FFA levels. Furthermore, Ncb5or a cytosolic [11] oxidoreductase providing an alternative electron transfer route for Scd1 [30] has been also shown to strengthen the anti-lipotoxic defense of the cells [31]. It is an intriguing question, however, whether the inner desaturating power of the cell is determined by the expression level of Scd1 enzyme itself or by the conductivity of the associated electron transfer chains.

Overexpression of Scd1 enzyme alone in the transfected cells caused a remarkable shift in the ratio of unsaturated and saturated FAs, which indicates the enhancement of desaturase activity without any metabolic tension. However, the cells, in which the components of the classic electron transfer chain (i.e. Cyb5 and/or Cyb5R) or the alternative electron supplier Ncb5or were overexpressed, did not present any sign of accelerated desaturation as the unsaturated over saturated FA ratio remained unchanged. This pattern indicates that the wild type cell employs Scd1 at its full capacity, and the substrate and electron supply allows a more intense desaturation as soon as the amount of Scd1 enzyme increases. This in turn is only consistent with the rate-limiting role of Scd1 in the overall process of Δ9 desaturation. We assumed, however, that the electron supply provided by Cyb5 and Cyb5R or by Ncb5or may be exhausted by a higher demand, which derives from an enhanced Scd1 activity; and hence in the palmitate-treated or Scd1 transfected cells, the electron transfer might become rate-limiting. However, even these conditions did not allow overexpression of the electron transfer proteins to intensify desaturation in the cells.

We concluded, therefore, that in our cellular model, Scd1 enzyme itself catalyzes the rate-limiting step of the overall process of FA Δ^9 desaturation, and this essential metabolic function cannot be facilitated by reinforcing the electron supply of the enzyme in this cell line. Our findings are in accordance with the observations that Scd1 expression is increased upon palmitate treatment in carious cells, such as cultured myotubes [24], L6GLUT4myc myoblasts [32], MIN6 insulinoma cells [33] and in HepG2 cells [34]. No data is available on a similar palmitate dependent or other saturated FA dependent induction of Cyb5, Cyb5R or Ncb5or. Cells might use Scd1 induction as a defense mechanism in saturated FA overload since an elevated Scd1 enzyme level on its own accelerates acyl-CoA desaturation and helps restoring a balanced intracellular FA pool.

Acknowledgments

We thank Ms. Valéria Mile for skillful technical assistance. This work was supported by the Hungarian National Research, Development and Innovation Office (NKFIH grant number: K 125201) and by the Higher Education Excellence Program of the Ministry of Human Capacities in the frame of Biotechnology research area of Budapest University of Technology and Economics (BME FIKP-BIO). Éva Kereszturi is a grantee of the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

References

1. Zambo, V., Simon-Szabo, L., Szelenyi, P., Kereszturi, E., Banhegyi, G. & Csala, M. (2013) Lipotoxicity in the liver, *World journal of hepatology.* **5**, 550-7.
2. Sarnyai, F., Donko, M. B., Matyasi, J., Gor-Nagy, Z., Marczi, I., Simon-Szabo, L., Zambo, V., Somogyi, A., Csizmadia, T., Low, P., Szelenyi, P., Kereszturi, E., Toth, B. & Csala, M. (2019) Cellular toxicity of dietary trans fatty acids and its correlation with ceramide and diglyceride accumulation, *Food Chem Toxicol.* **124**, 324-335.
3. Bosma, M., Dapito, D. H., Drosatos-Tampakaki, Z., Huiping-Son, N., Huang, L. S., Kersten, S., Drosatos, K. & Goldberg, I. J. (2014) Sequestration of fatty acids in triglycerides prevents endoplasmic reticulum stress in an in vitro model of cardiomyocyte lipotoxicity, *Biochimica et biophysica acta.* **1841**, 1648-55.
4. Zhang, Y., Dong, L., Yang, X., Shi, H. & Zhang, L. (2011) alpha-Linolenic acid prevents endoplasmic reticulum stress-mediated apoptosis of stearic acid lipotoxicity on primary rat hepatocytes, *Lipids Health Dis.* **10**, 81.
5. Iwai, T., Kume, S., Chin-Kanasaki, M., Kuwagata, S., Araki, H., Takeda, N., Sugaya, T., Uzu, T., Maegawa, H. & Araki, S. I. (2016) Stearoyl-CoA Desaturase-1 Protects Cells against Lipotoxicity-Mediated Apoptosis in Proximal Tubular Cells, *International journal of molecular sciences.* **17**.
6. Cai, D., Fan, J., Ma, D., Wu, Y. & Lu, Y. (2014) [SCD1 over-expression inhibits palmitic acid-induced apoptosis of rat BRL hepatocytes], *Zhonghua gan zang bing za zhi = Zhonghua ganzangbing zazhi = Chinese journal of hepatology.* **22**, 48-52.
7. Green, C. D. & Olson, L. K. (2011) Modulation of palmitate-induced endoplasmic reticulum stress and apoptosis in pancreatic beta-cells by stearoyl-CoA desaturase and Elovl6, *American journal of physiology Endocrinology and metabolism.* **300**, E640-9.
8. Ntambi, J. M. & Miyazaki, M. (2004) Regulation of stearoyl-CoA desaturases and role in metabolism, *Progress in lipid research.* **43**, 91-104.
9. Larade, K. & Bunn, H. F. (2006) Promoter characterization and transcriptional regulation of Ncb5or, a novel reductase necessary for pancreatic beta-cell maintenance, *Biochimica et biophysica acta.* **1759**, 257-62.
10. Zhu, H., Qiu, H., Yoon, H. W., Huang, S. & Bunn, H. F. (1999) Identification of a cytochrome b-type NAD(P)H oxidoreductase ubiquitously expressed in human cells, *Proceedings of the National Academy of Sciences of the United States of America.* **96**, 14742-7.
11. Zambo, V., Toth, M., Schlachter, K., Szelenyi, P., Sarnyai, F., Lotz, G., Csala, M. & Kereszturi, E. (2016) Cytosolic localization of NADH cytochrome b(5) oxidoreductase (Ncb5or), *FEBS letters.* **590**, 661-71.
12. Miyazaki, M. & Ntambi, J. M. (2003) Role of stearoyl-coenzyme A desaturase in lipid metabolism, *Prostaglandins, leukotrienes, and essential fatty acids.* **68**, 113-21.
13. Guillou, H., Zadravec, D., Martin, P. G. & Jacobsson, A. (2010) The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice, *Progress in lipid research.* **49**, 186-99.
14. Mantzaris, M. D., Tsianos, E. V. & Galaris, D. (2011) Interruption of triacylglycerol synthesis in the endoplasmic reticulum is the initiating event for saturated fatty acid-induced lipotoxicity in liver cells, *The FEBS journal.* **278**, 519-30.
15. Goni, F. M. & Alonso, A. (1999) Structure and functional properties of diacylglycerols in membranes, *Progress in lipid research.* **38**, 1-48.
16. Calder, P. C., Yaqoob, P., Harvey, D. J., Watts, A. & Newsholme, E. A. (1994) Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity, *The Biochemical journal.* **300 (Pt 2)**, 509-18.

17. Diakogiannaki, E., Welters, H. J. & Morgan, N. G. (2008) Differential regulation of the endoplasmic reticulum stress response in pancreatic beta-cells exposed to long-chain saturated and monounsaturated fatty acids, *The Journal of endocrinology.* **197**, 553-63.
18. Xu, S., Nam, S. M., Kim, J. H., Das, R., Choi, S. K., Nguyen, T. T., Quan, X., Choi, S. J., Chung, C. H., Lee, E. Y., Lee, I. K., Wiederkehr, A., Wollheim, C. B., Cha, S. K. & Park, K. S. (2015) Palmitate induces ER calcium depletion and apoptosis in mouse podocytes subsequent to mitochondrial oxidative stress, *Cell death & disease.* **6**, e1976.
19. Banhegyi, G., Csala, M., Mandl, J., Burchell, A., Burchell, B., Marcolongo, P., Fulceri, R. & Benedetti, A. (1996) Fatty acyl-CoA esters and the permeability of rat liver microsomal vesicles, *The Biochemical journal.* **320 (Pt 1)**, 343-4.
20. Egnatchik, R. A., Leamy, A. K., Jacobson, D. A., Shiota, M. & Young, J. D. (2014) ER calcium release promotes mitochondrial dysfunction and hepatic cell lipotoxicity in response to palmitate overload, *Molecular metabolism.* **3**, 544-53.
21. Lee, J. Y., Cho, H. K. & Kwon, Y. H. (2010) Palmitate induces insulin resistance without significant intracellular triglyceride accumulation in HepG2 cells, *Metabolism: clinical and experimental.* **59**, 927-34.
22. Volmer, R., van der Ploeg, K. & Ron, D. (2013) Membrane lipid saturation activates endoplasmic reticulum unfolded protein response transducers through their transmembrane domains, *Proceedings of the National Academy of Sciences of the United States of America.* **110**, 4628-33.
23. Thorn, K., Hovsepyan, M. & Bergsten, P. (2010) Reduced levels of SCD1 accentuate palmitate-induced stress in insulin-producing beta-cells, *Lipids in health and disease.* **9**, 108.
24. Peter, A., Weigert, C., Staiger, H., Machicao, F., Schick, F., Machann, J., Stefan, N., Thamer, C., Haring, H. U. & Schleicher, E. (2009) Individual stearoyl-coa desaturase 1 expression modulates endoplasmic reticulum stress and inflammation in human myotubes and is associated with skeletal muscle lipid storage and insulin sensitivity in vivo, *Diabetes.* **58**, 1757-65.
25. Matsui, H., Yokoyama, T., Sekiguchi, K., Iijima, D., Sunaga, H., Maniwa, M., Ueno, M., Iso, T., Arai, M. & Kurabayashi, M. (2012) Stearoyl-CoA desaturase-1 (SCD1) augments saturated fatty acid-induced lipid accumulation and inhibits apoptosis in cardiac myocytes, *PloS one.* **7**, e33283.
26. Peter, A., Weigert, C., Staiger, H., Rittig, K., Cegan, A., Lutz, P., Machicao, F., Haring, H. U. & Schleicher, E. (2008) Induction of stearoyl-CoA desaturase protects human arterial endothelial cells against lipotoxicity, *American journal of physiology Endocrinology and metabolism.* **295**, E339-49.
27. Ikeda, J., Ichiki, T., Takahara, Y., Kojima, H., Sankoda, C., Kitamoto, S., Tokunou, T. & Sunagawa, K. (2015) PPARgamma Agonists Attenuate Palmitate-Induced ER Stress through Up-Regulation of SCD-1 in Macrophages, *PloS one.* **10**, e0128546.
28. Rogowski, M. P., Flowers, M. T., Stamatikos, A. D., Ntambi, J. M. & Paton, C. M. (2013) SCD1 activity in muscle increases triglyceride PUFA content, exercise capacity, and PPARdelta expression in mice, *Journal of lipid research.* **54**, 2636-46.
29. Ntambi, J. M., Miyazaki, M., Stoehr, J. P., Lan, H., Kendziorski, C. M., Yandell, B. S., Song, Y., Cohen, P., Friedman, J. M. & Attie, A. D. (2002) Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity, *Proceedings of the National Academy of Sciences of the United States of America.* **99**, 11482-6.
30. Larade, K., Jiang, Z., Zhang, Y., Wang, W., Bonner-Weir, S., Zhu, H. & Bunn, H. F. (2008) Loss of Ncb5or results in impaired fatty acid desaturation, lipoatrophy, and diabetes, *The Journal of biological chemistry.* **283**, 29285-91.

31. Zhang, Y., Larade, K., Jiang, Z. G., Ito, S., Wang, W., Zhu, H. & Bunn, H. F. (2010) The flavoheme reductase Ncb5or protects cells against endoplasmic reticulum stress-induced lipotoxicity, *Journal of lipid research.* **51**, 53-62.
32. Voss, M. D., Beha, A., Tennagels, N., Tschank, G., Herling, A. W., Quint, M., Gerl, M., Metz-Weidmann, C., Haun, G. & Korn, M. (2005) Gene expression profiling in skeletal muscle of Zucker diabetic fatty rats: implications for a role of stearoyl-CoA desaturase 1 in insulin resistance, *Diabetologia.* **48**, 2622-30.
33. Busch, A. K., Gurisik, E., Cordery, D. V., Sudlow, M., Denyer, G. S., Laybutt, D. R., Hughes, W. E. & Biden, T. J. (2005) Increased fatty acid desaturation and enhanced expression of stearoyl coenzyme A desaturase protects pancreatic beta-cells from lipoapoptosis, *Diabetes.* **54**, 2917-24.
34. Rojas, C., Pan-Castillo, B., Valls, C., Pujadas, G., Garcia-Vallve, S., Arola, L. & Mulero, M. (2014) Resveratrol enhances palmitate-induced ER stress and apoptosis in cancer cells, *PloS one.* **9**, e113929.

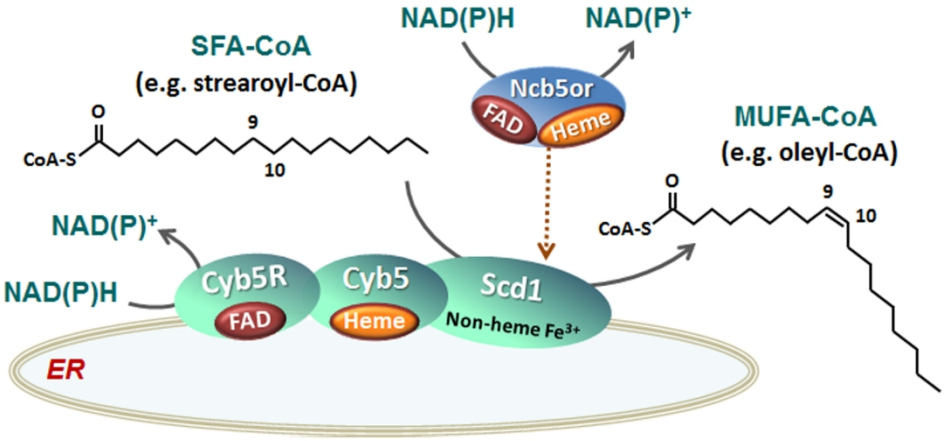

Fig. 1. Electron transfer chains of microsomal fatty acyl-CoA desaturation. Saturated fatty acids can be desaturated by integral enzymes of the endoplasmic reticulum (ER) membrane. The first double bond is formed at carbon 9 in the saturated acyl-CoAs by a non-heme iron containing protein, stearoyl-CoA desaturase (Scd1). Scd1 converts saturated acyl-CoA-s (SFA-CoA), such as stearoyl-CoA (C18:0) or palmitoyl-CoA (C16:0) to Δ9 mono-unsaturated derivatives (MUFA-CoA), oleyl-CoA (C18:1 cis Δ9) or palmitoleyl-CoA (C16:1 cis Δ9), respectively. Cytosolic NAD(P)H feeds the process with electrons either through the flavoprotein cytochrome b5 reductase (Cyb5R) and the hemoprotein cytochrome b5 (Cyb5) or through the Cyb5R-like and Cyb5-like domains of NADH cytochrome b5 oxidoreductase (Ncb5or).

199x93mm (300 x 300 DPI)

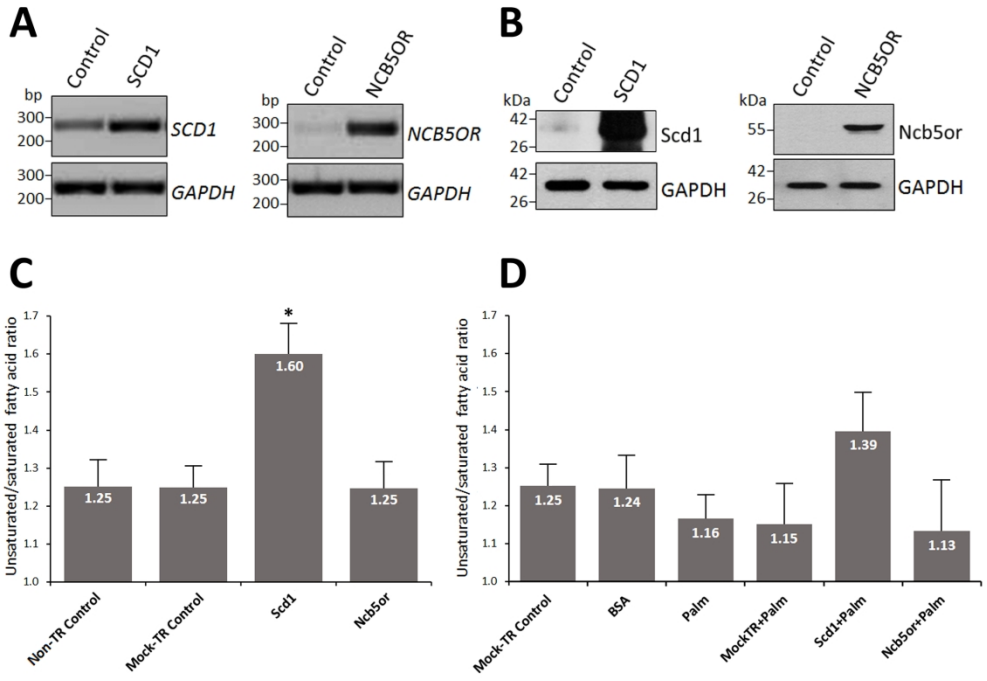

Fig. 2. Changes in the fatty acid profile of the Scd1 or Ncb5or transfected HEK293T cells. A. SCD1 and NCB5OR mRNA levels were monitored in transiently transfected HEK293T cells by using semi-quantitative RT-PCR with gene specific primers and by using GAPDH as a housekeeping reference gene. A representative ethidium bromide-stained agarose gel image is shown. B. The amount of Scd1 and Ncb5or proteins was assessed by Western blot analysis of cell lysates using specific antibodies and GAPDH as a loading and internal control. A representative immunoblot is shown. C. Cells were mock-transfected or transfected with the plasmid expressing SCD1 or NCB5OR genes. They were harvested and FA methyl esters were produced by saponification and methylation 24 h after transient transfection. D. The cells (control or 24 h transfected) were treated with BSA or BSA-conjugated palmitate (50 μM) for 15 h. Cells were then harvested, and FA methyl esters were produced by saponification and methylation. C and D. The amount of saturated (C16:0, C18:0) and mono-unsaturated (C16:1 cis Δ9, C18:1 cis Δ9) fatty acids was measured by GC-FID, and the ratio of unsaturated/saturated fatty acids was calculated. Data are shown in the diagrams as mean values ± S.D.; n=3; *P < 0.01 vs. mock transfected control.

199x145mm (300 x 300 DPI)

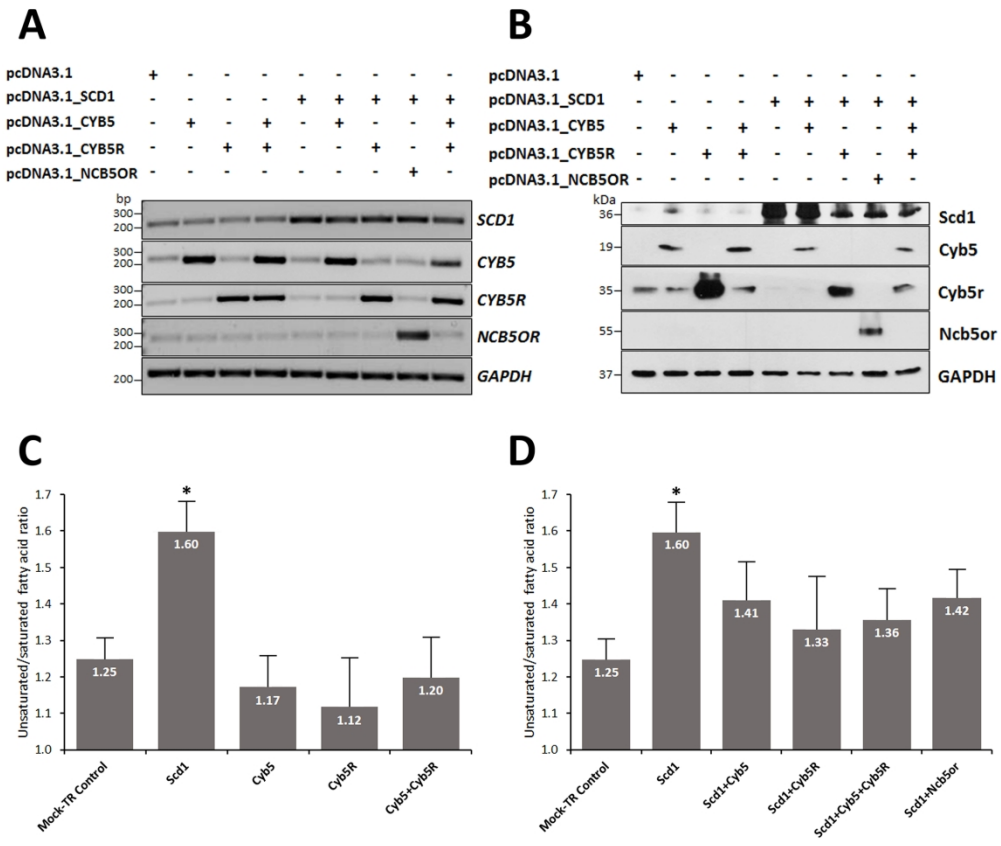

Fig. 3. Fatty acid desaturation in the cells overexpressing various components of the related electron transfer chains. HEK293T cells were mock-transfected or transfected with plasmids expressing SCD1, CYB5, CYB5R or NCB5OR genes and their combinations. A. The mRNA levels were compared by using semi-quantitative RT-PCR with gene specific primers and by using GAPDH as a housekeeping reference gene. A representative ethidium bromide-stained agarose gel of three independent experiments is shown. B. The amount of Scd1, Cyb5, Cyb5R and Ncb5or proteins was assessed by Western blot analysis of cell lysates using specific antibodies and GAPDH as a loading and internal control. A representative immunoblot of three independent experiments is shown. C and D. Cells were mock-transfected or transfected with the expression constructs as indicated. They were harvested and FA methyl esters were produced by saponification and methylation 24 h after transient transfection. The amount of saturated (C16:0, C18:0) and mono-unsaturated (C16:1 cis Δ9, C18:1 cis Δ9) fatty acids was measured by GC-FID, and the ratio of unsaturated/saturated fatty acids was calculated. Data are shown in the diagrams as mean values ± S.D.; n=3; *P < 0.01 vs. mock transfected control.

199x176mm (300 x 300 DPI)

	Amount of fatty acid (μg/mg)			
	C16:0 (palmitate)	C16:1 (palmitoleate)	C18:0 (stearate)	C18:1 (oleate)
Non-TR control	35.34 ± 2.64	15.06 ± 0.99	14.42 ± 1.17	47.23 ± 2.72
Mock-TR control	36.73 ± 2.13	16.36 ± 1.13	14.79 ± 1.11	48.01 ± 3.03
Scd1	34.57 ± 3.05	25.52 ± 1.84*	14.56 ± 1.32	53.08 ± 3.63
Ncb5or	37.12 ± 3.12	16.27 ± 1.34	15.22 ± 1.28	48.95 ± 3.03
BSA	37.59 ± 2.28	15.32 ± 1.01	15.09 ± 1.02	50.17 ± 2.94
Palm	44.35 ± 3.44	18.49 ± 1.33	16.57 ± 1.63	52.42 ± 3.86
Mock-TR+Palm	42.36 ± 4.56	18.95 ± 1.46	14.50 ± 1.78	46.36 ± 3.62
Scd1+Palm	44.63 ± 4.16	30.40 ± 2.39*#	15.03 ± 1.82	52.66 ± 3.16
Ncb5or+Palm	48.75 ± 4.33*	21.28 ± 1.57	16.57 ± 1.35	52.59 ± 4.06
Cyb5	33.98 ± 2.14	14.20 ± 1.20	13.19 ± 1.11	41.08 ± 3.11
Cyb5R	38.91 ± 3.19	12.78 ± 1.29	14.14 ± 1.51	46.50 ± 3.85
Cyb5+Cyb5R	32.45 ± 2.82	12.68 ± 1.79	12.24 ± 0.99	40.89 ± 3.08
Scd1+Cyb5	33.44 ± 2.45	21.79 ± 1.86	13.96 ± 1.01	45.11 ± 3.75
Scd1+Cyb5R	36.40 ± 2.96	21.83 ± 1.58	15.45 ± 1.53	47.24 ± 3.23
Scd1+Cyb5+Cyb5R	32.07 ± 3.03	19.06 ± 1.84	11.67 ± 0.89	40.31 ± 2.98
Scd1+Ncb5or	39.83 ± 3.20	25.38 ± 1.38*	16.42 ± 1.13	54.48 ± 3.27

*P < 0.01 vs. mock transfected control; #P < 0.01 vs. palmitate-treated mock transfected cells.