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Obesity

α-Lipoic Acid Reduces Fatty Acid Esterification and Lipogenesis in Adipocytes from Overweight/Obese Subjects

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Objective: α-Lipoic acid (α-LA) is a natural occurring antioxidant with beneficial effects on obesity. The aim of this study was to investigate the putative effects of α-LA on triglyceride accumulation and lipogenesis in subcutaneous adipocytes from overweight/obese subjects and to determine the potential mechanisms involved.

Methods: Fully differentiated human subcutaneous adipocytes were treated with α-LA (100 and 250 μM) during 24 h for studying triglyceride content, *de novo* lipogenesis, and levels of key lipogenic enzymes. The involvement of AMP-activated protein kinase (AMPK) activation was also evaluated.

Results: α-LA down-regulated triglyceride content by inhibiting fatty acid esterification and *de novo* lipogenesis. These effects were mediated by reduction in fatty acid synthase (FAS), stearoyl-coenzyme A desaturase 1, and diacylglycerol O-acyltransferase 1 protein levels. Interestingly, α-LA increased AMPK and acetyl CoA carboxylase phosphorylation, while the presence of the AMPK inhibitor Compound C reversed the inhibition observed on FAS protein levels.

Conclusions: α-LA down-regulates key lipogenic enzymes, inhibiting lipogenesis and reducing triglyceride accumulation through the activation of AMPK signaling pathway in human subcutaneous adipocytes from overweight/obese subjects.

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Introduction

Alpha-lipoic acid (5-(1,2-dithiolan-3-yl)-pentanoic acid; α-LA) is a natural occurring antioxidant compound with demonstrated anti-obesity properties in rodents (1,2). While the body weight lowering actions of α-LA in humans remain controversial, some recent clinical trials in overweight/obese humans have revealed that α-LA moderately reduced body weight, fat mass, and BMI (3,4), which was accompanied by a decrease in total cholesterol (4), inflammatory markers such as interleukin 6 (IL-6), tumor necrosis factor-α or CRP (3), and haemoglobin-A1c in obese individuals with type 2 diabetes (5). The anti-obesity actions of α-LA have been related to its ability to reduce food intake and to increase energy expenditure (6). Furthermore, several studies have revealed that adipose tissue is a key target in the body weight lowering and insulin-sensitizing actions of α-LA (1,7). Thus, in a previous study, our group demonstrated that α-LA stimulates lipolysis rates in adipocytes, which could contribute to adiposity reduction (8). Other mechanism that could also be involved in α-LA fat mass lowering properties is the modulation of triglyceride accumulation in adipocytes. In this sense, triglycerides that are going to be stored in adipose tissue are mainly acquired from circulating lipoproteins, chylomicrons, and VLDL. These triglycerides require intravascular hydrolysis by lipoprotein lipase to liberate free fatty acids (FFA) which are then uptaken by adipocytes through specific FA transporters such as FATP, FABP, or CD36 (9). Once the FFA are inside the adipocyte they are esterified into triglycerides and stored in the lipid droplet. Traditionally, a minor

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Author contributions: MF-G carried out experiments, analyzed data and prepared the first draft of the manuscript, MJM-A designed research, raised grants, analyzed data and coordinated the project, PP-M designed research and analyzed data, PLP-H, MH, NS and ML-Y carried out experiments, JAM and DL helped in the implementation of the experimental design. All authors were involved in writing and critical review of the paper and have approved the final version of the manuscript. Additional Supporting Information may be found in the online version of this article.

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contribution to whole body lipid stores in human diet-induced obesity has been attributed to adipose tissue lipogenesis (10). However, recent studies have demonstrated that the inhibition of related lipogenic enzymes could be an effective strategy against obesity and diabetes conditions, having a signaling function beyond the generation of lipid stores (11). In fact, it has been reported that *de novo* lipogenesis not only contributes to the rapid recovery of fat in adipose tissue, but also acts as a glucose sink that allows glycaemia to be maintained within the range of physiological values (12). Thus, mice with targeted deletion of adipose tissue fatty acid synthase (FAS) showed increased energy expenditure and decreased adiposity, which was accompanied by an enhanced insulin sensitivity and glucose tolerance when fed on a high-fat diet (11). Although α-LA has revealed important anti-lipogenic actions in liver in rodents (13,14), there is no information available about the effects of α-LA on lipogenesis in human adipocytes. Thus, the aim of this study was to evaluate the putative effects of α-LA on triglyceride accumulation and lipogenesis in subcutaneous adipocytes from overweight/obese subjects; the potential underlying mechanisms were also examined.

Methods

Cell culture and differentiation of human subcutaneous pre-adipocytes

Commercially available cryopreserved human subcutaneous pre-adipocytes from nondiabetic overweight-obese female donors (BMI: 26.85-33.37 kg/m^2) were purchased from Zen-Bio (Research Triangle Park, NC) and differentiated according to manufacturer's instructions. Briefly, cryopreserved pre-adipocytes were plated in 12 wells plates (Nunc A/S; Roskilde, Denmark) at 40,000 cells/cm^2 and cultured in an incubator set up to 37°C in a humidified 5% CO_2 atmosphere in pre-adipocyte medium (PM-1; DMEM/Ham's F-12 medium, HEPES, FBS, penicillin, streptomycin, amphotericin B; Zen-Bio). Cells were fed every other day with 1 ml of PM-1 until confluent. To induce differentiation, PM-1 medium was replaced with 1 ml of differentiation medium (DM2; Zen-Bio) including biotin, pantothenate, human insulin, dexamethasone, isobutylmethylxanthine, and a PPARγ agonist (days 0-7). After 7 days, 600 µl of DM-2 medium were removed and 800 µl of adipocyte medium (AM1; Zen-Bio), which included PM-1, biotin, pantothenate, human insulin, and dexamethasone was added. Cells were incubated for additional 2 days, and 800 µl of media were replaced by 800 µl of fresh AM1. By day 14 of incubation, cells contained large lipid droplets and were considered mature adipocytes.

Treatments

Before treatments, cell media was removed and replaced with 1 ml of fresh AM1. α-LA (Sigma; St. Louis, MO) was dissolved in ethanol. The AMP-activated protein kinase (AMPK) activator AICAR (Sigma) was dissolved in ultrapurified water and the selective AMPK antagonist Compound C in DMSO. About 1000× stocks were prepared and 1 µl/ml of media was added. When Compound C (Calbiochem; San Diego, CA) (20 µM) was used, cells were pre-incubated during 1 h. Control cells were treated with the same amount of the corresponding vehicle.

Triglyceride measurement

After 24 h of treatment with α-LA, adipocytes were homogenized in lysis buffer and diluted at a final protein concentration of 50 mg/ml. Homogenates were diluted (1:1) in 1% deoxycholate (Sigma) and incubated at 37°C for 5 min. Triglyceride content was evaluated by using the Infinity Triglycerides Liquid Stable Reagent (Thermo Electron Corporation, Louisville, CO, USA) following manufacturer's instructions. Data were normalized by protein content and expressed as fold change of control.

Fatty acid incorporation to triglycerides

Fatty acid incorporation to triglycerides in human subcutaneous adipocytes was carried out as previously described (15). Briefly, differentiated human adipocytes were treated during 24 h with or without α-LA in AM-1, then cells were washed with PBS and incubated for 4 h in Krebs-Ringer buffer without glucose, containing 125 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.25 mM KH_2PO_4, 1.25 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$) pH 7.8 containing 3% fatty acid-free BSA pH 7.8, 2 mM L-carnitine, 80 μM palmitic acid (Sigma), and 20 μM ^{14}C-palmitic acid (58 μCi/μmol, Perkin Elmer; Waltham, MA). Then, cells were washed with PBS and scraped in cold buffer (0.25 M sucrose; 10 mM Tris HCl; 1 mM EDTA; 1 mM dithiothreitol, pH 7.4). Neutral lipids were extracted by adding 5 vol chloroform/methanol (2:1) and 0.4 vol 1 M KCl/HCl. Lipids were separated by thin-layer chromatography to measure labeled palmitate incorporation into triglycerides using heptane-isopropylether-acetic acid (60:40:4, v/v/v) as developing solvent. Results were normalized to total protein content of cell extracts.

Glucose incorporation into triglycerides

For glucose incorporation into triglycerides, cells were incubated for 4 h in Krebs-Ringer buffer containing 3% BSA, 10 mM HEPES, 2 mM glucose, and 0.5 μCi/ml ^{14}C-D-glucose (56.3 mCi/mmol; American radiolabeled chemicals, St. Louis, MO). After 4 h of incubation, cells were washed with cold PBS and then scraped in cold buffer (0.25 M sucrose; 10 mM Tris HCl; 1 mM EDTA; 1 mM dithiothreitol, pH 7.4). Neutral lipids were extracted by adding 5 vol chloroform/methanol (2:1). Lipids were separated by thin-layer chromatography to measure labeled glucose incorporation into triglycerides using heptane-isopropylether-acetic acid (60:40:4, v/v/v) as developing solvent. The results were normalized to total protein content of cell lysates.

Western blot analysis

Western blot analysis was performed in 14 days post-differentiation adipocytes. Cells were incubated in AM-1 with or without the appropriate treatment during 24 h. Lysates were then obtained by the addition of a buffer containing: 2 mM Tris HCl (pH 8); 137 mM NaCl; 2 mM EDTA; 1% protease inhibitor cocktail 1 (Sigma); 1 mM Sodium orthovanadate; and 1 mM PMSF. Protein extracts were collected after sample centrifugation. Proteins were quantified with the BCA method (Pierce-Thermo Scientific, Rockford, IL) according to the supplier's instructions. Total proteins were resolved in SDS-PAGE minigels and electroblotted onto PVDF membranes (GE Healthcare Europe GmbH, Barcelona, Spain). The membranes were blocked and incubated with specific antibodies against FAS, AMPK, phospho-Thr^{172} AMPK, Acetyl CoA carboxylase (ACC), phospho-Ser^{79} ACC (Cell signaling, Beverly, MA); Stearoyl-Coenzyme A desaturase 1 (SCD1), Diacylglycerol O-Acyltransferase 1 (DGAT1) (Abcam, Cambridge, UK); and Actin (Sigma). Secondary antibody was horseradish peroxidase goat anti-rabbit IgG-HRP (Bio Rad Laboratories, Hercules, CA). The immunoreactive proteins were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA). Band intensities were quantified using a GS-800 calibrated densitometer (Bio Rad Laboratories). In some cases, infrared fluorescent secondary antibodies (Cell signaling) were used and quantitated using an Odyssey scanner (LI-COR Biosciences, Lincoln, USA).

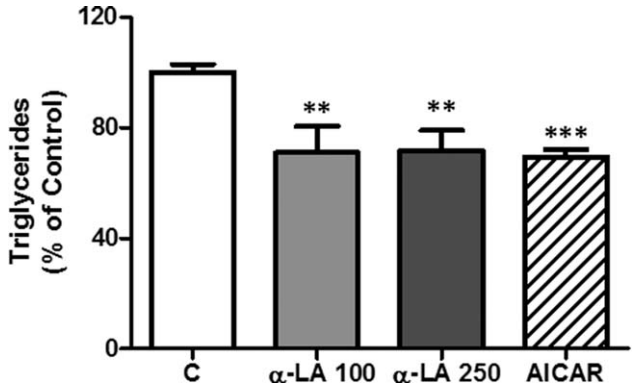

Figure 1 α-LA reduces triglyceride accumulation in human subcutaneous adipocytes from overweight/obese subjects. Mature adipocytes were treated with α-LA (100 and 250 μM) or AICAR (2 mM) during 24 h; cells were lysed and intracellular triglyceride content was assayed. Data are expressed as mean ± S.E. of at least six independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control (vehicle-treated cells). Abbreviations: α-LA, α-lipoic acid.

Data analysis

Data are expressed as mean with standard errors (SE). Differences were set up as statistically significant at $P < 0.05$. Comparisons between the values for different variables were analyzed by one-way ANOVA, followed by Bonferroni post hoc tests, or by Student's *t* test or *U*-Mann Whitney as appropriate after testing the normality with the Kolmogorov-Smirnoff and Shapiro-Wilk tests. SPSS 19.0 version for Windows (SPSS, Chicago, IL) and GraphPad Prism 5.0 (Graph-Pad Software, San Diego, CA) were used for the statistical analysis.

Results

α-LA reduces triglyceride content in human subcutaneous adipocytes

A significant decrease on triglyceride content was found in abdominal subcutaneous adipocytes from overweight/obese subjects after 24 h of treatment with α-LA (100 and 250 μM; $P < 0.01$). A similar reduction ($P < 0.001$) on triglyceride content was observed in adipocytes treated with the AMPK activator AICAR (Figure 1).

α-LA inhibits lipogenesis in human subcutaneous adipocytes

To evaluate if the α-LA triglyceride-lowering effects were caused by an inhibition of lipogenesis, both fatty acid esterification and glucose incorporation into triglycerides were evaluated. Twenty-four hours of treatment with α-LA (100 and 250 μM) induced an inhibition of ^{14}C-palmitic acid incorporation into triglycerides ($P < 0.05$ and 0.01, respectively; Figure 2A). Moreover, α-LA also caused a significant reduction in *de novo* lipogenesis, which was analyzed by the ^{14}C-glucose incorporation into triglycerides ($P < 0.05$; Figure 2B).

α-LA represses lipogenic enzymes in human subcutaneous adipocytes

For a better understanding of the mechanisms involved in the inhibition of triglyceride accumulation induced by α-LA, several key

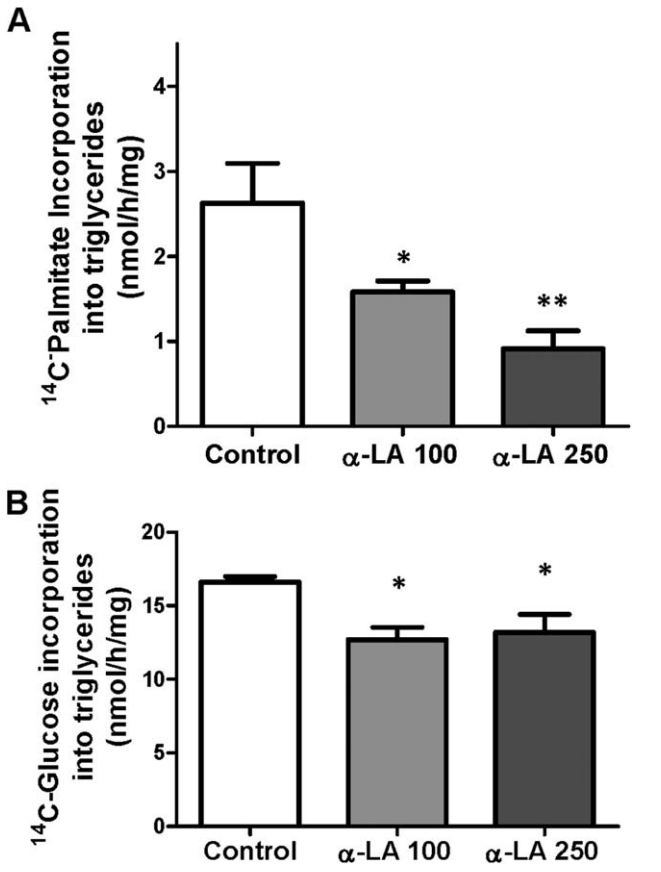

Figure 2 α-LA inhibits lipogenesis in human subcutaneous adipocytes from overweight/obese subjects. Effects of α-LA on (**A**) fatty acid esterification, evaluated by measuring labeled palmitate incorporation into triglycerides, and (**B**) *de novo* lipogenesis assessed after the fate of [^{14}C]glucose into triglycerides. Data are expressed as mean ± S.E. of at least 4 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control (vehicle-treated cells). Abbreviations: α-LA, α-lipoic acid.

enzymes of the lipogenic pathway were analyzed. Protein levels of DGAT1, one of the acyltransferases responsible for the esterification of triglycerides in adipose tissue were diminished following treatment with α-LA (250 μM, $P < 0.01$) (Figure 3A). However, α-LA did not modify DGAT2 expression (Supporting Information Figure S1). Moreover, α-LA (100 and 250 μM) caused a dose-dependent inhibition of FAS ($P < 0.01$) and SCD1 protein levels ($P < 0.01$ to 0.001), the two rate-limiting enzymes in *de novo* lipogenesis (Figure 3B).

Activation of AMPK signaling pathway is involved in the anti-lipogenic actions of α-LA in human subcutaneous adipocytes

AMPK is an enzyme involved in cellular energy homeostasis that switches off pathways which consume energy such as lipogenesis and fatty acid incorporation into triglycerides (16). Because of that, we tested the effects of α-LA on AMPK pathway. The results revealed that 250 μM α-LA significantly increased ($P < 0.05$) AMPK phosphorylation (Figure 4A). One of the first proteins identified as a target of AMPK is acetyl-CoA carboxylase (ACC), a key enzyme of the lipogenic pathway. In fact, AMPK phosphorylates and subsequently inhibits ACC (16). In this context, in parallel with

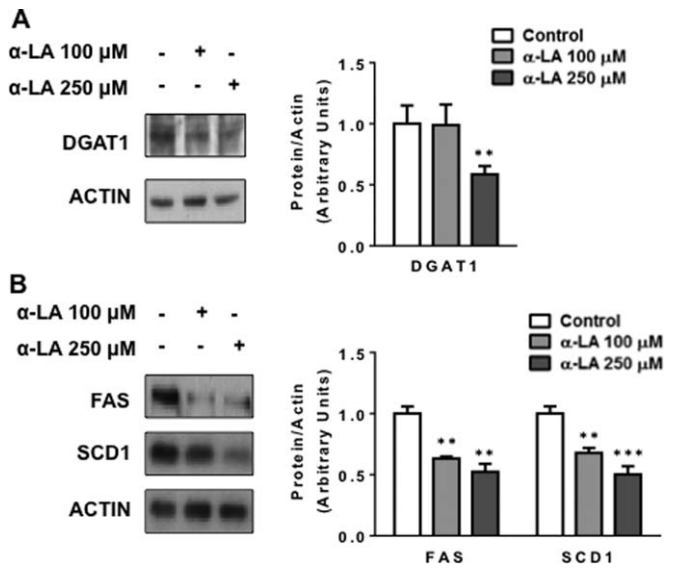

Figure 3 α-LA down-regulates lipogenic enzymes in human subcutaneous adipocytes from overweight/obese subjects. The effects of α-LA on (**A**) DGAT1 and (**B**) FAS and SCD1 protein levels were analyzed after 24 h of α-LA (100 and 250 μM) treatment. Data are expressed as mean ± S.E. of at least four independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control (vehicle-treated cells). Abbreviations: α-LA, α-lipoic acid; DGAT1, diacylglycerol O-acyltransferase 1; FAS, fatty acid synthase; SCD1, stearoyl-coenzyme A desaturase 1.

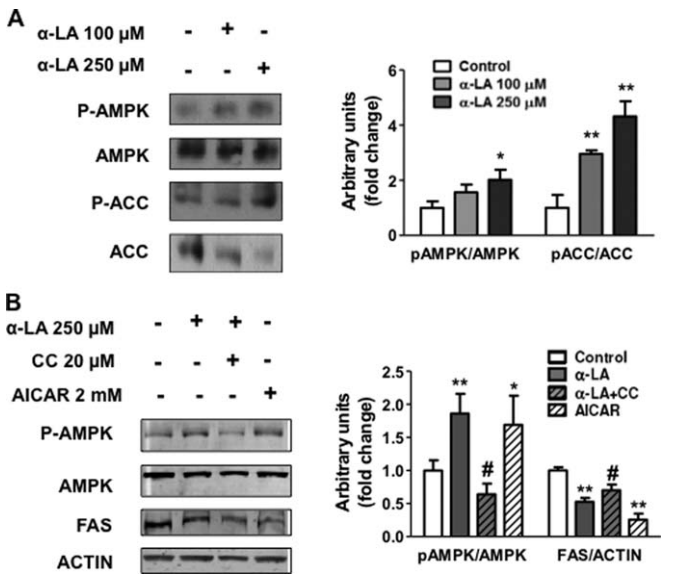

Figure 4 AMPK signaling pathway is involved in α-LA anti-lipogenic effects in human subcutaneous adipocytes from overweight and obese subjects. (**A**) Effects of α-LA (100 and/or 250 μM) treatment during 24 h on AMPK and ACC phosphorylation. (**B**) Effects of the AMPK selective inhibitor Compound C (CC) on α-LA actions on AMPK phosphorylation and FAS protein levels. The effects of the AMPK activator AICAR are also shown. Data are expressed as mean ± S.E. of at least four independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control (vehicle-treated cells); $^{\#}P < 0.05$ vs. α-LA-treated adipocytes. Abbreviations: α-LA, α-lipoic acid; AMPK, 5′ AMP-activated protein kinase; pAMPK, Phospho Thr^{172} 5′ AMP-activated protein kinase; ACC, acetyl CoA carboxylase; pACC, Phospho Ser^{79} acetyl CoA carboxylase; CC, Compound C; FAS, fatty acid synthase.

the activation of AMPK, α-LA increased ACC phosphorylation on Ser^{79}, being significant at 100 and 250 μM ($P < 0.01$; Figure 4A). Furthermore, to better characterize the involvement of AMPK activation on the effects of α-LA on lipogenesis, we assessed the actions of Compound C, a selective inhibitor of AMPK, on lipogenic enzymes. Thus, Compound C reversed the stimulatory effect of α-LA on AMPK and partly prevented the inhibitory effect of α-LA on FAS protein levels ($P < 0.05$; Figure 4B). Although a similar tendency was observed on SCD1 and DGAT1, no statistical significance was reached (data not shown). As a positive control, adipocytes were also treated with the AMPK activator AICAR, which stimulated AMPK phosphorylation and, in parallel, reduced FAS protein levels (Figure 4B).

The effects of α-LA on leptin and adiponectin, two adipokines that have been shown to regulate AMPK and/or lipogenic genes in adipose tissue (17,18), were also evaluated. Our data showed that α-LA inhibited both mRNA and secretion levels of leptin and adiponectin (Supporting Information Figure S2A and 2B).

Discussion

Our data provide novel evidence concerning the ability of α-LA to decrease triglyceride content in human subcutaneous adipocytes obtained from the abdominal region of overweight/obese subjects. Adipose tissue triglyceride content is determined by the balance between triglyceride synthesis (lipogenesis) and hydrolysis (lipolysis). We have previously reported the ability of α-LA to promote lipolysis in adipocytes (8), and the current data demonstrate that α-LA is also able to reduce triglyceride synthesis in mature adipocytes from overweight/obese subjects. These effects of α-LA on adipocyte lipid metabolism are not likely to be secondary to effects on adipocyte differentiation, as a previous study from our group has shown no effects of α-LA (250 μM) on master genes involved in this process, such as PPARγ, C/EBPα and C/EBPβ, in cultured mature adipocytes (8).

Lipogenesis occurs either as a consequence of esterification of FFA with glycerol or *de novo* synthesis of fatty acids. Concerning fatty acid incorporation into triglycerides, DGAT catalyzes the final acylation step to yield triglycerides. The key role of DGAT1 in triglyceride accumulation in adipose tissue was clearly demonstrated by the fact that DGAT1-deficient mice have reduced adiposity and are resistant to diet-induced obesity (19). Furthermore, DGAT activity has also been involved in the regulation of FFA uptake/storage in adipose tissue in humans (20). Therefore, pharmacological inhibition of DGAT1 has been proposed as a feasible therapeutic strategy for human obesity and type 2 diabetes. Our data show for the first time the ability of α-LA to decrease DGAT1 protein content in adipocytes from overweight/obese subjects, suggesting that DGAT1 inhibition could contribute to the anti-obesity properties of α-LA. Apart from DGAT1, another isozyme of DGAT has been identified, DGAT2 (21). $Dgat2^{-/-}$ mice exhibit severe reductions of triglyceride content in the body, suggesting a critical role of DGAT2 in lipogenesis; however, the physiological roles of DGAT2 in adult mice remain unclear because $Dgat2^{-/-}$ mice die shortly after birth (22). In our study, we have also evaluated the effects of α-LA on DGAT2 expression and no significant changes were observed between control and treated-adipocytes. Differential regulation of both DGAT isoforms has been described in adipose tissue in obesity (23).

Moreover, it has been shown that central leptin administration decreased DGAT2 expression in adipose tissue independently of DGAT1 (23). It has been suggested that increased DGAT2 activity has a role in steatosis (24). In this context, a previous study from our group (14) and data from others (25) have observed that dietary supplementation with α-LA reduced DGAT2 gene expression and liver triglyceride accumulation. Taking together, these data suggest that the regulation of DGAT1 and DGAT2 by α-LA seems to be tissue specific.

De novo lipogenesis is involved in fatty acid biosynthesis and in the regulation of the triglyceride storage capacity of adipose tissue (26). Indeed, it has been suggested that *de novo* lipogenesis may account for up to 20% of lipid turnover within adipose tissue (27). Our present data clearly show that the intracellular lipid-lowering effects of α-LA are associated with suppressed *de novo* lipogenesis, which could be triggered in part by the inhibition of some of the main enzymes regulating this pathway such as FAS and SCD1. FAS catalyzes the first committed step in *de novo* lipogenesis, and adipose tissue FAS has been implicated in obesity and insulin resistance in humans (28). Here, we demonstrate that α-LA diminishes FAS protein level. Targeted deletion of FAS in adipose tissue has been shown to decrease adiposity in mice, which are resistant to diet-induced obesity (11). SCD1 is a key enzyme involved in the control of *de novo* lipogenesis by catalyzing the rate-limiting step in the synthesis of monounsaturated fatty acids (29). SCD1 deficiency reduces lipogenesis and protects mice from diet-induced obesity (30). We have found that α-LA treatment also caused a strong inhibition in SCD1 protein level in our model of human adipocytes. A recent study has shown that the inhibition of SCD1, induced by sterculic acid or by conjugated linoleic acid (CLA), reduces *de novo* lipogenesis and down-regulates lipogenic genes such as ACC or FAS in primary bovine adipocytes (31). Our data revealed that in addition to FAS and SCD1 decrease, α-LA also reduced ACC protein levels, a key enzyme of the lipogenic pathway which mediates the initial step of the fatty acid synthesis. ACC activity is regulated mainly by phosphorylation, which causes enzyme inactivation (32). AMPK, a major cellular regulator of lipid metabolism, has been shown to phosphorylate and inactivate ACC in adipocytes (33). In this way, several of our findings suggest that AMPK mediates the α-LA inhibitory effects on lipogenic enzymes in human adipocytes from overweight/obese subjects. Thus, an increase in AMPK phosphorylation was observed after α-LA treatment, which was accompanied by the subsequent increase of ACC phosphorylation. In addition, the use of the AMPK antagonist Compound C reversed the α-LA-mediated down-regulation observed in FAS protein levels in parallel with the reduction of AMPK phosphorylation. These results are in agreement with the observations of Chen et al. (13), reporting that FAS inhibition is an important consequence of AMPK activation mediated by α-LA in C_2C_{12} myotubes.

On the other hand, it has been reported that leptin activates AMPK, increases ACC phosphorylation and lowers the expression of lipogenic genes in rat adipose tissue (17,18). Similar to previous observations in rodents (34), our current data in human adipocytes also revealed that α-LA inhibits both leptin mRNA and secreted levels, suggesting that leptin is not involved in the stimulatory action of α-LA on AMPK and the inhibition of lipogenic pathways. Adiponectin has also been reported to activate AMPK in adipocytes (17). A previous study from our group has shown that dietary supplementation with α-LA promotes AMPK phosphorylation and upregulates adiponectin in white adipose tissue (1), which could suggest that α-LA might reduce lipogenesis by increasing adiponectin. However, the present data argue against this possibility, as adiponectin production is reduced in α-LA-treated human adipocytes, as previously found in 3T3-L1 cells (35). AMPK activation has been related to the production of adiponectin, but the outcomes are controversial, and both increases and decreases of adiponectin production have been reported (17). On the other hand, the lack of parallelism between the *in vitro* inhibitory effect of α-LA and the stimulation of adiponectin described after *in vivo* supplementation with α-LA could also suggest that indirect mechanisms or some *in vivo* metabolic processing are involved.

While *in vitro* models have limitations, several studies have previously reported the ability of similar concentrations of α-LA (100-250 μM) to regulate glucose and lipid metabolism (8,36,37) and mitochondrial biogenesis (37) in murine adipocytes. These concentrations of α-LA are above physiological values, but therapeutic concentrations of α-LA fall within this micromolar range (38,39), suggesting that the effects of α-LA *in vitro* could be linked to its therapeutic effect *in vivo*. In this context, several studies in rodents have demonstrated that dietary supplementation with α-LA reduced lipogenesis in liver and muscle in part by AMPK-dependent pathways (13,14,40).

Taking together, the present study demonstrates the ability of α-LA to down-regulate key lipogenic enzymes, inhibiting both *de novo* lipogenesis and fatty acids esterification and reducing triglyceride accumulation in subcutaneous adipocytes from overweight/obese subjects through the activation of AMPK signaling pathway. These data suggest that the inhibition of adipose tissue lipogenesis could also contribute to the anti-obesity actions of α-LA in humans. O

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