Effects of lipoic acid on lipolysis in 3T3-L1 adipocytes

Marta Fernández-Galilea 1, Patricia Pérez-Matute 1,2, Pedro L Prieto-Hontoria 1, J Alfredo Martinez 1, Maria J Moreno-Aliaga 1

1Department of Nutrition, Food Science, Physiology and Toxicology. University of Navarra. Pamplona (Spain). 2HIV and Associated Metabolic Alterations Unit. Infectious Diseases Area. Center for Biomedical Research of La Rioja (CIBIR). Logroño (Spain)

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Corresponding author: María J. Moreno-Aliaga. Department of Nutrition, Food Science, Physiology and Toxicology. University of Navarra. Researching Building. C/ Irunlarrea, 1. 31008, Pamplona (Navarra) Spain. Tel.: +34948425600 ext. 6558; Fax number: 948 425 740. e-mail: mjmoreno@unav.es

Abbreviations: AdPLA, adipose-specific phospholipase A2; Akt, serine-threonine protein kinase Akt; AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; BHA, butylated hydroxyanisole; C/EBPα, CCAAT/enhancer-binding protein alpha; C/EBPβ, CCAAT/enhancer-binding protein beta; CGI-58, comparative gene identification 58; CILO, cilostamide; ERK 1/2, extracellular signal-regulated kinase; G0S2, G(0)/G(1) switch gene 2; HSL, hormone-sensitive lipase; JNK, c-Jun N-terminal kinase; LA, lipoic acid; LD, lipid droplet; LY, LY294002; MAGL, Monoacyl glycerol lipase; MAPK, mitogen-activated protein kinase; NAC, n-acetyl cysteine; PD, PD98059; PDE3B, phosphodiesterase 3B; PGE2, prostaglandin E2; PI3K, phosphatidylinositol 3 kinase; PKA, protein kinase A; PPARγ, peroxisome proliferator-activated receptor gamma; ROS, reactive oxygen species; SAA, serum amyloid A; SP, SP600125; TAG, triacyl glycerol; TNF-α, tumor necrosis factor alpha; WAT, white adipose tissue.
ABSTRACT

Lipoic acid (LA) is a naturally occurring compound with beneficial effects on obesity. The aim of this study was to evaluate its effects on lipolysis in 3T3-L1 adipocytes and the mechanisms involved. Our results revealed that LA induced a dose and time-dependent lipolytic action, which was reversed by pre-treatment with the JNK inhibitor SP600125, the PKA inhibitor H89 and the AMPK activator AICAR. In contrast, the PI3K/Akt inhibitor LY294002 and the PDE3B antagonist Cilostamide enhanced LA-induced lipolysis. LA treatment during 1 h did not modify total protein content of HSL, but significantly increased the phosphorylation of HSL both at Ser\(^{563}\) and at Ser\(^{660}\), which was reversed by H89. LA treatment also induced a marked increase in PKA-mediated perilipin phosphorylation. LA did not significantly modify either the protein levels of ATGL or its activator CGI-58 and inhibitor G0S2. Furthermore, LA caused a significant inhibition of AdPLA protein and mRNA levels in parallel with a decrease in the amount of PGE\(_2\) released and an increase in cAMP content. Together, these data suggest that the lipolytic actions of LA are mainly mediated by phosphorylation of HSL through cAMP-mediated activation of protein kinase A probably through the inhibition of AdPLA and PGE\(_2\).

Supplementary Key Words: Hormone Sensitive Lipase, Adipose Triglyceride Lipase, Adipose-specific phospholipase A2, prostaglandin E\(_2\).
INTRODUCTION

Lipoic acid (LA) or 1,2-dithiolane-3-pentaenoic acid is a naturally occurring compound that contains two thiol groups with diverse beneficial effects on health. The biological effects of LA were primarily associated with its antioxidant properties. In fact, LA is able to directly scavenge reactive oxygen species (ROS) and regenerate endogenous antioxidants such as glutathione, and vitamins E and C (1, 2). Moreover, several studies have described potential beneficial effects of LA on obesity and associated commorbidities such as insulin resistance, type 2 diabetes or fatty liver diseases. Thus, in rodents LA has been shown to cause profound weight loss by reducing food intake and enhancing energy expenditure (3) as well as by inducing a reduction on intestinal sugar absorption (4). More recently, two clinical trials in humans reported that LA caused significant reductions of body weight, body mass index (BMI), blood pressure and abdominal circumference in obese subjects (5, 6). LA also improved insulin sensitivity and plasma lipid profile possibly through amelioration of oxidative stress and chronic inflammatory status in obese patients with impaired glucose tolerance (7). Previous studies provided strong evidences that LA is able to deeply affect adipose tissue development and function by the inhibition of adipogenesis (8), the regulation of the secretion of several adipokines such as leptin (9) and apelin (10) and also by the promotion of mitochondrial biogenesis (11).

In this context, previous studies suggested that LA seems to stimulate the lipolytic response in an in vitro model of broiler chicken adipocytes (12). However, the molecular mechanisms that mediate these effects remain unclear. Lipolysis is a complex process highly regulated, which involves the co-ordinately participation of several lipases and lipid droplet (LD) proteins (13). Thus, the lipolytic process occurs through the consecutive action of three lipases: adipose triglyceride lipase (ATGL/desnutrin), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MAGL) (14). ATGL exhibits high substrate specificity for triacyl
glycerol (TAG) (15). Lipase activity of ATGL largely depends on its coactivation by CGI-58, while G0S2 acts as an inhibitor of ATGL activity and ATGL-mediated lipolysis (16). Recently it has been shown that ATGL is phosphorylated by AMPK at Ser\(^{406}\), increasing TAG hydrolase activity (17).

The activity of HSL is well known to be regulated post-transcriptionally by reversible phosphorylation. In murine adipocytes PKA phosphorylates HSL at several serine residues (563, 659, and 660) resulting in increased translocation of HSL to the lipid droplet surface and increased lipolytic activity (18). Furthermore, AMP-activated protein kinase (AMPK) phosphorylates HSL at Ser\(^{565}\), which prevents phosphorylation induced by PKA (19, 20). Activation of phosphodiesterase 3B (PDE3B) via the Akt-mediated phosphorylation of Ser\(^{273}\) attenuates PKA activity and thereby HSL activation and lipolysis (21, 22). In addition to the PKA-mediated phosphorylation, HSL may be also phosphorylated by other kinases such as ERK1/2, which activates HSL by phosphorylation on Ser\(^{600}\) (23). It has been also suggested that JNK could play a role in the regulation of lipolysis based on the fact that silencing of Jnk1 and Jnk2 accelerates basal lipolysis in mouse adipocytes (24).

Protein trafficking and specific protein-protein interactions at the surface of lipid droplets are also key factors in the regulation of lipolysis. Perilipin A is a lipid droplet scaffold protein that plays a central role in orchestrating interactions among lipolytic effector proteins (25). Under basal conditions, perilipin restricts the access of cytosolic lipases to LD, thereby maintaining a low rate of basal lipolysis. However, the phosphorylation of perilipin by PKA results in perilipin conformational changes that expose LD stores and facilitates the translocation of phosphorylated HSL to the LD, thereby increasing the lipolytic process (26).

Recently, a novel intracellular adipose-specific phospholipase A2 (AdPLA) has been identified (27). It was suggested that AdPLA could be another mediator in the regulation of lipolysis by generating arachidonic acid for the production of prostaglandins (28). In fact,
AdPLA null mice exhibited reduced adipose tissue prostaglandin E\(_2\) (PGE\(_2\)) production, and augmented HSL-phosphorylation leading to increased lipolysis, supporting that AdPLA is a major regulator of adipocyte lipolysis by regulating PGE\(_2\) abundance (28).

Previous studies have demonstrated the ability of LA to modulate ERK, JNK and Akt signaling pathways (8, 9, 29), as well as AMPK activity (30, 31) in different cell types. Moreover, LA stimulates cAMP production in purified human NK cells (32) and modulates the production of PGE\(_2\) in osteoblasts (33).

Based on these previous findings, we hypothesized that LA could be a key regulator of lipolysis in mammals through modulation of lipases and lipid droplet proteins activities. Therefore, the aim of this study was to characterize the lipolytic action of LA in cultured adipocytes and to elucidate the molecular mechanisms and signaling pathways involved.

**MATERIAL AND METHODS**

**Cell culture and differentiation of 3T3-L1 cells**

Murine 3T3-L1 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose, 10% calf bovine serum (Invitrogen, CA, USA) and 1% penicillin and streptomycin (Gibco, Invitrogen Corporation, CA, USA) and were maintained in an incubator set up to 37 °C and 5% of CO\(_2\). At confluence pre-adipocytes were induced to differentiate into adipocytes by culturing them for 48 h in DMEM containing 10% fetal bovine serum (FBS) (Invitrogen), antibiotics and supplemented with dexamethasone (1 mM; Sigma, St. Louis, MO, USA), isobutylmethylxantine (0.5 mM; Sigma) and insulin (10 mg/mL; Sigma). Then, cells were cultured with 10% FBS and insulin for 48 h. After that, media was replaced with 10% FBS in DMEM and antibiotics, but without insulin and this media was changed every 2 days up to day 8 post confluence, when cells were completely differentiated to adipocytes (34, 35).
Treatments

LA (α-Lipoic acid; Sigma) was dissolved in ethanol. The inhibitors SP600125 (SP) (Calbiochem, San Diego, CA, USA), PD98059 (PD) (Sigma), H89 (Santa Cruz, Santa Cruz, CA, USA), LY294002 (LY) (Sigma), Cilostamide (CILO) (Sigma), and L798106 (Tocris, Ellisville, MO) were dissolved in DMSO. The AMPK activator AICAR (Sigma) was dissolved in ultrapurified water. All compounds were prepared as 1000x stock solutions and added to the culture medium. Control cells were treated with the same amount of the corresponding vehicle.

Prior to the addition of the appropriate treatments, fully differentiated 3T3-L1 adipocytes were serum starved for at least 4 h (by switching to DMEM containing 2-2.5% fatty acid free-BSA or to DMEM with 1% FBS) and then treated with or without LA (1-500 µM) during different time intervals (30 min to 24 h). In order to analyze the signaling pathways involved in LA-actions, adipocytes were pre-incubated for 1h with the selective inhibitors or activators (20 µM SP, 50 µM PD, 1 µM H89, 50 µM LY, 2 µM CILO, 10 µM L798106 and 2 mmol/l AICAR).

Lipolysis measurement

Lipolysis was evaluated by measuring the amount of glycerol and free fatty acids released to the media. Glycerol was determined after 1 to 24 h of LA treatment using an autoanalyzer following the manufacturer instructions (Cobas-Mira, Roche Diagnostics, Basel, Swiss). Free fatty acids were quantified after 3 h of LA treatment by using the Lipolysis Assay KIT for Free Fatty Acids Detection (Zen-Bio Inc, Research Triangle Park, NC) according to the manufacturer’s instructions.
Analysis of mRNA levels

Total RNA was extracted from 3T3-L1 cells using TRIzol® reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentrations and quality were measured by Nanodrop Spectrophotometer ND1000 (Thermo Scientific, Wilminton, DE, USA). RNA was then incubated with the RNase-free kit DNAse (Ambion, Austin, TX, USA) for 30 min at 37 ºC. RNA (2 µg) was reverse-transcribed to cDNA using MMLV (Moloney Murine Leukaemia Virus) reverse transcriptase (Invitrogen). For the real time quantitative polymerase chain reaction analysis, 4.5 µl of 1/100 or 1/50 dilution of cDNA per reaction were used in a final reaction volume of 10 µl.

ATGL, HSL, perilipin, AdPLA, PPARγ, C/EBPa and C/EBPβ mRNA levels were determined using predesigned Taqman® Assays-on-Demand (Applied Biosystems, Foster City, CA, USA). Taqman Universal Master Mix was also provided by Applied Biosystems. The reaction conditions were followed according to manufacturer’s instructions.

Amplification and detection of specific products were performed using the ABI PRISM 7900HT Fast System Sequence Detection System (Applied Biosystems).

All mRNA levels were normalized by the housekeeping gene β-Actin obtained from Applied Biosystems. Samples were analyzed in duplicate. Ct values (the cycle where the emitted fluorescence signal is significantly above background levels and is inversely proportional to the initial template copy number) were generated by the ABI software. Finally, the relative expression level of each gene was calculated as $2^{-\Delta\Delta Ct}$ (36).

Western blot analysis

Western blot analyses were performed in 8 days post-differentiation adipocytes. Cells were incubated in serum-free DMEM overnight and then with or without the appropriate treatment. Lysates were 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 according to the supplier’s instructions (Pierce-Thermo Scientific, Rockford, IL, USA). 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 ATGL, HSL, HSL phospho Ser$^{565}$, HSL phospho Ser$^{563}$, HSL phospho Ser$^{660}$, perilipin, phospho (ser/thr) PKA substrate (p-perilipin), AMPK, AMPK phospho Thr$^{172}$, AKT, AKT phospho Ser$^{473}$, MAPK (ERK1/2), ERK1/2 phospho Thr$^{202/204}$, JNK and JNK phospho Thr$^{183/185}$ (from Cell Signaling Technologies, Beverly, MA, USA), AdPLA (from Cayman Chemical, Ann Arbor, MI, USA), CGI-58, G0S2 (from Santa Cruz) and Actin (from Sigma). Secondary antibody was horseradish peroxidase goat anti-rabbit IgG-HRP (Bio Rad Laboratories). The immunoreactive proteins were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, Illinois, USA). Band intensities were quantified using a GS-800 calibrated densitometer (Bio Rad Laboratories).

**Fatty acid oxidation determination**

Fatty acid oxidation to acid-soluble metabolites (ASM) was measured with radiolabeled $^{14}$C-palmitate (Perkin Elmer, Boston, MA) in mature 3T3-L1 adipocytes as previously described (37). ASM were extracted by addition of 1 ml cold 1 M HClO$_4$ (Panreac, Barcelona, Spain). After centrifugation (10 min, 1800 g), radioactivity in the supernatant was measured by scintillation counting by using a Wallac 1409 liquid scintillation counter (EG&G Company, Turku, Finland). Protein content in parallel cultures of vehicle- and LA-treated cells was analyzed using a BCA method.
ELISA assays

Prostaglandin E\(_2\) (PGE\(_2\)) concentration in the media was determined after 24 h of LA (250 µM) treatment by using a PGE\(_2\) Enzyme Immunoassay kit (Arbor assays, Ann Arbor, MI, USA). The amount of intracellular cAMP was quantified after 1 and 24 h of LA (250 µM) treatment by using the cAMP Direct EIA kit (Arbor Assays).

Data analysis

Data are expressed as mean ± 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 once the normality with the Kolmogorov-Smirnoff and Shapiro-Wilk tests was screened. SPSS 19.0 version for Windows (SPSS, Chicago, IL, USA) and GraphPad Prism 5.0 (Graph-Pad Software INC. San Diego, CA, USA) were used for the statistical analysis.

RESULTS

Effects of LA on lipolysis in 3T3-L1 adipocytes

A dose-dependent significant increase in the amount of glycerol released into the media was observed in those adipocytes treated with LA (250-500 µM, \(P<0.01\) and \(P<0.001\)) for 24 h (Fig. 1A). Moreover, the lipolytic effect of LA was time-dependent. Thus, the significant increase in glycerol release was observed after 1 h of treatment \((P<0.05)\) and it became more prominent after 3 and 6 h of treatment (250-500 µM, \(P<0.001\)) (Fig. 1B). Furthermore, LA also induced a concentration-dependent increase in the amount of free fatty acids released after 3 h treatment (100-500 µM, \(P<0.001\)) (Fig. 1C). We also tested the effects of LA on isoproterenol-induced lipolysis and the data revealed that LA did not have any additional
effect on the lipolytic effect of isoproterenol (Supplementary Fig. I). In order to rule out if the lipolytic effect of LA was caused by a global down-regulation of adipocyte differentiation markers, PPARγ, C/EBPα and C/EBPβ gene expression levels were analyzed after 24 h of LA (250 µM) treatment and no differences were observed when compared with control cells (Supplementary Fig. II). Moreover, to test if the lipolytic actions of LA were also shared by other molecules with antioxidant properties, the effects of vitamin C, resveratrol, N-acetyl cysteine (NAC) and butylhydroxyanisole (BHA) on glycerol release were evaluated. The data showed that, at the concentration tested, resveratrol and BHA, but not NAC or Vitamin C were able to stimulate lipolysis in 3T3-L1 adipocytes (Supplementary Fig. III).

**Signaling pathways involved in the lipolytic actions of LA**

In order to evaluate the ability of LA to modify some signaling pathways involved in the regulation of lipolysis, the phosphorylation levels of JNK, ERK1/2, AMPK and PI3K/AKT were analyzed both after a short (30 min-1h) and long-term treatment (24 h).

No effects were observed in JNK Thr\(^{183}\)/Tyr\(^{185}\) phosphorylation after 30 min of treatment with LA (250 µM), while a significant \((P<0.05)\) reduction of JNK phosphorylation was observed after 24 h of treatment (Fig. 2A). In contrast, the significant increase on ERK1/2 Thr\(^{202}\)/Tyr\(^{204}\) phosphorylation \((P<0.01)\) induced by LA (250 µM) after 1 h was reversed to basal levels after 24 h of treatment (Fig. 2B). Moreover, the stimulatory effect \((P<0.05)\) of LA on AMPK Thr\(^{172}\) phosphorylation was only observed in long-term treated (24 h) adipocytes (Fig. 2C). Regarding the PI3K/AKT signaling pathway, LA (250 µM) treatment caused a significant inhibition of AKT Ser\(^{473}\) phosphorylation both at short \((P<0.05)\) and long-term \((P<0.01)\) treatments (Fig. 2D).

For a better understanding of the potential signaling pathways involved in the lipolytic action of LA, the effects of specific kinase inhibitors or activators on LA-induced glycerol release
were studied. Basal lipolysis was significantly enhanced by the PI3K/Akt inhibitor LY294002 ($P<0.001$) and the PDE3B antagonist Cilostamide ($P<0.01$), and decreased by the PKA inhibitor H89 and the AMPK activator AICAR ($P<0.001$). Interestingly, our data revealed that the lipolytic actions of LA were reversed by pre-treatment with the JNK inhibitor SP600125 ($P<0.01$), the PKA inhibitor H89 and the AMPK activator AICAR ($P<0.001$). Interestingly, the stimulatory effects of LA on lipolysis were significantly enhanced ($P<0.01$ and $P<0.001$) in adipocytes treated with the PI3K/AKT inhibitor LY294002 and the PDE3B antagonist Cilostamide (Fig. 2E).

**Effects of LA treatment on HSL, ATGL, Perilipin, CGI-58 and G0S2 levels**

In contrast to the LA lipolytic actions, a significant ($P<0.05$) decrease on total protein content of the two main lipases ATGL and HSL was observed in LA-treated (250 µM for 24 h) adipocytes (Fig. 3A). Accordingly, gene expression levels of both ATGL and HSL were also significantly downregulated ($P<0.05$) by LA treatment for 24 h (Fig. 3B). Perilipin mRNA levels were also reduced in LA-treated adipocytes while no changes in perilipin protein content were observed (Fig. 3A and 3B).

The inhibitory actions of LA treatment on ATGL gene expression were not observed in presence of the JNK inhibitor SP600125 and ERK1/2 inhibitor PD98059 ($P<0.05$) (Fig. 3C). Similarly, the inhibition of the ERK1/2 signaling pathway was able to reverse the down-regulation of HSL and perilipin gene expression observed after LA treatment ($P<0.05$) (Fig. 3D and E).

HSL activity is regulated by reversible phosphorylation in serine residues. PKA phosphorylates HSL at Ser$^{563}$, and Ser$^{660}$, which stimulates HSL activity. Thus, to better elucidate the mechanisms underlying the lipolytic actions of LA, we next investigated the effects of LA on HSL phosphorylation both in Ser$^{563}$ and Ser$^{660}$. LA treatment (250 µM)
during 1 h did not modify total protein content of HSL, but significantly increased \((P<0.05)\) the phosphorylation of HSL both at Ser\(^{563}\) (Fig. 4A) and at Ser\(^{660}\) (Fig. 4B). However, LA did not modify the AMPK-induced phosphorylation of HSL at Ser\(^{565}\) (Supplementary Fig. IV). These data suggest that LA stimulates lipolysis by increasing PKA activity. Perilipin phosphorylation is also PKA-dependent. Using a perilipin-specific antibody and a phospho-PKA-motif-specific substrate antibody, we found that LA treatment induced a marked increase \((P<0.01)\) in PKA-mediated perilipin phosphorylation (Fig. 4C). In fact, the LA-induced phosphorylation of HSL at Ser\(^{563}\) and Ser\(^{660}\) as well as of perilipin was dramatically blunted in the presence of the PKA inhibitor H89. We also found that AMPK activation disrupted the LA-induced phosphorylation of HSL at both Ser\(^{563}\) and Ser\(^{660}\) (Fig. 4A and 4B), without modifying the p-PKA substrate/perilipin ratio (Fig. 4C). Interestingly, the inhibition of the JNK pathway induced a significant increase in the phosphorylation of HSL at Ser\(^{660}\), both in the absence and presence of LA, and in PKA-mediated perilipin phosphorylation (Fig. 4B and 4C). Moreover, the ERK1/2 inhibitor PD98059 prevented the LA-induced phosphorylation of HSL at Ser\(^{563}\) without modifying LA-effects on Ser\(^{660}\) and p-PKA substrate/perilipin ratio. All these data suggest that LA stimulates lipolysis mainly through the PKA-mediated phosphorylation of perilipin and HSL. However, LA treatment during 1 h did not significantly modify the protein levels of ATGL. Neither CGI-58 nor G0S2, the activator and inhibitor of ATGL activity respectively, were significantly altered after 1 or 24 h of LA treatment (Fig. 5A and B respectively).

**Effects of LA on AdPLA levels, and on PGE\(_2\) and cAMP production**

AdPLA has been described as the major phospholipase A2 in adipose tissue with a key role in the regulation of lipolysis through the modulation of PGE\(_2\) levels. As shown in Fig. 6A, LA
treatment during 1 and 24 h (250 µM) caused a significant inhibition ($P<0.05$) of AdPLA protein content as well as on mRNA levels (Figure not shown).

We next aimed to evaluate the effects of LA on the major AdPLA product, PGE$_2$, which binds the G$\alpha_i$-coupled receptor EP3, and down-regulates lipolysis by inhibiting cAMP production. Our data showed that the amount of PGE$_2$ released to the media was significantly reduced in LA-treated adipocytes at 24 h of treatment ($P<0.05$) (Fig. 6B) and also at shorter (4 and 8 h) periods of treatment (data not shown). In parallel, a significant increase in cAMP levels was found in LA-treated adipocytes for 1 and 24 h (Fig. 6C). Moreover, the lipolytic effect of LA was partially reversed by co-treatment with PGE$_2$, an effect that was not observed in the presence of the EP3-receptor antagonist L798106 (Fig. 6D).

**DISCUSSION**

Our current data demonstrate the lipolytic action of LA in cultured adipocytes in a concentration and time-dependent manner. It is important to note that the doses able to induce lipolysis were similar to those that inhibited adipogenesis in 3T3-L1 preadipocytes (8) and no toxicity was observed. Previous studies in broiler chickens also support the lipolytic action of LA both *in vitro* and *in vivo* models (12, 38). However, the mechanisms of action remain uncertain. In the present study we tested if the lipolytic effects of LA were shared by other compounds with antioxidant properties. Our data revealed that resveratrol and BHA, but not Vitamin C or NAC were able to stimulate lipolysis, suggesting that the lipolytic actions seem to be independent of the antioxidant capacities.

Moreover, our data showed that despite the stimulatory effects of LA on lipolysis, both HSL and ATGL gene expression and protein levels were inhibited after 24 h of LA treatment, together with a decrease in perilipin mRNA levels. These effects of LA on HSL, ATGL and perilipin were reversed by the presence of the ERK1/2 inhibitor PD98059 in the media. A
down-regulation of ATGL, HSL and perilipin gene expression together with increased lipolysis has also been described after TNF-α treatment in adipocytes (39-41). Moreover, it was observed that the administration of Trecadrine, a beta-3 adrenergic agonist that stimulates lipolysis (42), induced a decrease in HSL mRNA levels in abdominal WAT, whereas an increase in HSL activity was observed (43). Furthermore, a recent study reported that serum amyloid A (SAA) also stimulated lipolysis in parallel with a reduced HSL protein content. However, SAA caused a significant increase of PKA-mediated HSL phosphorylation (44), suggesting opposite trends in HSL expression and activity. In this context, the mechanisms controlling HSL activity have been thoroughly studied, showing that reversible phosphorylation at several serine sites is a hallmark of HSL regulation. Indeed, HSL is activated by PKA-induced phosphorylation at Ser\(^{563}\) and Ser\(^{660}\). Moreover, the lipid droplet protein perilipin is also phosphorylated by PKA, and upon phosphorylation, perilipin shifts to the cytoplasm and the accessibility of HSL to the lipid surface is promoted and the lipolysis enhanced (45-47). The results of our study suggest a key role of PKA-induced lipolysis in the lipolytic actions of LA because of i) LA increased HSL phosphorylation both at Ser\(^{563}\) and Ser\(^{660}\); ii) PKA-induced perilipin phosphorylation was increased by LA treatment; iii) the PKA inhibitor H89 completely blunted the lipolytic action of LA as well as the LA-induced phosphorylation of phospho-PKA substrates. Taking together, these data suggest an important role of PKA-mediated phosphorylation of perilipin and HSL in the lipolytic effect of LA.

ATGL plays a governing role in both basal and adrenergically stimulated TAG breakdown in adipocytes (14). However, our data suggest that ATGL activation is not importantly involved in the lipolytic action of LA as concluded from the findings that no significant changes were observed either on the levels of the ATGL co-activator protein called CGI-58 or the inhibitory protein G0S2 (16, 48).
PI3K/AKT is a major player of insulin action and its activation increases PDE3B activity, and hydrolysis of cAMP leading to a net dephosphorylation of HSL and inhibition of lipolysis (49). In our experimental cell model, LA inhibits AKT phosphorylation both at 30 min and 24 h of treatment, and both the PI3K/AKT inhibitor LY294002 and the PDE3B antagonist Cilostamide potentiated the stimulatory effects of LA on basal lipolysis. Therefore, the present results suggest that the lipolytic effects of LA could be mediated by decreasing AKT activation, which might increase cAMP, and lipolysis mediated by HSL and perilipin activation.

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that regulate various cellular activities, including lipolysis. Regarding the role of JNK activation in the regulation of lipolysis, it was described that JNK1/JNK2-deficiency drastically enhanced basal lipolysis (24). In this context, our data show that incubation with the JNK inhibitor SP600125 (2 h) stimulates the phosphorylation of HSL at Ser$^{563}$ and Ser$^{660}$ as well as phospho-PKA substrate/perilipin ratio, supporting the idea that JNK inhibition leads to increased lipolysis. However, our current data and previous studies show that the amount of glycerol released into the media is not modified or even reduced by longer-term incubation with SP600125 (41, 50), suggesting that the effects of JNK inhibition on lipolysis might be time-dependent. Our results demonstrated that LA induced a time-dependent inhibition of JNK phosphorylation, which might suggest the involvement of this pathway in the lipolytic actions of LA. Thus, preincubation with SP600125 for 1 h potentiated the phosphorylation of HSL at Ser$^{660}$ observed after 1 h of treatment with LA. However, co-incubation with the JNK inhibitor SP600125 partially reversed the stimulatory effect on lipolysis and the inhibition induced by LA on ATGL gene expression after 24 h of treatment, suggesting that the involvement of JNK on LA-induced lipolysis is complex and seems also to be time-dependent. On the other hand, the fact that pretreatment with the ERK1/2 inhibitor PD98059...
reversed the downregulation of ATGL, HSL and perilipin gene expression induced by LA treatment during 24 h might suggest the involvement of this pathway in LA-induced lipolysis. However, our data evidenced that ERK1/2 phosphorylation is not affected by LA after 24 h of treatment and that pretreatment with PD98059 was not able to reverse the lipolytic action of LA, arguing against the involvement of this pathway.

AMPK has been also involved in the regulation of lipolysis (51, 52). Thus, it has been reported that phosphorylation of HSL at Ser^{565} by AMPK prevents activation by PKA, inhibiting lipolysis (19, 53, 54). Moreover, the negative regulation of AMPK activity by PKA has been shown to be important for converting a lipolytic signal into an effective lipolytic response (55). However, it has been recently reported that ATGL is phosphorylated/activated by AMPK to increase lipolysis (17). Thus, the effects described for AMPK activators on lipolysis are controversial showing both inhibition (56, 57) and activation of lipolysis (17, 58), and it has been suggested that the effects of AMPK activation on lipolysis might be time-dependent (59). Our present data show that LA treatment stimulates AMPK phosphorylation and promotes lipolysis. However, the lipolytic effects of LA were already observed after 1 h of treatment when AMPK phosphorylation was not induced, suggesting that AMPK is not involved in the short-term lipolytic effects of LA. On the contrary, the presence of the AMPK activator AICAR inhibited LA-stimulated lipolysis at 24 h of treatment, according with the remarkable increase of AMPK phosphorylation observed at this period of time. Taking together, these data suggest that the lipolytic action of LA is not mediated by the activation of AMPK in the first stages but it could contribute to the regulation of the long-term lipolytic effects of LA.

Recently it has been described and functionally characterized a new adipocyte phospholipase A2 called AdPLA (27). Afterwards, it was demonstrated that AdPLA ablation increased lipolysis by reducing PGE$_2$ levels and thereby stimulating cAMP and phosphorylation of
HSL through cAMP-mediated activation of PKA (28). Our results showed for the first time that AdPLA gene expression is down-regulated by LA treatment as well as PGE$_2$ levels, accompanied by an increase in cAMP levels, which could also contribute to the increased phosphorylation of HSL at Ser$^{563}$ and Ser$^{660}$ and thereby to the lipolytic effects of LA. In support of this, our data revealed that co-incubation with PGE$_2$ was able to partially reverse the stimulatory effect of LA on lipolysis, while this effect of PGE$_2$ was not observed in the presence of an EP3 antagonist.

All these data suggest that the ability of LA to stimulate lipolysis in adipocytes could also contribute to its antiobesity properties. It is important to take into consideration that increased lipolysis and FFA release from adipose tissue has been associated with the development of insulin resistance (60). However, recent findings have demonstrated that, surprisingly, increasing lipolysis in adipose tissue does not necessarily increase serum FFA levels because increasing lipolysis in adipose tissue causes a shift within adipocytes towards increased FA utilization and energy expenditure and thus protects against obesity. Therefore, it has been suggested an activation of lipolysis may be a promising therapeutic target for the treatment of obesity (13, 61). In this context, we and others have demonstrated that dietary supplementation with LA reduces weight loss and fat mass without increasing circulating FFA and improves insulin resistance both in rodents (10, 62, 63) and humans (7), and as previously suggested, this could be associated to LA-induced fatty acid oxidation. In this context, our experimental data support the notion about the ability of LA to promote fatty acid oxidation in 3T3-L1 adipocytes (Supplementary Fig. V). A recent study have also evidenced that LA subsequently increased AMPK and ACC phosphorylation, leading to increased palmitate β-oxidation in myotubes (64). Moreover, studies of our group also have shown that LA supplementation prevents the downregulation of genes involved in mitochondrial and peroxisomal β-oxidation in liver of high fat-induced obese rats (65).
In summary, the present data demonstrate the ability of LA to stimulate lipolysis in 3T3-L1 adipocytes and suggest that these lipolytic actions of LA are mainly mediated by the phosphorylation of HSL through cAMP-mediated activation of PKA probably through the inhibition of AdPLA and PGE\(_2\).

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REFERENCES


FIGURE LEGENDS

Fig. 1. LA stimulates lipolysis in 3T3-L1 adipocytes. Mature 3T3-L1 adipocytes were treated with LA (0-500 μM) for the indicated times (1, 3, 6 or 24 h). A: Lipolysis was assessed by the amount of glycerol released into media in adipocytes treated for 24 h. B: Time-dependent effects of LA (250 and 500 μM) on glycerol release. C: Concentration-dependent effects of LA on fatty acid release in adipocytes treated during 3 h. Data are expressed as mean ± S.E. of 6 independent experiments. *P <0.05, **P<0.01 and ***P<0.001 vs. Control (vehicle-treated cells).

Fig. 2. Signaling pathways involved in the lipolytic effects of LA. A-D: Effects of LA on the phosphorylation of (A) JNK, (B) ERK1/2, (C) AMPK and (D) PI3K/AKT. Band intensities for each phosphorylated species were normalized to their respective total fractions. E: Effects of LA treatment during 24 h on glycerol release in the presence or absence of the JNK inhibitor SP600125 (SP), the AMPK activator AICAR, the ERK1/2 inhibitor PD98059 (PD), the PKA inhibitor H89, the PI3K/AKT inhibitor LY294002 (LY) and the PDE3B inhibitor Cilostamide (CILO). Data are expressed as mean ± S.E. of at least 3 independent experiments. *P <0.05, **P<0.01 and ***P<0.001 vs. Basal Control (vehicle-treated cells) #P<0.05, ##P<0.01 and ###P<0.001 vs. respective control, bP<0.01 and cP<0.001 vs. basal LA-treated adipocytes.

Fig. 3. Long-term LA treatment downregulates total HSL, ATGL and perilipin transcripts. The effects of LA (250 μM) on total ATGL, HSL and perilipin protein (A) and mRNA (B) levels were assessed in 3T3-L1 adipocytes after 24 h of treatment. C-E: Effects of the JNK inhibitor SP600125 (SP), the ERK1/2 inhibitor PD98059 (PD), the PKA inhibitor H89, the AMPK activator AICAR and the PI3K/AKT inhibitor LY294002 (LY) on (C) ATGL, (D)
HSL and (E) Perilipin mRNA levels in control and LA-treated 3T3-L1 adipocytes. Data are expressed as mean ± S.E. of at least 3 independent experiments. *P<0.05, **P<0.01 and ***P<0.01 vs. Basal Control (vehicle-treated cells). #P<0.05, ##P<0.01 vs. respective control. aP<0.05 vs. basal LA-treated adipocytes.

Fig. 4. LA stimulates PKA-mediated phosphorylation of HSL and perilipin. A-B: Representative Western blots for (A) Ser\(^{563}\)-phosphorylated HSL and (B) Ser\(^{660}\)-phosphorylated HSL in differentiated 3T3-L1 adipocytes treated with LA (250 \(\mu\)M) for 1 h in the presence or absence of the JNK inhibitor SP600125 (SP), the AMPK activator AICAR, the ERK1/2 inhibitor PD98059 (PD) and the PKA inhibitor H89. Band intensities were normalized to total HSL. C: Adipocyte lysates were also immunoblotted using a phospho-PKA-motif-specific antibody and then the blots were stripped and reprobed with antiperilipin antibodies to detect native perilipins. The density of the protein bands was quantified and the data (mean ± S.E.) were expressed as p-PKA substrate/perilipin ratio. (n ≥ 3 independent experiments). *P<0.05 and ***P<0.001 vs. Basal Control (vehicle-treated cells). #P<0.05 vs. respective control. aP<0.05, bP<0.01 and cP<0.001 vs. basal LA-treated cells.

Fig. 5. LA does not modify the levels of the ATGL co-activator CGI-58 and ATGL inhibitor G0S2. A-B: Lysates from 3T3-L1 adipocytes treated with LA (250 \(\mu\)M) for 1 h (A) and 24 h (B) were immunoblotted for ATGL, CGI-58, G0S2 and Actin antibody. Band intensities for ATGL, CGI-58 and G0S2 were normalized to Actin. Data are expressed as mean ± S.E. of at least 5 independent experiments. *P<0.05 vs. Control (vehicle-treated cells).

Fig. 6. LA reduces AdPLA levels and PGE\(_2\) secretion and increases intracellular cAMP levels in 3T3-L1 adipocytes. A: AdPLA protein levels at 1 and 24 h of treatment with LA
B: PGE$_2$ released to the media in 3T3-L1 adipocytes treated with LA (250 µM) during 24 h. C: Intracellular cAMP levels at 1 and 24 h of treatment with LA (250 µM). D: Effects of PGE$_2$ (0.5 ng/ml) on the lipolytic action of LA (250 µM) in the presence or absence of the EP3 antagonist L78106 (10 µM). Data are expressed as mean ± S.E. of at least 3 independent experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.01$ vs. Control (vehicle-treated cells). $^\dagger$$P<0.05$ vs. PGE$_2$-treated cells. $^a$$P<0.05$ vs. basal LA-treated cells.
Figure 1

A

Glycerol (µmol/L)

B

Glycerol (%)

0
100
200
300
400
500
80
100
120
140
160
180

0 1 2 3 4 5 6 7

Length of treatment (h)

B

Basal
LA 250 µM
LA 500 µM

C

FFA (µmol/L)

0
500
1000
1500

0 1 10 100 250 500 LA (µM)

**
***

Glycerol (µmol/L)

0 1 10 100 250 500 LA (µM)

***

Glycerol (%)

Basal
LA 250 µM
LA 500 µM

* *

Glycerol (%)

Basal
LA 250 µM
LA 500 µM

**
***

Glycerol (µmol/L)

0 1 10 100 250 500 LA (µM)

**
***
Figure 2

A

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**P-ERK1/2/ERK1/2,** **P-JNK/JNK,** **P-AMPK/AMPK,** **P-AKT/AKT**
Figure 3

A

B

C

D

E

Protein/Actin

mRNA/β Actin

ATGL mRNA/β Actin

HSL mRNA/β Actin

Perilipin mRNA/β Actin

Control

LA 250 µM

Basal SP AICAR PD H89 LY

ATGL HSL Perilipin

Control

LA 250 µM

Basal SP AICAR PD H89 LY

ATGL HSL Perilipin

Control

LA 250 µM

Basal SP AICAR PD H89 LY

ATGL HSL Perilipin

Control

LA 250 µM

Basal SP AICAR PD H89 LY

ATGL HSL Perilipin

Control

LA 250 µM

Basal SP AICAR PD H89 LY

ATGL HSL Perilipin

Control

LA 250 µM

Basal SP AICAR PD H89 LY

ATGL HSL Perilipin
Figure 4

A

LA 250 µM

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<td>-</td>
<td>+</td>
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P-HSL\textsuperscript{563}

B

LA 250 µM

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P-HSL\textsuperscript{660}

C

LA 250 µM

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P-PKA substrate/Perilipin
Figure 5

A

1h

LA (250 µM) - +

ATGL
CGI-58
G0S2
ACTIN

B

24h

LA (250 µM) - +

ATGL
CGI-58
G0S2
ACTIN
Figure 6

A. Western blot analysis of AdPLA and Actin levels at 1 h and 24 h in the presence or absence of LA 250 µM. 

B. Prostaglandin E2 (PGE2) levels (pg/ml) in Control and LA-treated samples at 1 h and 24 h.

C. cAMP levels (% of Control) in Control and LA-treated samples at 1 h and 24 h.

D. Glycerol levels (%) in Control, LA, PGE2, PGE2 + L78106, LA + PGE2, and LA + PGE2 + L78106 treatments.