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

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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 pretreatment with the c-Jun N-terminal kinase inhibitor SP600125, the PKA inhibitor H89, and the AMP-activated protein kinase activator AICAR. In contrast, the PI3K/Akt inhibitor LY294002 and the PDE3B antagonist cilostamide enhanced LA-induced lipolysis. LA treatment for 1 h did not modify total protein content of hormone-sensitive lipase (HSL) but significantly increased the phosphorylation of HSL 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 the protein levels of adipose triglyceride lipase or its activator comparative gene identification 58 (CGI-58) and inhibitor G(0)/G(1) switch gene 2 (G0S2). Furthermore, LA caused a significant inhibition of adipose-specific phospholipase A2 (AdPLA) protein and mRNA levels in parallel with a decrease in the amount of prostaglandin E_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 prostaglandin E_2.—Fernández-Galilea, M., P. Pérez-Matute, P. L. Prieto-Hontoria, J. A. Martinez, and M. J. Moreno-Aliaga. **Effects of lipoic acid on lipolysis in 3T3-L1 adipocytes.** ***J. Lipid Res.*** **2012.** 53: **2296–2306.**

Supplementary key words hormone-sensitive lipase • adipose triglyceride lipase • adipose-specific phospholipase A2 • prostaglandin E_2

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 its associated comorbidities, 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, 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 have provided strong evidence 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 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 that is highly regulated and

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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; ERK1/2, extracellular signal-regulated kinase; FFA, free fatty acid; 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; MAPK, mitogen-activated protein kinase; NAC, n-acetyl cysteine; PD, PD98059; PDE3B, phosphodiesterase 3B; PGE_2, prostaglandin E2; PI3K, phosphatidylinositol 3 kinase; PKA, protein kinase A; PPARγ, peroxixome proliferator-activated receptor gamma; ROS, reactive oxygen species; SP, SP600125; TAG, triacyl glycerol; TNF-α, tumor necrosis factor alpha.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of five figures.

involves the coordinated 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 (14). ATGL exhibits high substrate specificity for triacyl glycerol (TAG) (15). Lipase activity of ATGL largely depends on its coactivation by comparative gene identification 58 (CGI-58), whereas G(0)/G(1) switch gene 2 (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 phosphorylated by other kinases, such as extracellular signal-regulated kinase (ERK1/2), which activates HSL by phosphorylation on Ser^{600} (23). It has been suggested that c-Jun N-terminal kinase (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.

MATERIALS AND METHODS

Cell culture and differentiation of 3T3-L1 cells

Murine 3T3-L1 cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM containing 25 mM glucose, 10% calf bovine serum (Invitrogen, Carlsbad, CA), and 1% penicillin and streptomycin (Gibco, Invitrogen Corp.) and were maintained in an incubator set up to 37°C and 5% of CO_2. At confluence, preadipocytes were induced to differentiate into adipocytes by culturing them for 48 h in DMEM containing 10% FBS (Invitrogen) and antibiotics and supplemented with dexamethasone (1 mM; Sigma, St. Louis, MO), 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 after 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), PD98059 (PD) (Sigma), H89 (Santa Cruz Biotechnology, Santa Cruz, CA), 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 1,000× stock solutions and added to the culture medium. Control cells were treated with the same amount of the corresponding vehicle.

Before 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). To analyze the signaling pathways involved in LA actions, adipocytes were preincubated for 1 h 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 (FFAs) released to the media. Glycerol was determined after 1 to 24 h of LA treatment using an autoanalyser following the manufacturer instructions (Cobas-Mira; Roche Diagnostics, Basel, Switzerland). 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). RNA was then incubated with the RNase-free kit DNase (Ambion, Austin, TX) for 30 min at 37°C. RNA (2 μg) was reverse-transcribed to cDNA using Moloney Murine Leukaemia

Virus reverse transcriptase (Invitrogen). For the real-time quantitative PCR 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, CCAAT/enhancer-binding protein alpha (C/EBPα), and CCAAT/enhancer-binding protein beta (C/EBPβ) mRNA levels were determined using predesigned Taqman® Assays-on-Demand (Applied Biosystems, Foster City, CA). Taqman Universal Master Mix was also provided by Applied Biosystems. The reaction conditions were followed according to the 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 adipocytes 8 days after differentiation. 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). Total proteins were resolved in SDS-PAGE minigels and electroblotted onto polyvinylidene difluoride 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}/Tyr^{185} (Cell Signaling Technologies, Beverly, MA); AdPLA (Cayman Chemical, Ann Arbor, MI); and CGI-58, G0S2 (Santa Cruz), and Actin (from Sigma). Secondary antibody was HRP goat anti-rabbit IgG-HRP (Bio Rad Laboratories). The immunoreactive proteins were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). Band intensities were quantified using a GS-800 calibrated densitometer (Bio Rad Laboratories).

Fatty acid oxidation determination

Fatty acid oxidation to acid-soluble metabolites was measured with radiolabeled ^{14}C- palmitate (Perkin Elmer, Boston, MA) in mature 3T3-L1 adipocytes as previously described (37). Acid-soluble metabolites were extracted by addition of 1 ml cold 1 M $HClO_4$ (Panreac, Barcelona, Spain). After centrifugation (10 min, 1,800 *g*), radioactivity in the supernatant was measured by scintillation counting by using a Wallac 1409 liquid scintillation counter (EG and G Co., Turku, Finland). Protein content in parallel cultures of vehicle- and LA-treated cells was analyzed using a BCA method.

ELISA assays

PGE_2 concentration in the media was determined after 4, 8, and 24 h of LA (250 µM) treatment by using a PGE_2 Enzyme Immunoassay kit (Arbor Assays, Ann Arbor, MI). 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 error (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 Mann-Whitney U test once the normality with the Kolmogorov-Smirnoff and Shapiro-Wilk tests was screened. SPSS 19.0 version for Windows (SPSS, Chicago, IL) and GraphPad Prism 5.0 (Graph-Pad Software Inc. San Diego, CA) were used for 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 induced a concentration-dependent increase in the amount of FFAs released after 3 h treatment (100–500 µM; $P < 0.001$) (Fig. 1C). We also tested the effects of LA on

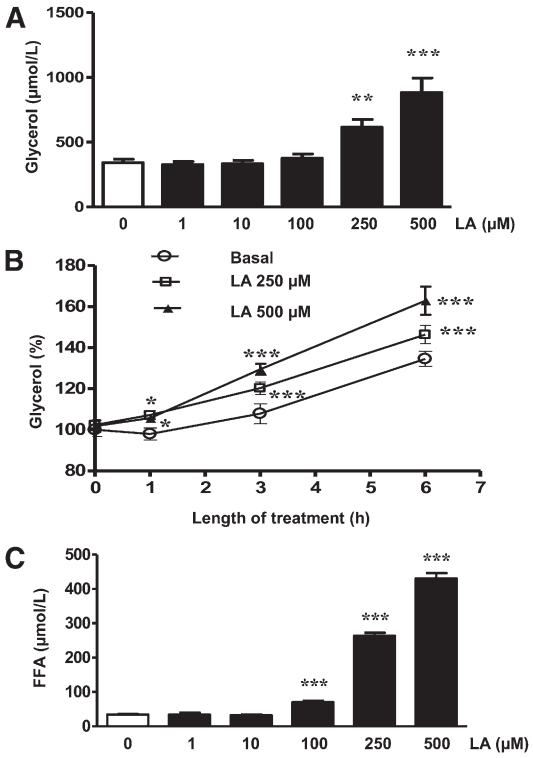

Fig. 1. LA stimulates lipolysis in 3T3-L1 adipocytes. Mature 3T3-L1 adipocytes were treated with LA (0–500 µM) for the indicated times (1, 3, 6, or 24 h). A: Lipolysis was assessed by the amount of glycerol released into media in adipocytes treated for 24 h. B: Time-dependent effects of LA (250 and 500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

isoproterenol-induced lipolysis and the data revealed that LA did not have any additional effect on the lipolytic effect of isoproterenol (supplementary Fig. I). 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 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

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 after short-term (30 min to 1 h) 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), but 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 after short-term ($P < 0.05$) 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 ($P < 0.001$). Our data revealed that the lipolytic actions of LA were reversed by pretreatment with the JNK inhibitor SP600125 ($P < 0.01$), the PKA inhibitor H89, and the AMPK activator AICAR ($P < 0.001$). 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 ATGL and HSL were also significantly down-regulated ($P < 0.05$) by LA treatment for 24 h (Fig. 3B). Perilipin mRNA levels were also reduced in LA-treated adipocytes, whereas no changes in perilipin protein content were observed (Fig. 3A, B).

The inhibitory actions of LA treatment on ATGL gene expression were not observed in the presence of the JNK inhibitor SP600125 and the 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, 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 investigated the effects of LA on HSL phosphorylation 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 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 Ser^{563} and Ser^{660} (Fig. 4A, B) without modifying the p-PKA substrate/perilipin ratio (Fig. 4C). The inhibition of the JNK pathway induced a significant increase in the phosphorylation of HSL at Ser^{660} in the absence and presence of LA and in PKA-mediated perilipin phosphorylation (Fig. 4B, C). Moreover, the ERK1/2 inhibitor PD98059 prevented the LA-induced phosphorylation of HSL at Ser^{563} without modifying LA effects on Ser^{660} and the 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 for 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 for 1 and 24 h (250 μM) caused a significant inhibition ($P < 0.05$) of AdPLA protein content as well as on mRNA levels (data not shown).

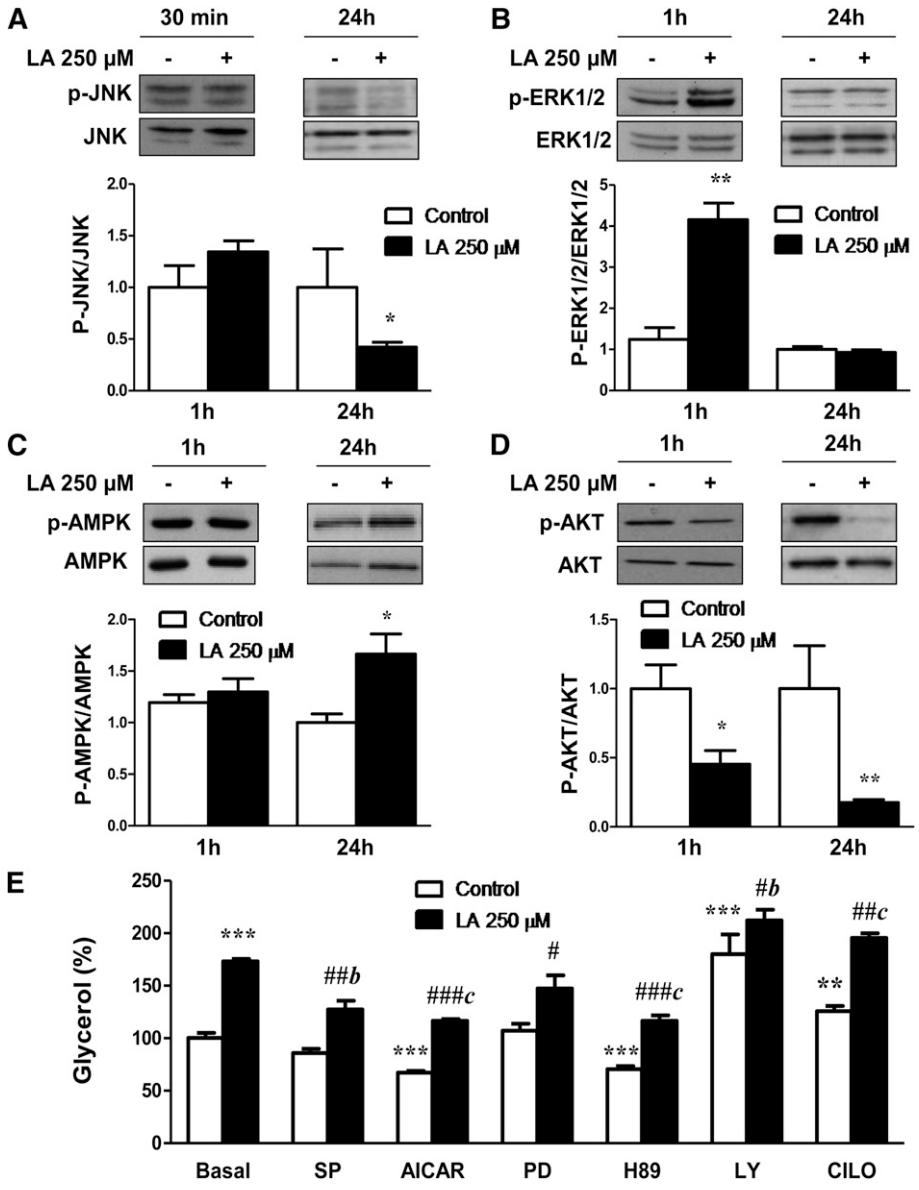

Fig. 2. Signaling pathways involved in the lipolytic effects of LA. A–D: Effects of LA on the phosphorylation of JNK (A), ERK1/2 (B), AMPK (C), and PI3K/AKT (D). Band intensities for each phosphorylated species were normalized to their respective total fractions. E: Effects of LA treatment for 24 h on 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. Data are expressed as mean ± SE of at least three 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. $^{b}P < 0.01$ and $^{c}P < 0.001$ vs. basal LA-treated adipocytes.

We next evaluated the effects of LA on the major AdPLA product, PGE_2, which binds the Gαi-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 cotreatment 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. 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 in vitro and in vivo (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

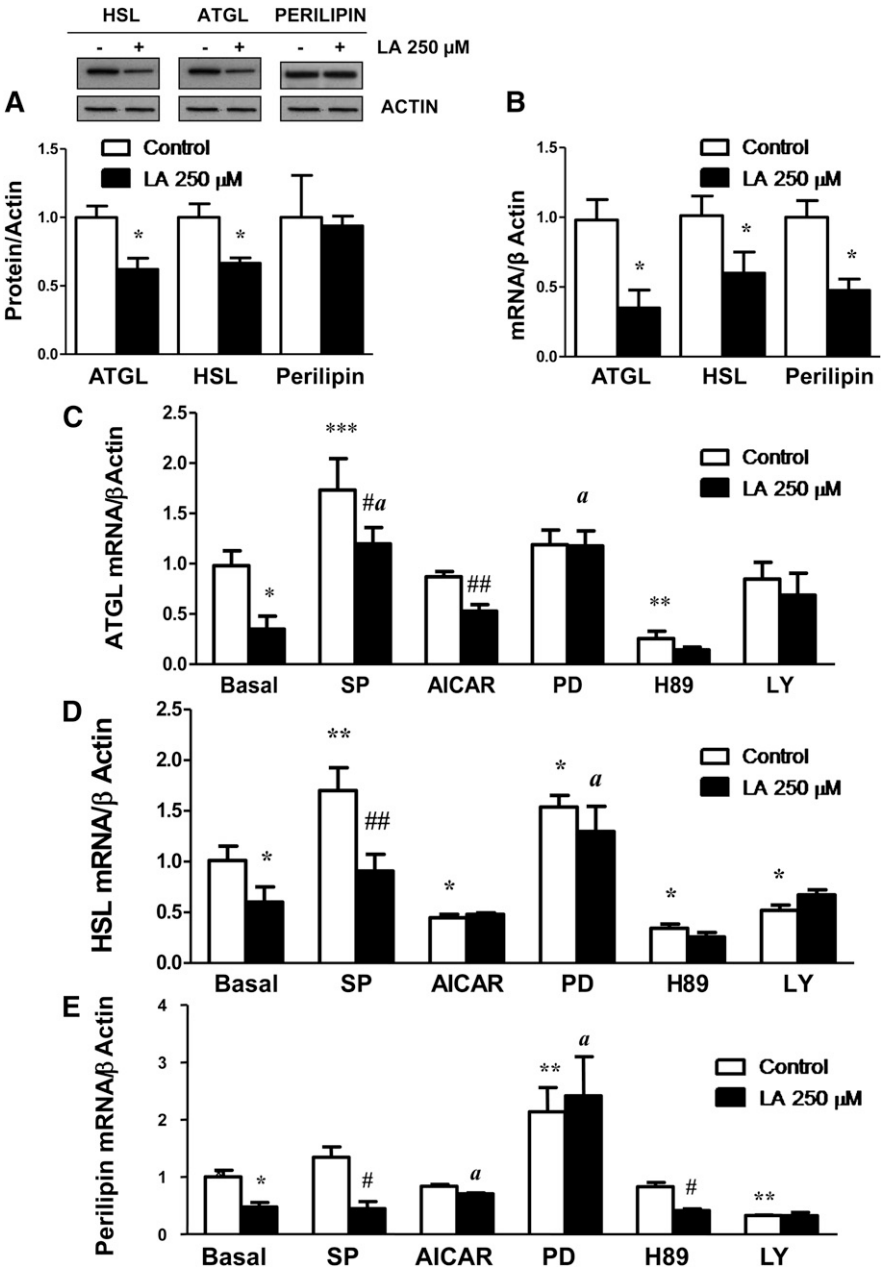

Fig. 3. Long-term LA treatment down-regulates total HSL, ATGL, and perilipin transcripts. The effects of LA (250 μM) on total ATGL, HSL, and perilipin protein (A) and mRNA (B) levels were assessed in 3T3-L1 adipocytes after 24 h of treatment. C–E: Effects of the JNK inhibitor SP600125 (SP), the ERK1/2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

able to stimulate lipolysis, suggesting that the lipolytic actions are independent of the antioxidant capacities.

Our data showed that despite the stimulatory effects of LA on lipolysis, 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 β-3 adrenergic agonist that stimulates lipolysis (42), induced a decrease in HSL mRNA levels in abdominal white adipose tissue, whereas an increase in HSL activity was observed (43). Furthermore, a recent study reported that serum amyloid A stimulated lipolysis in parallel with a reduced HSL protein content. However, serum amyloid A caused a significant increase of PKA-mediated HSL phosphorylation (44), suggesting opposite trends in HSL

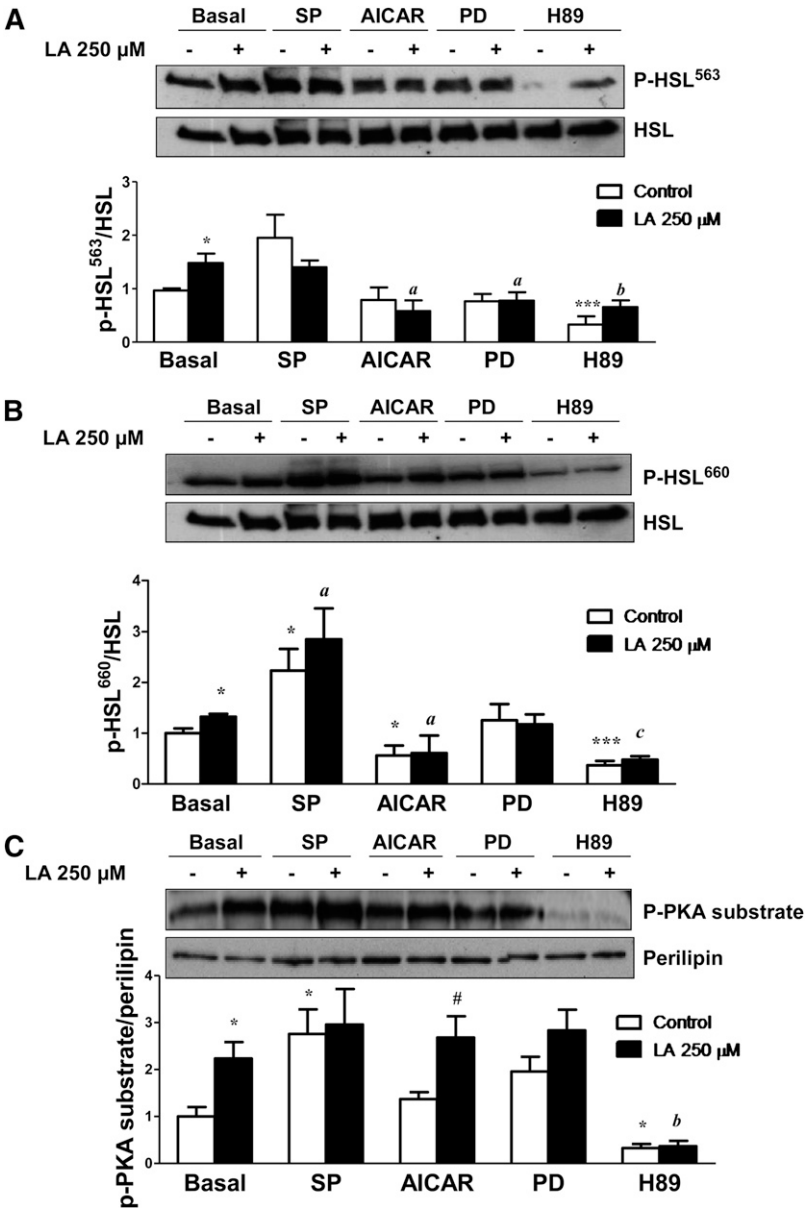

Fig. 4. LA stimulates PKA-mediated phosporylation of HSL and perilipin. A and B: Representative Western blots for Ser^{563}-phosphorylated HSL (A) and Ser^{660}-phosphorylated HSL (B) in differentiated 3T3-L1 adipocytes treated with LA (250 μM) for 1 h in the presence or absence of the JNK inhibitor 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 immunoblotted using a phospho-PKA-motif-specific antibody, and 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 ± SE) 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. $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ vs. basal LA-treated cells.

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. Upon phosphorylation, perilipin shifts to the cytoplasm, accessibility of HSL to the lipid surface is promoted, and lipolysis is enhanced (45–47). The results of our study suggest a key role of PKA-induced lipolysis in the lipolytic actions of LA because *i*) LA increased HSL phosphorylation at Ser^{563} and Ser^{660}, *ii*) PKA-induced perilipin phosphorylation was increased by LA treatment, and *iii*) the PKA inhibitor H89 completely blunted the lipolytic action of LA as well as the LA-induced phosphorylation of phospho-PKA substrates. Taken 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 basal and adrenergically stimulated TAG breakdown in adipocytes (14). However,

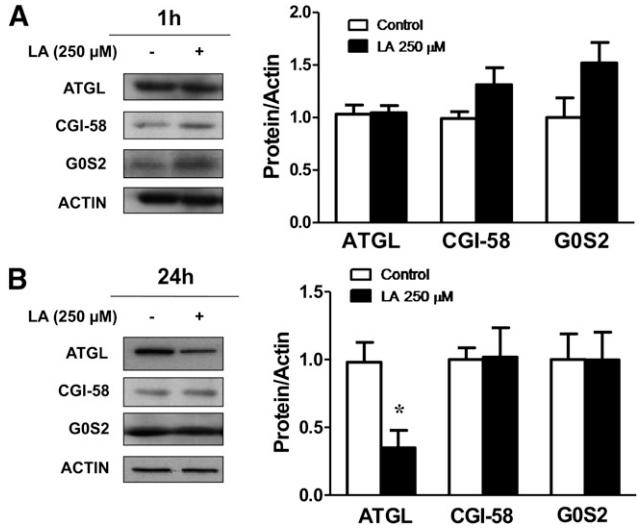

Fig. 5. LA does not modify the levels of the ATGL coactivator CGI-58 or the ATGL inhibitor G0S2. A and B: Lysates from 3T3-L1 adipocytes treated with LA (250 μM) for 1 h (A) and 24 h (B) were immunoblotted for ATGL, CGI-58, G0S2, and actin antibody. Band intensities for ATGL, CGI-58, and G0S2 were normalized to actin. Data are expressed as mean ± SE of at least five independent experiments. $*P < 0.05$ vs. control (vehicle-treated cells).

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 on the levels of the ATGL coactivator protein CGI-58 or on 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 inhibited AKT phosphorylation both at 30 min and 24 h of treatment, and 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 that lipolysis could be 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, coincubation 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 to be time dependent. On the other hand, the fact that pretreatment with the ERK1/2 inhibitor PD98059 reversed the down-regulation 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 show 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 and activated by AMPK to increase lipolysis (17). Thus, the effects described for AMPK activators on lipolysis are controversial, showing 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 time. Taken together, these data suggest that the lipolytic action of LA is not mediated by the activation of AMPK in the first stages but could contribute to the regulation of the long-term lipolytic effects of LA.

A new adipocyte phospholipase A2, called AdPLA, has recently been described and functionally characterized (27). It has been 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 expression and PGE_2 levels were down-regulated by LA treatment, 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 coincubation with PGE_2 was able to partially reverse the stimulatory effect of LA on lipolysis, whereas this effect of PGE_2 was not observed in the presence of an EP3 antagonist.

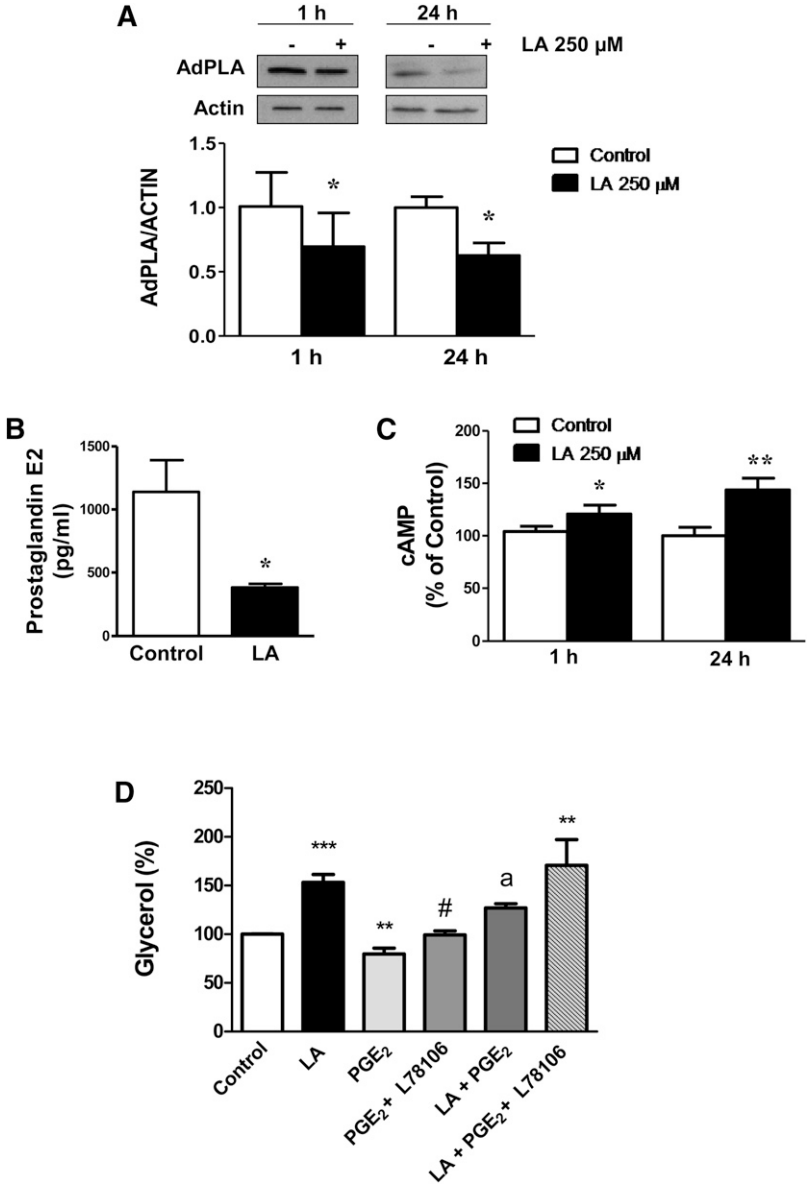

Fig. 6. LA reduces AdPLA levels and PGE_2 secretion and increases intracellular cAMP levels in 3T3-L1 adipocytes. A: AdPLA protein levels at 1 and 24 h of treatment with LA (250 μM). B: PGE_2 released to the media in 3T3-L1 adipocytes treated with LA (250 μM) for 24 h. C: Intracellular cAMP levels at 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 ± SE of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.01$ vs. control (vehicle-treated cells). $^{\#}P < 0.05$ vs. PGE2-treated cells. $^{a}P < 0.05$ vs. basal LA-treated cells.

All these data suggest that the ability of LA to stimulate lipolysis in adipocytes could also contribute to its antiobesity properties. Increased lipolysis and FFA release from adipose tissue has been associated with the development of insulin resistance (60). However, recent findings have demonstrated that increasing lipolysis in adipose tissue does not necessarily increase serum FFA levels because increasing lipolysis in adipose tissue causes a shift within adipocytes toward increased FA utilization and energy expenditure and thus protects against obesity. Therefore, it has been suggested that 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 in rodents (10, 62, 63) and in humans (7), and, as previously suggested, this could be associated with 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 shown that LA increased AMPK and ACC phosphorylation, leading to increased palmitate β-oxidation in myotubes (64). Moreover, studies of our group have shown

that LA supplementation prevents the down-regulation 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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