ORIGINAL RESEARCH

Lipoic Acid Inhibits Leptin Secretion and Sp1 Activity in Adipocytes

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Abbreviations: FBS, foetal bovine serum; LA, lipoic acid; LASY, lipoic acid synthase; PF, pair-fed; PP1, protein phosphatase 1; SE, standard error; WAT, white adipose tissue.

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Abstract

Lipoic acid (LA) is an antioxidant with therapeutic potential on several diseases like diabetes and obesity. Hyperleptinemia and oxidative stress play a major role in the development of obesity-linked diseases. The aim of this study was to examine *in vivo* and *in vitro* the effects of LA on leptin production, as well as to elucidate the mechanisms and signaling pathways involved in LA actions. Dietary supplementation with LA decreased both circulating leptin, and adipose tissue leptin mRNA in rats. Treatment of 3T3-L1 adipocytes with LA caused a concentration-dependent inhibition of leptin secretion and gene expression. Moreover, LA stimulated the anaerobic utilization of glucose to lactate, which negatively correlated with leptin secretion. Furthermore, LA enhanced phosphorylation of Sp1 and inhibited Sp1 transcriptional activity in 3T3-L1 adipocytes. Moreover, LA inhibited Akt phosphorylation, a downstream target of PI3K. Treatment with the PI3K inhibitor LY294002 mimicked LA actions, dramatically inhibiting both leptin secretion and gene expression and stimulating Sp1 phosphorylation. All of these data suggest that the phosphorylation of Sp1 and the accompanying reduced DNA-binding activity are likely to be involved in the inhibition of leptin induced by LA, which could be mediated in part by the abrogation of the PI3K/Akt pathway.
1 Introduction

Obesity is a disease with serious public health implications, associated with insulin resistance, type 2 diabetes, hypertension, dyslipemia and atherosclerosis. A growing body of evidence support that obesity is linked to a state of chronic oxidative stress, which may result from a combination of adipokine imbalance, and reduced antioxidant defenses [1].

White adipose tissue (WAT) dysfunction plays a critical role in the development of obesity-associated disorders. Indeed, WAT is a complex and metabolically active organ, with an important relevance in regulating whole-body metabolism. In addition to its primary role as a fuel reservoir, WAT has been confirmed as a major endocrine organ, that synthesizes and secretes an array of sex steroids, and bioactive peptides termed ‘adipokines’, involved in the physiological regulation of fat storage, energy metabolism, food intake, insulin sensitivity, and immune function among others [2].

Leptin is an adipokine that plays an important role regulating food intake and energy balance. Although leptin deficiency leads to severe obesity in rodents and humans, it is clearly established that serum leptin concentrations are proportional to the amount of adipose tissue mass and therefore, most frequently obesity is accompanied by hyperleptinemia, associated with resistance to the actions of this adipokine [3]. Hyperleptinemia itself has also been suggested to be involved in the pathogenesis of the co-morbidities associated to obesity such as type 2 diabetes mellitus, hypertension, cardiovascular disease, and cancer [4]. Leptin has been also shown to induce perturbations in the anti-oxidative defence system and increase oxidative stress in both animals and humans [5]. In fact, hyperleptinemia is associated with oxidative stress in type 2 diabetes mellitus, and decreases insulin secretion from the pancreatic β cells [6].
Therefore, antioxidant therapy has been proposed as an useful strategy for attenuation of oxidative stress and hyperleptinemia in obesity [1, 7].

Lipoic acid (LA; 1,2-dithiolane-3-pentanoic acid) is a naturally occurring short chain fatty acid with sulfhydryl groups, which is a necessary cofactor for mitochondrial enzymes [8, 9]. Aside from its enzymatic role, in vitro and in vivo studies suggest that LA also acts as a powerful micronutrient with diverse pharmacological and antioxidant properties [10]. A recent study has demonstrated that down-regulation of lipoic acid synthase (LASY), the enzyme involved in the endogenous synthesis of LA, reduced endogenous levels of LA as well as critical components of the antioxidant defence network, increasing oxidative stress. Moreover, this down-regulation of LASY induced a significant loss of mitochondrial membrane potential and decreased insulin-stimulated glucose uptake in skeletal muscle cells along with an increased inflammatory response which, in turn, leads to increased insulin resistance, mitochondrial dysfunction and inflammation [11]. Thus, LA can be considered as a promising therapeutic approach for chronic inflammatory diseases such as diabetes and obesity [12, 13]. Thus, it has been reported the ability of LA to reduced body weight gain in rodents by suppressing food intake and increasing energy expenditure [8, 14-16]. Studies of our group has also described that LA decreased feed efficiency and intestinal sugar transport, which could explain at least in part its reducing effects on body weight gain despite of high fat diet feeding [14]. Moreover, a recent study in pre-obese and obese human subjects have evidenced that LA (800 mg/day for 4 months) induced significant reductions of body weight, BMI, blood pressure, and abdominal circumference, together with a decrease in inflammatory markers [17].

Some of these studies have described that the reduction of body weight and adiposity induced by LA is accompanied by a decrease in plasma leptin levels [8, 18,
However, it still remains to be elucidated if this reduction in circulating leptin levels is merely a consequence of reduced fat mass or if LA treatment can directly inhibit leptin production. Therefore, the first aim of the present study was to examine the effects of LA supplementation on leptin production in lean and high-fat fed rats. Moreover, we investigate the in vitro effects of LA on leptin secretion and gene expression in cultured adipocytes. We also aimed to find out the potential molecular mechanisms involved. Because of the importance that glucose metabolism and Sp1 transcription factor plays in the regulation of leptin secretion by the adipocytes [20-22], we tested the effects of LA on the anaerobic utilization of glucose to lactate and on Sp1 activity and its regulation by phosphorylation. Moreover, we also analyzed the potential signaling pathways underlying the effects of LA on leptin and Sp1.

2 Materials and methods

2.1 Animal and diets

Six-week-old growing male Wistar rats (n=54) were obtained from the Centre of Applied Pharmacology (CIFA, Pamplona, Spain). Animals were housed in polycarbonate cages (3-4 rats per cage) in temperature-controlled rooms (22 ± 2 °C) with a 12 hour light–dark cycle, fed a pelleted chow diet and given deionised water ad libitum for an adaptation period of 5 days.

Rats were then assigned into 6 experimental groups for 8 weeks. Control, CLIP and PF-CLIP groups were fed with a standard diet (Harlan Tekland Global Diets) containing 16.7 % of energy as proteins, 78.6 % as carbohydrates and 4.6 % as lipids per dry weight. The Obese, OLIP, and PF-OLIP groups were fed with a high fat diet (OpenSource diets Research Diets Inc) containing 60 % of energy as lipids, 20 % as carbohydrates and 20 % as proteins per dry weight, which has been widely used to
induce obesity in rodents [23]. The diet of the subgroups CLIP and OLIP was supplemented with racemic α-Lipoic acid (Sigma-Aldrich, St Louis, MO) in a proportion of 0.25 g LA/100 g of diet as previously described [8]. LA was thoroughly and homogeneously mixed with both diets (chow and high fat) using a blender. Food intake was measured 3 times per week before the onset of the dark period, and the two Pair-Fed groups (PF-CLIP and PF-OLIP) received then the same amount of food ingested by the groups CLIP or OLIP, respectively, but without LA supplementation. These two groups are necessary to distinguish what proportion of the LA actions is independent of LA-effects on food intake.

Body weight and food intake were recorded every 2-3 days. At the end of the experimental period (56 days), rats were killed by decapitation and blood and tissue samples including WAT depots (epididymal, retroperitoneal, mesenteric and subcutaneous) were collected as previously described [24]: Thus, epididymal WAT includes prominent bilateral intra-abdominal visceral depots in male rats attached to the epididymides; retroperitoneal WAT, bilateral depots in abdominal cavity behind the peritoneum on the dorsal side of the kidney; subcutaneous fat, bilateral superficial subcutaneous WAT between the skin and muscle fascia posterior to the lower segment of the upper limbs; mesenteric fat, is outlined by the two peritoneal leaflets holding the intestine against the posterior abdominal wall. All fat depots were weighed and kept at –80 °C for subsequent analysis. Visceral WAT depot was estimated by the sum of epididymal, retroperitoneal and mesenteric depot weights. All experimental procedures were performed according to National and Institutional Guidelines for Animal Care and Use with the approval of the Ethical Committee for Animal Care and Use at the University of Navarra.
2.2 Cell culture and differentiation of 3T3-L1 cells

Mouse preadipose cell line 3T3-L1 was purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/l glucose, calf serum 10 % (v/v) and antibiotics 1 % (v/v), and were maintained in a water-jacketed incubator set to 37 ºC and 5 % carbon dioxide. Confluent cells were induced to differentiate by incubating for 48 h with differentiation medium containing 1 µmol/l dexamethasone (Sigma, St Louis, MO), 0.5 µmol/l isobutylmethylxanthine (Sigma), 10 µg/ml insulin (Sigma), 10 % (v/v) foetal bovine serum (FBS) and antibiotics (1 %) in DMEM (Invitrogen, Grand Island, NY). Then, cells were cultured with 10 % (v/v) FBS and 10 µg/ml insulin and antibiotics (1 %) in DMEM for 48 h. After 2 days, media was replaced with 10 % FBS in DMEM and antibiotics, without insulin, and changed every 2 days. Different treatments were added to differentiated 3T3-L1 adipocytes (day 7-8 postconfluence) for a period time of 24 hours [25].

2.3 Adipocyte isolation and primary culture

Adipocytes were isolated under sterile conditions from epididymal fat depots of eight-week-old male Wistar rats (250-280 g) fed a pelleted chow diet and given deionised water ad libitum [25]. Briefly, adipose tissue fragments were digested with type I collagenase at 37 ºC with gentle shaking for 30 min. The resulting cell suspension was diluted in Hepes buffer (5 mmol/l D-glucose, 2 % BSA, 135 mmol/l NaCl, 2.2 mmol/l CaCl₂ · 3H₂O, 1.25 mmol/l MgSO₄ · 7H₂O, 0.45 mmol/l KH₂PO₄, 2.17 mmol/l Na₂HPO₄ and 10 mmol/l Hepes, pH 7.4) and then filtered through a 400 µm nylon mesh. The isolated adipocytes were washed three times and resuspended in DMEM (5
mmol/l glucose) supplemented with 1 % (v/v) FBS and incubated for 30–40 min at 37 °C.

The isolated adipocytes were plated on 500 μl of a collagen matrix (Purecol; Inamed Biomaterials) in six-well culture plates and, after 50 min incubation, culture medium containing the different treatments was added and cells were cultured for up 48 h. At the end of the incubation period samples were collected to measure leptin.

2.4 Treatments

α-Lipoic acid (Sigma Aldrich) was dissolved in ethanol. LY294002 (Sigma), SP600125 (Biomol), PD98059 (Sigma) and Troglitazone (Cayman Chemical, Michigan, USA) were dissolved in DMSO. Control cells were treated with the same proportion of the corresponding vehicle (ethanol and/or DMSO in proportion 0.01 %). When the selective PI3K inhibitor LY294002 (50 μM), JNK inhibitor SP600125 (20 μM), and MAPK inhibitor PD98059 (50 μM) were used, adipocytes were pre-incubated for 1 hour with these inhibitors prior to the addition of LA (250 μM).

2.5 Biochemical measurements

Leptin concentrations in both serum and culture media were determined by a Rat/Mouse Leptin ELISA kit (Linco Research, Missouri, USA). The lowest level of leptin that can be detected by this assay is 0.05 ng/mL.

Glucose and lactate concentrations in the media were measured using an Autoanalyzer (Cobas Roche Diagnostic, Basel, Switzerland) as previously described [26]. The amount of carbon released as lactate per amount of carbon taken up as glucose was calculated as Δ[lactate]/Δ[glucose], where Δ is the difference, and expressed as a percentage.
2.6 Nuclear extracts and Sp1 binding activity

Nuclear extracts were prepared from fully differentiated 3T3-L1 adipocytes as previously described [27] with some modifications. The entire procedure was carried out at 4°C. Cells were collected and washed once with cold PBS and then with 200 µl buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT (dithiothreitol), 0.5 mM PMSF with 0.6 % Nonidet P40) and kept on ice for 15 min. After centrifugation in a bench-top centrifuge at 800 g for 30 seconds, the cell pellet was resuspended in 1 ml of buffer B (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), and centrifuged at 800 g for 30 seconds. Then, the cell pellet was resuspended in 38 µl of buffer C (10 mM HEPES pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and shaked on 4 ºC at 1500 rpm for 30 min with occasional mixing. The suspension was then centrifuged at 10000 g for 10 min. Insoluble material was removed by centrifugation, and aliquots were kept at −80 ºC.

Nuclear extracts were left untreated or treated with Protein Phosphatase 1 (1.25 U or 5 U), and dephosphorylation reactions were performed at 30°C for 15 min according to the protocol recommend by the supplier (New England Biolabs, UK). Nuclear protein was then quantified using Bradford assay method. A total of 6 µl of nuclear protein and 6 µl nuclear extract dilution buffer from each treatment were analyzed for Sp1 activity using the Transcription Factor ELISA kit, which assesses Sp1 protein-DNA binding activity (Panomics, Fremont, CA).

2.7 Western Blotting

3T3-L1 adipocytes were cultured and induced to differentiate as described [25]. Cells were serum-starved overnight and then incubated with the appropriate treatment.
Nuclear extracts were prepared as previously reported. Briefly, equal amounts of protein samples were separated by 12 % SDS-PAGE and transferred into PVDF membrane. Once blocked the membrane was probed with primary antibodies against phospho-Sp1 (Thr 453) (Abcam plc, Cambridge, UK) and Sp1 (H-225) (Santa Cruz Biotechnology, USA). After further washings, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, California, USA). The immunoreactive proteins were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, USA). Band intensities were quantified using a GS-800 calibrated densitometer (Bio-Rad Laboratories).

2.8 Real-time PCR

Total RNA was extracted from epididymal fat depots and from 3T3-L1 cells using TRIzol® reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions, and then the RNA was incubated with RNAse-free DNase kit (Ambion, Austin, TX). Two μg of RNA were reverse-transcribed to cDNA using MMLV (Moloney Murine Leukaemia Virus) reverse transcriptase (Invitrogen). Leptin mRNA levels were determined using predesigned TaqMan® Assays-on-Demand (Rn00565158_m1* and Mm00434759_m1*, Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using the ABI PRISM 7000HT and ABI PRISM 7900HT Fast System Sequence Detection System (Applied Biosystems, Foster City, CA, USA) Sequence as described previously [25]. Leptin mRNA levels were normalized by two different housekeeping genes Cyclophilin (Rn00690933_m1*, Mm02342430_g1) and/or Ubiquitin C (Rn01789812_g1, Applied Biosytems). Fold changes of gene expression were calculated by the \(2^{-\Delta\Delta C_t}\) method.
2.9 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 analysed by one-way ANOVA, followed by Bonferroni post hoc test, or by Student’s t test or U-Mann Whitney after testing the normality with the Kolmogorov-Smirnov and Shapiro-Wilk tests. Furthermore, Pearson correlation analysis was performed to screen potential association between two variables. SPSS 15.0 version for Windows (SPSS, Chicago, IL, USA) and GraphPad Prism 4.0 (Graph- Pad Software Inc., San Diego, CA, USA) were used for the statistical analyses.

3. Results

3.1 Effects of LA on leptin circulating levels and gene expression in vivo

Table 1 shows that treatment with LA decreased food intake, body weight gain and adipose tissue size in both control or high fat-fed rats. Moreover, the group fed on a high fat diet and treated with LA (OLIP group) had a lower body weight gain and reduced visceral fat weight than its corresponding Pair-Fed (PF) group ($p<0.05$), which received the same amount of food than LA-treated animals but without supplementation with LA. As expected, leptin circulating levels were significantly higher in the obese group in comparison with the control group ($p<0.001$). On the contrary, LA treatment significantly decreased leptin plasma levels ($p<0.001$ for CLIP and $p<0.01$ for OLIP) (Fig. 1A). The patterns of LA effects on leptin gene expression were similar to those observed in plasma levels (Fig. 1B). The decrease in leptin was also observed in the PF-groups, but it was less pronounced than in the LA-supplemented groups, and no statistically differences were found when comparing both groups (Fig. 1A and 1B).

As expected, leptin levels were positively correlated with the size of total white
adipose tissue ($p<0.001$) (Fig. 1C).

### 3.2 Effects of LA on leptin secretion and gene expression in 3T3-L1 adipocytes

Treatment of 3T3-L1 adipocytes with LA caused a concentration-dependent inhibition of basal leptin secretion (Fig. 2A), which was only statistically significant at the highest concentrations tested (-69 and -91% for 250 and 500 µM respectively, $p<0.01$). Basal leptin gene expression was also significantly inhibited by the presence of LA at concentrations of 100 and 250 µM (-81-91%, $p<0.05-0.001$, respectively) (Fig. 2B). Moreover, we also tested the effects of LA treatment on insulin-stimulated leptin secretion in a model of primary rat adipocytes anchored to a collagen matrix, which responds to more physiological concentrations of insulin than 3T3-L1 adipocytes. Fig. 2C shows that LA was also able to partially inhibit the stimulatory effect of insulin on leptin secretion.

### 3.3 Effects of LA on glucose uptake, lactate production and the percentage of glucose metabolized to lactate in 3T3-L1 adipocytes

Glucose uptake was not affected by LA-treatment (Fig. 3A). However, LA increased lactate production by 44% at the highest concentration tested (500 µM, $p<0.01$), whereas lower concentrations did not show any effect (Fig. 3B). Treatment of 3T3-L1 adipocytes with LA (0-500 µM) significantly increased in a concentration-dependent manner, the percentage of glucose that is metabolized to lactate ($p<0.01- p<0.001$; 100-500 µM) (Fig. 3C). Furthermore, the percentage of glucose metabolized to lactate was inversely correlated with leptin secretion, as can be observed in Figure 3D ($r=-0.448; p<0.001$).
3.4 Effects of LA on Sp1-DNA binding activity

To define the mechanisms of LA inhibitory action on leptin, we study LA effects on the transcription factor Sp1, which has been shown to mediate the activation of leptin promoter in response to insulin-stimulated glucose metabolism [21]. As shown in Figure 4A, nuclear extracts from LA-treated adipocytes for 24 h exhibited a decreased abundance of Sp1-DNA complexes ($p<0.01$), suggesting a significant inhibitory effect of LA on Sp1 transcription factor activity.

Several studies have demonstrated that changes in the phosphorylation status of Sp1 are controlling the ability of this transcription factor to bind to DNA [28]. Our data showed that the phosphorylation of Sp1 in Thr453 was significantly increased ($p<0.05$) in LA-treated adipocytes (Fig. 4B). Moreover, we found that treatment of nuclear extracts with protein phosphatase 1 (PP1) in order to dephosphorylate Sp1 abolished the inhibitory effect of LA on Sp1 activity (Fig. 4C).

3.5 Effects of PI3K, ERK1/2 and JNK inhibitors on LA-inhibitory actions on leptin production and Sp1 phosphorylation in 3T3-L1 adipocytes

The effects of several inhibitors of different signaling pathways on both Sp1 phosphorylation and on leptin secretion and gene expression were tested.

Our data evidenced that treatment with the PI3K inhibitor LY294002 (50 $\mu$mol/l) mimicked LA actions, and dramatically reduced both leptin secretion (Fig. 5A) and gene expression (Fig. 5B), as well as increased Sp1 phosphorylation (Fig. 5C). Moreover, we also found that LA treatment caused a significant ($p<0.05$) inhibition of Akt Ser437 phosphorylation in 3T3-L1 adipocytes (Fig. 5D). The JNK inhibitor (SP600125) also stimulated Sp1 phosphorylation in adipocytes, but without affecting the LA actions on phosphorylation of Sp1 or leptin secretion. Moreover, the MAPK
inhibitor (PD98059) was able to reverse the LA-induced phosphorylation of Sp1, without modifying the inhibitory action of LA on leptin secretion (Figs. 5A-5C).

4. Discussion

Previous studies of our group and others (using the same and higher doses than the used in the present study) have demonstrated that the antiobesity effects of dietary supplementation with LA are secondary, at least in part, to its inhibitory actions on food intake [8, 14]. Moreover, it has been evidenced that leptin and its receptor are not essential for α-LA-induced anorexia since the reduction of food intake and body weight was also observed in leptin deficient or leptin receptor–deficient mice [8].

In addition, it was reported LA given in food not only decreases food intake but also stimulates whole-body energy expenditure [8], and decreases intestinal alpha-methylglucoside (alpha-MG) absorption both in lean and obese rats [14]. Moreover, other studies showed that dietary supplementation with lower doses of LA that not affect food intake is able to improve glucose metabolism in diabetic rats [29]. All of these data suggest peripheral metabolic actions of LA in different tissues independently of its central actions on food intake.

In the present trial we have evaluated only the effects of LA on male rats, but it would be interesting to test if a similar or a differential response is observed in females. In this context, previous studies have described that LA stimulates glucose transport activity and insulin signaling in skeletal muscle of lean and obese female Zucker rats [30, 31], as observed in other model of obese male rats [32].

Our present data show that dietary supplementation with LA decreases adiposity as well as both circulating levels of leptin, and gene expression in WAT. Moreover, the
observed changes in leptin levels were positively correlated with the changes in the size of adipose tissue. These results are in agreement with other studies [8, 18, 19, 33] also showing that the body and adipose weight reduction induced by LA treatment is accompanied by a parallel decrease in circulating leptin levels. Moreover, the fact that leptin levels were always slightly lower in LA-treated animals than in their corresponding pair-fed groups suggests a potential direct inhibitory effect of LA on the ability of adipocytes to secrete leptin. However, this possibility had not been yet addressed.

In the present study, we demonstrated for the first time a direct inhibitory effect of LA on both basal and insulin-stimulated leptin secretion in adipocytes. This effect was concentration-dependent, being significant at 250 and 500 µM. Moreover, similar effects of LA were observed on leptin gene expression (from 100 µM), suggesting that LA-inhibition of leptin is mediated, at least in part, at transcriptional level. A previous study considered in vitro treatment with 50 µM LA as a physiological relevant dose [34]. Moreover, similar concentrations of LA that the used in our present trial have been shown to be effective in regulating other biological and metabolic functions including adipocyte differentiation [35], mitochondrial biogenesis [36] and glucose uptake [37] in the same adipocyte line. We have not measured the circulating levels of LA reached after dietary supplementation, and therefore it is difficult to compare the correspondence between the doses used in the in vivo and in vitro approaches. However, our findings suggest that the observed decrease in plasma leptin levels after dietary supplementation with LA, are likely to be not only due to a decrease in the size of WAT depots induced by LA, but also a direct inhibitory effect of LA on the ability of adipocytes to secrete leptin could be contributing.
Several *in vitro* and *in vivo* studies have demonstrated that glucose metabolism is a major determinant of leptin production in adipocytes [20, 22, 38]. The ability of LA treatment (2.5 mM) for 2-60 minutes to stimulate glucose uptake in 3T3-L1 cells by inducing a redistribution of GLUT1 and GLUT4 transporters has been described [39]. Furthermore, another study in 3T3-L1 cells treated with 250 µM LA has also shown a stimulation of glucose uptake during the first 6 h of treatment. However, a longer preincubation period with LA (24-48 h) inhibited glucose uptake into adipocytes, suggesting that the time of treatment with LA is a key factor [37]. Our data show that LA treatment (1-500 µM) during 24 h did not modify basal glucose uptake in 3T3-L1 adipocytes. Therefore, the different results between our present data and previous studies could be explained by the different concentrations tested and the period of treatment evaluated.

It has been suggested that uptake of glucose by itself is not the main determinant of the regulation of leptin production, but subsequent metabolic utilization of glucose is playing an important regulatory role of leptin levels [20, 22]. Indeed, leptin secretion has been found to be inversely proportional to the amount of glucose anaerobically metabolized to lactate [40, 41, 26]. In this context, our data demonstrate that LA significantly increased in a concentration-dependent manner, the percentage of glucose that is metabolized to lactate. Moreover, a negative correlation between leptin secretion and the percentage of glucose anaerobically converted to lactate was observed, suggesting that this increase in the anaerobic utilization of glucose could explain, at least in part, the observed inhibitory effects of LA on leptin.

Sp1 is a ubiquitous transcription factor that may function as a cellular glucose sensor [42]. Furthermore, Sp1 has been identified as an important transcription factor involved in the regulation of leptin gene. Thus, the site centered at -97 pb of the leptin
promoter is conserved in evolution and binds Sp1 present in adipocyte nuclear extracts and contributes to promoter leptin activity [43]. In fact, mutation of this Sp1 site in the proximal human and murine leptin promoters reduced promoter activity [43, 44]. Moreover, previous studies of our group have demonstrated that Sp1 is involved in the induction of leptin by insulin-stimulated glucose metabolism [21]. Our results demonstrated that LA treatment (250 μM for 24 h) inhibited Sp1-DNA binding activity in mature 3T3-L1 adipocytes. In support of these data, a previous study demonstrated that LA decreased the TNF-α and or IL-1β-induced Sp1 binding activity in human umbilical vein endothelial cells [45]. Moreover, a recent trial has also observed that LA inhibited in a dose-dependent manner the activation of Sp1 binding induced by TGF-β in HepG2 cells [46]. In contrast, Cho et al. [35] observed in adipocytes at the early stage of differentiation that LA (250 μM, 3 h of treatment) did not modify the transcriptional activity of Sp1 in the absence or presence of insulin. Taking together the inhibitory action of LA on Sp1 binding activity and leptin production suggests that the inhibition of Sp-1 mediated transcription is likely to be involved in the LA-induced reduction of leptin production. However, with our present data we can not rule out the possibility that leptin production could be inhibited by LA through other mechanisms independently of Sp1 activity.

A growing body of evidence suggest that the DNA-binding and transcription activity of Sp1 may increase or decrease in response to changes in phosphorylation in many cell types [47]. Our present data clearly demonstrated the ability of LA to phosphorylate Sp1, and that dephosphorylation by PP1 abolished the inhibitory effect of LA on Sp1 transcriptional activity, strongly suggesting that phosphorylation of Sp1 is likely to be involved in the inhibitory action of LA on leptin gene expression and protein secretion. In this context, previous studies of our group showed that okadaic
acid, a potent phosphatase inhibitor, inhibited the stimulation of leptin secretion and mRNA levels induced by insulin-stimulated glucose metabolism [21], suggesting the involvement of Sp1 dephosphorylation. Moreover, dephosphorylation of Sp1 by protein phosphatase 1 has been involved in the glucose-mediated activation of several genes including acetyl-CoA carboxylase, aldolase and pyruvate kinase [48, 49].

We also analyzed the potential signal transduction pathways mediating the inhibitory effect of LA on Sp1 phosphorylation and leptin secretion and gene expression. Several kinases including ERK1/2 and PI3K have been involved in the phosphorylation of Sp1 by different factors such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) [50, 51]. Moreover, it has been shown that the insulin-induced up-regulation of leptin in breast cancer cells is regulated by PI3K and ERK1/2 and depends on Sp1 interaction with specific regions of the leptin promoter [52]. Our data show that treatment with the PI3K inhibitor LY294002 mimicked and potentiated LA actions on the phosphorylation of Sp1. Moreover, treatment with the PI3K inhibitor dramatically reduced both basal leptin secretion and gene expression, suggesting an important role of this pathway in controlling basal leptin secretion. This finding is in agreement with the observations of Maeda and Horiuchi [53]. However, other studies using different cell types did not observe an inhibitory action of LY294002 on basal leptin secretion. Taking together our data strongly suggest that the inhibition of the PI3K pathway by LA could be involved in the LA-induced phosphorylation of Sp1 as well as on the inhibitory effect of LA on basal leptin secretion. Indeed, we demonstrated here that LA treatment inhibited Akt phosphorylation, a downstream target of PI3K, in 3T3-L1 adipocytes. Shi et al. (2008) [54] also described that LA (5 mM) causes Akt inhibition in hepatoma cell lines. In contrast, other studies have reported that LA remediates the age-associated impairment of Akt phosphorylation in primary rat
hepatocytes [34]. Moreover, it has been described that LA improves insulin-stimulated Akt phosphorylation in soleus muscle from high-fat fed [32] and from the insulin resistant Goto-Kakizaki rats [55]. Therefore, the effect of LA on PI3K/Akt pathway is complex and seems to be dependent on the cell type, as well as on the characteristics of the treatment (dose/concentrations and duration) tested.

Previous studies have evidenced the ability of LA to interfere with the MAPK/ERK pathway. Some trials have shown that LA strongly activated ERK in adipocytes at early stages of differentiation [35], while others found that LA inhibited the TGF-β-induced phosphorylation of ERK in AML-12 cells [46]. Our present data show that inhibition of ERK1/2 was able to reverse the LA-induced phosphorylation of Sp1, but not the inhibition of leptin secretion, suggesting that this pathway is not involved in the inhibitory actions of LA on leptin production.

Here we show the ability of antioxidant molecules like LA to inhibit leptin secretion. In the same way, other antioxidants such as Vitamin C and resveratrol also inhibit leptin secretion by adipocytes [56, 57]. However, prooxidant agents (exposure to H₂O₂) have also been shown to alter adipokine gene expression in adipocytes, including the inhibition of leptin secretion [58]. JNKs are activated by reactive oxygen species, and are well known for regulating transcription factors through phosphorylation. Moreover, H₂O₂ has also been shown to induce JNK pathway and to phosphorylate Sp1 and reduce Sp1 binding to DNA, and JNK inhibitors are able to attenuate H₂O₂ – induced Sp1 phosphorylation in human alveolar epithelial cells [59]. However, Min et al. (2010) [46] described that LA inhibited the phosphorylation of JNK and also the TGF-β-stimulated Sp1-DNA binding activity in HepG2 cells. In the same way, we have observed that in adipocytes LA is also able to induce Sp1 phosphorylation and decrease the Sp1 binding activity. Moreover, we also reported that inhibition of JNK pathway
with SP600125 also stimulated Sp1 phosphorylation in adipocytes, without affecting the LA-induced phosphorylation of Sp1. In addition, the inhibition of JNK pathway was not able to reverse the LA actions on leptin secretion. Taking together all of these data, it can be hypothesized that the activation of JNK pathway is not likely to be involved either in the LA-induced phosphorylation of Sp1 or in the inhibitory action of LA on leptin secretion.

Taking together our present results in cultured adipocytes and previous data of other groups in Hep G2 [46, 23] and HUVEC [45] cells suggest a role of Sp1 in mediating some of the actions observed for LA in \textit{in vitro} models. However, it still remains to be addressed the potential physiological involvement of Sp1 in the effects of LA after dietary supplementation in animal models.

On the other hand, the fact that LA directly alters leptin secretion by adipocytes raises the possibility that LA could also regulate other bioactive adipokines (such as adiponectin, apelin and visfatin) that directly regulate nutrient metabolism and insulin sensitivity, opening future research perspectives to better understand the mechanisms of LA actions.

In summary, the present data clearly demonstrate that LA inhibits leptin secretion and gene expression, and suggest that increased anaerobic metabolism of glucose may be contributing to these effects. The phosphorylation of Sp1 and the reduced DNA-binding activity of this transcription factor are also likely to be involved in the inhibition of leptin induced by LA in adipocytes. Moreover, these effects of LA seem to be mediated in part by PI3K/Akt pathway. Furthermore, the findings provided in this study suggest that LA could be a potential therapeutic agent for the treatment of some of the metabolic complications associated to obesity in which hyperleptinemia is involved, including cancer. In fact, it has been suggested that insulin-stimulated leptin
may promote breast cancer progression and that this process requires Sp1 and is partially regulated by the PI3K [52].

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Author Disclosure Statement
The authors declare no conflict of interest concerning this research.

5 References


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Figure legends

FIG. 1. LA supplementation inhibits leptin in control and high-fat fed rats. (A) Effects of LA (0.25 g/100 g diet during 56 days) on leptin circulating levels and (B) leptin gene expression in epididymal fat. (C) Pearson’s correlation between leptin circulating levels and total White Adipose Tissue. Data are expressed as mean ± SE. (n=10-12 for Control, CLIP, Obese and OLIP; n=6 for PF-CLIP and PF-OLIP). ***p<0.001, *p<0.05 vs. Control; ###p<0.001, ##p<0.01 vs. Obese.

FIG. 2. LA inhibits leptin production in 3T3-L1 adipocytes. Effects of different concentrations of LA (1-500 μM) on (A) basal leptin secretion and (B) leptin gene expression in 3T3-L1 adipocytes, and (C) on insulin-stimulated leptin secretion in primary cultured epididymal rat adipocytes treated over 48 h. Data are expressed as mean ± SE of 3-6 independent experiments, ***p<0.001, **p<0.01, *p <0.05 vs Control (vehicle-treated cells); #p<0.05 vs. insulin-treated cells.

FIG. 3. LA increases the anaerobical utilization of glucose in adipocytes. Effects of different concentrations of LA (1-500 μM) on (A) glucose uptake, (B) lactate productions and (C) the percentage of glucose metabolized to lactate in 3T3-L1 adipocytes treated over 24 h. Data are expressed as mean ± SE (n=6). (D) Relationship between leptin secretion levels and the percentage of glucose metabolized to lactate. ***p<0.001, **p<0.01 vs Control.

FIG. 4. LA inhibits Sp1 activity through phosphorylation. (A) Sp1-DNA binding activity in nuclear extracts from LA (250 μM)-treated and untreated 3T3-L1 adipocytes during 24 h. (B) Effects of LA (250 μM) on Sp1 phosphorylation in 3T3-L1 adipocytes.
(C) Protein Phosphatase 1 (PP1) abolishes the inhibitory effect of LA on Sp1 activity. Dephosphorylation reactions were performed by incubating with PP1 at 30°C for 15 min the nuclear extracts obtained from control and LA-treated cells. Data are expressed as mean ± SE. (n=3). *p<0.05, **p<0.01 vs. Control.

FIG. 5. Analysis of the signaling pathways involved in LA actions on leptin and Sp1 phosphorylation. Effects of the PI3K inhibitor LY294002, the JNK inhibitor SP600125, and the ERK1/2 inhibitor PD98059 on LA-induced inhibition of (A) leptin secretion and (B) leptin gene expression, and (C) Sp1 phosphorylation in 3T3-L1 adipocytes. (D) Analysis of Akt (Ser-473) activation in mature 3T3-L1 adipocytes after treatment for 30 min with LA (250 μM). Data are expressed as mean ± SE. (n=3-8 independent experiments). ***p<0.001, **p<0.01, *p<0.05 vs. respective Control. ###p<0.001, ##p<0.01, #p<0.05 vs. Basal-Control (vehicle-alone treated adipocytes). †††p<0.001, ††p<0.01, †p<0.05 vs. Basal-LA-treated cells.