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Alpha-Lipoic Acid Inhibits Glycogen Synthesis and Modifies Glucose Metabolism and Signaling Pathways in Soleus Muscles from Healthy Rats

Aliza H. Stark, Erez Ilan, Bracha Timar, Diana Borenshtein and Zecharia Madar†

The Hebrew University of Jerusalem, Faculty of Agricultural, Food and Environmental Quality Sciences, Institute of Biochemistry, Food Science and Nutrition, Rehovot 76100, Israel

Abstract

Alpha-lipoic acid is a known hypoglycemic agent that may be useful in the treatment of diabetes. The objective of this study was to investigate the fate of glucose in isolated muscles incubated with lipoic acid by determining its direct effects on specific metabolic and signaling pathways. Soleus muscles from healthy rats were incubated with lipoic acid in the absence or presence of insulin. Glucose transport, glycogen synthesis, glucose oxidation and lipid synthesis were determined and affects on major pathways associated with insulin signaling were evaluated. Glucose transport was not significantly altered by the addition of lipoic acid to the incubation medium. However, lipoic acid decreased glycogen synthesis in comparison to controls. Glucose oxidation was moderately increased while de-novo lipid synthesis from glucose was inhibited. Wortmannin repressed insulin stimulation of glucose incorporation into glycogen, an effect that was augmented by the combined treatment of wortmannin and lipoic acid. Basal and insulin-stimulated serine phosphorylation of Akt was not changed by the addition of lipoic acid to the incubation medium. These data show that in this *in vitro* model, lipoic acid did not significantly affect glucose uptake but dramatically modified pathways of glucose metabolism within muscle tissue.

Key words: alpha-lipoic acid, glucose metabolism, wortmannin, Akt, rats

INTRODUCTION

Alpha-lipoic acid (LPA), a potent antioxidant and a co-factor of mitochondrial dehydrogenase complexes, has been shown to improve glucose metabolism in diabetic subjects (1-3). Oral administration of LPA for a four week period increased insulin sensitivity as measured by an isoglycemic glucose-clamp (1). Another mechanism by which LPA can act as a hypoglycemic agent is through stimulation of basal and insulin-activated glucose uptake. This has been documented in L6 myotubes (4), cardiac myocytes (5) and in isolated rat muscles (6-8). Increased transport in response to LPA appears to be dependent on phosphatidylinositiol 3-kinase activity and a redistribution and/or increase of glucose transporters. In obese Zucker (fa/fa) rats, Jacob et al. (7). found that parenteral treatments of LPA improved insulin-mediated 2-deoxyglucose uptake, glucose oxidation and glycogen synthesis in epitrochlearis muscles. It was hypothesized that in muscles from Sprague-Dawley rats, similar to fa/fa rats, LPA treatment would lead to increased glucose transport, increased incorporation of glucose into glycogen and potentially, increased lipid synthesis. Although LPA has been considered for use in treating diabetic subjects, an unequivocal understanding of its mechanisms of action has not been established. The current study investigated the effects of LPA on glucose metabolism using an *in vitro* model of isolated soleus muscles. The objective was to investigate the fate of glucose in muscles of healthy rats treated with LPA, by determining its affects on specific metabolic and signaling pathways.

MATERIALS AND METHODS

Animals: Male Sprague Dawley rats (Harlan, Israel) weighing 100~120 g were housed in a controlled environment (22~24 and 12 h light-12 h dark) with food and water freely available. All animals were cared for under the guidelines set forth by the Animal Care Committee of the Hebrew University, Jerusalem, Israel.

Experimental protocols: All materials were purchased from Sigma (Rehovot, Israel) unless otherwise noted.

Glucose transport was measured in soleus muscles dissected from anaesthetized rats by a modified method of Wallberg-Henriksson et al. (9). Incubations were performed in sealed glass vials in a 37°C shaking water bath,

†Corresponding author. E-mail: madar@agri.huji.ac.il
Phone: 972-8-948-9008. Fax: 972-8-936-3208

under continuous flow of 95% O_2 : 5% CO_2. Pre-incubation was carried out for 20 min in 2.5 mmol/L oxygenated Krebs-Henseleit bicarbonate buffer supplemented with 0.1% bovine serum albumin (BSA), 8 mmol/L glucose and 32 mmol/L mannitol. Muscles were then washed for 10 min in 2.5 mmol/L glucose-free medium containing 40 mmol/L mannitol. The samples were transferred to 2.5 mL incubation medium containing 3-O-methyl D-glucose (1 mmol/L), (0.7 uC/mL, Amersham, UK) and mannitol (40 mmol/L) (0.1 uC/mL, Amersham, UK), in the presence or absence of insulin (120 nmol/L) with or without 2.4 mmol/L LPA (Asta Medica, Frankfurt, Germany). Commonly, for one rat, one leg muscle was incubated with insulin and the contralateral muscle incubated without insulin. At the completion of the 20 min incubation period, muscles were removed from the vials, blotted on filter paper and dissolved in 0.75 mL 1 mol/L NaOH overnight. Radioactivity was measured and glucose transport rates determined.

Glucose incorporation into glycogen was determined in soleus muscles surgically excised and incubated in a shaking water bath. Tissues were placed in sealed vials containing oxygenated Krebs-Henseleit bicarbonate buffer supplemented with 0.1% BSA, 5 mmol/L HEPES and 2 mmol/L pyruvate (10). After preincubation for 30 min, muscles were transferred to a second set of vials. One muscle from each rat was placed in medium containing glucose (5 mmol/L) and (^{14}C-U) glucose (0.5 μC/mL), and the contralateral muscle was incubated with glucose and insulin (120 nmol/L). In addition, LPA (2.4 mmol/L) was also added. After 60 min of incubation under 95% O_2 : 5% CO_2 muscles were blotted and rapidly frozen in liquid nitrogen. The frozen muscle specimens were weighed and glycogen isolated in a modified method of Rigden et al. (11). Tissues were boiled for 30 min in 7.7 mmol/L KOH and precipitated at 4°C in 3 volumes of ethanol and Na_2SO_4. After centrifugation, radioactivity was determined in the precipitates. Where noted, wortmannin (50 nmol/L) or dantrolene (100 μmol/L) were added to incubation medium.

Glucose oxidation: Soleus muscles were incubated as described above, and $^{14}CO_2$ production from (^{14}C-U) glucose was measured to determine glucose oxidation (12).

De novo lipid synthesis: Soleus muscles were incubated as described above with 5 mmol/L and (^{14}C-U) glucose (0.5 μCi/mL) in the presence or absence of 2.4 mmol/L LPA and insulin (120 nmol/L). Muscle lipids synthesized during the incubation process were measured by weighing and powdering frozen muscle tissue (20～50 mg) followed by homogenization in 750 μL of Krebs-Henseleit bicarbonate buffer. Muscle homogenates were extracted by the method of Folch (13) using chloroform-methanol (2:1). Samples were extracted overnight, vortexed and centrifuged for 5 min at 3,000 × g, the lower lipid phase was collected and radioactivity determined.

Western blots: Muscle samples were homogenized in lysis buffer containing 10% glycerol, 20 mmol/L Tris, 145 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton, 0.05% NP-40, 0.2 μmol/L PMSF, 0.2 mmol/L $NaVO_4$, 10 mmol/L NaF and protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Homogenates were centrifuged, protein concentration of the supernatant determined and standard electrophoresis (SDS-PAGE) and blotting procedures carried out. Protein identification was performed by incubation with specific antibodies to the phosphorylated form of AKT protein (Cell Signaling Technology, Beverly, MA), followed by ECL detection. Band intensities were quantified using scanning densiometry and expressed as arbitrary units.

Statistical analysis: Data are expressed as mean ± SE. Each experiment was repeated 2～4 times with 12 rats per experiment and each treatment carried out in triplicate or quadruplicate. Differences among means were determined by analysis of variance (ANOVA) and two-way ANOVA was used in multivariable analyses. Differences were considered significant at probability levels of $p \leq 0.05$ using the Fisher,s protected least significant difference method.

RESULTS

Isolated soleus muscles from healthy rats demonstrated a 32% increase in glucose transport when incubated with insulin (Fig. 1). When 2.4 mmol/L LPA was added to the incubation medium, glucose transport increased slightly but this difference did not attain statistical significance. Following LPA treatment, insulin no longer caused a significant stimulation of glucose uptake. In contrast, addition of LPA resulted in a dose-dependent de-

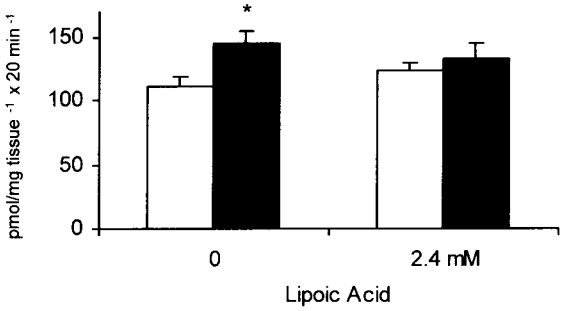

Fig. 1. Glucose transport measured in isolated rat soleus muscles in the presence of 2.4 mmol/L lipoic acid without insulin (□) and with added insulin (120 nmol/L) (■). Three sets of experiments were carried out with 4～6 replicates per treatment. *Represents a significant difference from control rats ($p < 0.05$).

crease in glucose incorporation into glycogen (Fig. 2). Glycogen production was increased 1~2 fold by insulin in control muscles ($p<0.002$). After LPA treatment insulin was still able to stimulate glucose flux into glycogen, but the absolute magnitude of the stimulation was reduced. Glucose oxidation was increased by 16~24% by LPA, but this trend did not reach significant levels (Table 1). Addition of insulin also tended to increase glucose oxidation in comparison to the same treatment without added insulin. When muscles were treated with both LPA (2.4 mmol/L) and insulin, a significant increase in glucose oxidation was observed in comparison to control or insulin alone. De novo lipid synthesis from glucose was also measured in isolated muscles (Table 1). Exposure to insulin increased baseline levels of lipid synthesis by 54%. LPA addition dramatically inhibited lipid in comparison to control levels ($p<0.001$). The combined treatment of LPA and insulin also led to a significant decrease in de novo lipid synthesis compared to treated controls, though levels were higher than with LPA alone.

Wortmannin, a phosphatidylinositol 3-kinase (PI 3 kinase) inhibitor, had a small, non-significant inhibitory effect on basal levels of glucose incorporation into glycogen in soleus muscles while totally inhibiting the stimulatory effect of insulin on glycogen synthesis (Fig. 3). When both LPA and wortmannin were added to the incubation medium, basal glycogen synthesis was 64% lower than with the wortmannin alone and 33% lower than LPA treatment. When insulin was added to LPA+wortmannin incubations, glycogen synthesis was significantly lower than either LPA + insulin or wortmannin + insulin (by 39% and 37% respectively) yet there was still a significant insulin stimulation of glycogen synthesis ($p<0.01$).

Dantrolene (inhibitor of Ca^{+2}release from the sarcoplasmic reticulum) and LPA were both able to inhibit basal glycogen synthesis (Fig. 4). While absolute values of insulin-stimulated glycogen synthesis were lower following either dantrolene or LPA treatment, the incremental effect of insulin was preserved. It should be noted that the combined treatment of both dantrolene and LPA had a stronger inhibitory effect than either compound alone;

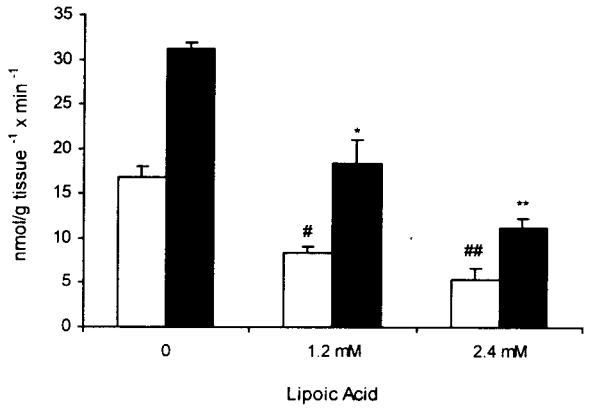

Fig. 2. Glucose incorporation into glycogen in soleus muscles isolated from healthy rats using two concentrations of lipoic acid (LPA) without insulin (□) or in the presence of insulin (■). Four sets of experiments were carried out with 4~6 replicates per treatment. Insulin significantly increased glycogen synthesis within each treatment.
#,##Represent significant differences among treatments in the absence of insulin ($p<0.05$).
*,**Represents significant differences among treatments following insulin stimulation ($p<0.05$).

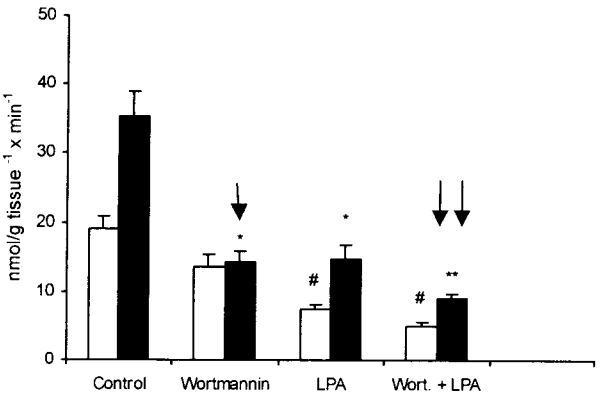

Fig. 3. Glucose incorporation into glycogen in isolated muscles from healthy rats using lipoic acid (2.4 mmol/L) and/or wortmannin (50 nmol/L) without insulin (□) or in the presence of insulin (■). Three sets of experiments were carried out with 3~6 replicates per treatment. Insulin significantly increased glycogen synthesis within each treatment with the exception of wortmannin. #Represents significant differences from basal levels ($p<0.05$). *,**Represents significant differences among treatments following insulin stimulation ($p<0.05$).
↓ marks the inhibition of insulin-stimulated glycogen synthesis by wortmannin. ↓↓ indicates interaction between lipoic acid (LPA) and wortmannin in the presence of insulin.

Table 1. Glucose oxidation and de novo lipid synthesis from glucose (nmol · g tissue^{-1} · min^{-1}) in soleus muscles isolated from healthy rats and incubated with lipoic acid (LPA), in the presence or absence of insulin

	Control		1.2 mmol/L LPA		2.4 mmol/L LPA	
	- insulin	+ insulin	- insulin	+ insulin	- insulin	+ insulin
Glucose oxidation	1.33±0.16	1.57±0.31	1.58±0.40	1.89±0.19	1.74±0.28	2.31±0.40*
Lipid synthesis	4.00±0.95	6.14±1.02	N.D.	N.D.	0.83±0.19#	1.35±0.09**

Data presented as Mean±SE. Experiments were repeated 2~4 times with 4~6 replicates per treatment. N.D. = not determined.
*Significantly different from control + insulin ($p<0.05$). #Significantly different from control ($p<0.001$).
**Significantly different from control + insulin ($p<0.001$).

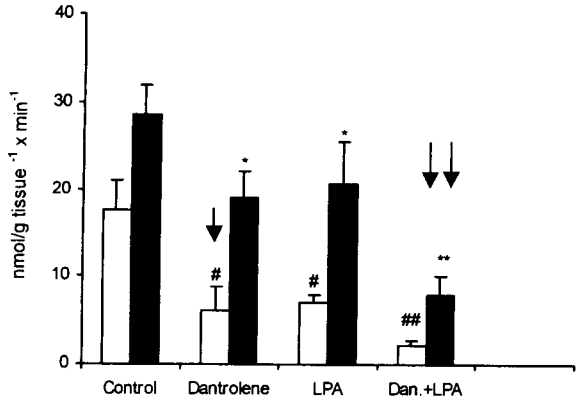

Fig. 4. Glucose incorporation into glycogen in isolated muscles from healthy rats using combinations of lipoic acid (LPA) (2.4 mmol/L) and/or dantrolene (100 μmol/L) without insulin (□) or in the presence of insulin (■). Two experiments were carried out with 4~6 replicates per treatment. Insulin significantly increased glycogen synthesis within each treatment. #,##Represents significant differences afrom basal levels ($p < 0.05$). *Represents significant differences following insulin stimulation ($p < 0.05$). ↓ marks the significant inhibition of basal glycogen synthesis by dantrolene. ↓↓ marks the additive effect of dantrolene plus LPA on glycogen synthesis.

this result was seen in either the absence or the presence of insulin (Fig. 4).

The extent of phosphorylation of Akt (protein kinase B), an insulin sensitive serine/theronine kinase involved in the insulin signaling cascade, was significantly increased in soleus muscles following incubation with insulin. Addition of LPA to the incubation medium did not effect on Akt phosphorylation in control or insulin stimulated muscles (Fig. 5).

DISCUSSION

This study investigated the effects of LPA on metabolic processes and on important signaling pathways in isolated soleus muscles from healthy rats. LPA is a known hypoglycemic agent (14), but its mechanisms of action are not satisfactorily understood. Furthermore, it is not well established how LPA interacts with insulin signaling pathways. Although several studies report a significant increase in glucose uptake in muscle cells (4) and tissue (6-8) following treatment with LPA, this was only partially confirmed in the *in vitro* model of isolated soleus muscle used in this study. LPA did not significantly elevate glucose transport (Fig. 1). However, LPA significantly inhibited glycogen synthesis in soleus muscles isolated from healthy rats (Fig. 2). This result was unexpected as it was hypothesized that glycogen synthesis would be unaffected or increase in muscles exposed to LPA. Glucose oxidation tended to be greater following exposure to LPA, but this difference did not appear to be sufficient to account for the dramatic decrease in glucose incorporation into glycogen (Table 1). De-novo lipid synthesis was also inhibited by exposure to LPA (Table 1). The question of the fate of glucose entering the muscle tissue remains unclear. Hong et al. (15) reported that both stereoisomers of LPA, S and R, inhibited purified pyruvate dehydrogenase complex (PDC) activity, which potentially could impair carbohydrate metabolism. On the other hand, the same study reported that intact HepG2 cells exposed to 1~4 mmol/L R-LPA did not demonstrate changes in pyruvate decarboxylation. Konrad et al. (2) found that LPA treatment decreased serum lactate and pyruvate concentrations in lean and obese patients with type 2 diabetes. This indicates that PDH may not be the site of LPA action, as inhibiting PDH activity would presumably lead to accumulation of pyruvate or lactate and decrease glycolysis. In the current study, where racemic LPA was used, there is little evidence to suggest that PDC inhibition occurred. Glucose oxidation was not impaired by LPA and neither fatty acid synthesis nor glycogenesis increased. In a muscle cell line and in adipocytes, LPA has been shown to directly activate steps in the insulin signaling cascade (16). In the presence of wortmannin, LPA-stimulated glucose uptake observed in the cell models was strongly inhibited, suggesting that the PI 3-kinase activity is necessary for the insulin-like action of LPA. Ramrath et al. (5) reported that preincubation of cardiomyocytes with wortmannin completely abolished the effects of insulin and of LPA, strongly suggesting that LPA mimics insulin action by activating the signaling cascade at or before the level of PI3-K. In the present study, wortmannin inhibited insulin-stimulated glycogen synthesis while LPA-inhibited basal and insulin-stimulated gly-

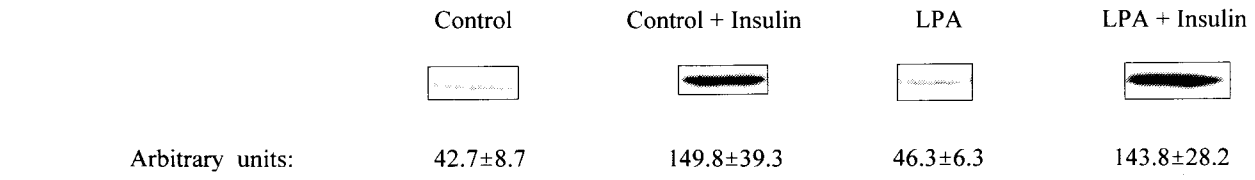

Fig. 5. Expression of the phosphorylated form of insulin sensitive serine/threonine kinase Akt in soleus muscles following incubation with lipoic acid (LPA) with or without insulin (120 nmol/L).

cogen synthesis. When both compounds were added to the incubation medium along with insulin, inhibition was greater than from either LPA or wortmannin alone. From this result, one can speculate that LPA is acting through inhibitory pathways that are independent of PI-3 kinase activation. On the other hand, it must be noted that wortmannin totally inhibited insulin stimulation of basal glycogen synthesis, but when LPA was added to the wortmannin + insulin incubation, a 60% increase of glucose incorporation into glycogen occurred in comparison to LPA + wortmannin. This suggests that LPA somewhat counteracts the inhibitory effect of wortmannin. LPA has been reported to act in an insulin-like manner, a theory supported by Tsakiridis et al. (16) and others (2,5) but not confirmed in this study. Unlike previous studies, our results showed an inhibitory effect of LPA on glycogen synthesis and a minimal affect on glucose transport. In addition, incubations with wortmannin did not provide definitive results regarding the action of LPA on insulin signaling pathways.

Dantrolene, an agent capable of preventing calcium release from the sarcoplasmic reticulum of skeletal muscle (17) had an additive inhibitory effect when used with LPA or added to LPA + insulin. This strongly suggests that the compounds are working through independent pathways and that LPA inhibition of glycogen synthesis in this model was not related to cytosolic calcium levels.

Recently, it has been proposed that Akt/PKB activation plays an essential role in insulin-induced glycogen synthesis (18,19). Phosphorylation and activation of Akt followed by serine phosphorylation and deactivation of glycogen synthase kinase-3 (GSK-3) may lead to dephosphorylation and activation of glycogen synthase. Yaworksy et al. (20) working in 3T3-L1 adipocytes, reported a rapid stimulation of glucose uptake in a wortmannin-sensitive manner and stimulation of Akt activity following exposure to LPA. In a separate study also using adipocytes, Rudich et al. (21) found that lipoic acid preserved insulin-stimulated protein kinase B/Akt serine phosphorylation and activity following exposure to oxidative stress. Both studies provide evidence for interaction between LPA and insulin signaling pathways. In a L6 myotube model, incubation with 2.5 mmol/L LPA led to a 4.9 fold increase in Akt1 activity and a 31 fold increase in PI 3-kinase activity (22). In our model Akt phosphorylation was not affected by exposure to LPA. Despite relatively unchanged Akt phosporylation, glucose incorporation into glycogen was depressed. This indirect method of measuring glycogen synthase activity suggests that inhibition occurs at the level of GSK-3 or at the level of dephosphorylation of glycogen synthase, rather than higher up on the signaling cascade. However, from results of separate study (data not shown) where GS activity was directly measured, LPA did not inhibit glycogen synthase activity and protein expression. At this time, we have no clear explanation as to the mechanism by which LPA inhibits glucose incorporation into glycogen in this *in vitro* model. It is possible that the concentrations used in this study, although similar to concentrations used in other *in vitro* work (15,20,22), may be considerably higher than physiological levels. Yaworsky et al. (20) estimated that the *in vitro* level of LPA (2.4 mmol/L) is at least two times higher than the concentration that could be reached in muscle cells in animals or humans treated with the compound. On the other hand, if exogenous lipoic acid is provided in large quantities as a daily supplement, it may be possible to raise LPA levels to a range similar to those used in cell culture and *in vitro* (15). It should also be noted that caution must be taken when extrapolating results from one model system to another.

In conclusion, the present report demonstrates that both metabolic and signaling pathways in soleus muscles are affected by LPA treatment. The processes may differ from those involved with insulin-stimulated glucose metabolism in aerobic muscle cells. This study attempts to elucidate mechanisms by which muscle tissues respond to exposure to LPA, but the results also raise new questions when compared to previous studies carried out in cell cultures and in whole animal models.

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