

## Lipoic Acid Prevents the Changes of Intracellular Lipid Partitioning by Free Fatty Acid

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**Background/Aims:** It is suggested that the hepatic lipid composition is more important than lipid quantity in the pathogenesis of non-alcoholic steatohepatitis. We examined whether lipoic acid (LA) could alter intrahepatic lipid composition and free cholesterol distribution. **Methods:** HepG2 cells were cultured with palmitic acid (PA) with and without LA. Apoptosis, changes of the mitochondrial structure, intracellular lipid partitioning, and reactive oxygen species (ROS) activity were measured. **Results:** Free fatty acid (FA) increased apoptosis, and LA co-treatment prevented this lipotoxicity (apoptosis in controls vs PA vs PA+LA, 0.5% vs 19.5% vs 1.6%,  $p < 0.05$ ). LA also restored the intracellular mitochondrial DNA copy number ( $553 \pm 33.8$  copies vs  $291 \pm 14.55$  copies vs  $421 \pm 21.05$  copies,  $p < 0.05$ ) and reversed the morphological changes induced by PA. In addition, ROS was increased in response to PA and was decreased in response to LA co-treatment (41,382 relative fluorescence unit [RFU] vs 43,646 RFU vs 41,935 RFU,  $p < 0.05$ ). LA co-treatment increased the monounsaturated and polyunsaturated FA concentrations and decreased the total saturated FA fraction. It also prevented the movement of intracellular free cholesterol from the cell membrane to the cytoplasm. **Conclusions:** LA opposes free FA-generated lipotoxicity by altering the intracellular lipid composition and free cholesterol distribution. (**Gut Liver 2013;7:221-227**)

**Key Words:** Thioctic acid; Lipotoxicity; Lipid partitioning; Non-alcoholic steatohepatitis; Liver cirrhosis

### INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is caused by the ex-

tensive accumulation of lipids in the liver, which gradually leads to lipotoxicity and oxidative stress, and eventually to cell apoptosis.<sup>1-3</sup> Triglycerides, the dominant type of lipid in NASH patients, were thought to be the main cause of NASH.<sup>4-9</sup> However, recent studies have shown that they may actually protect the liver from inflammation and oxidative damage by storing excess fatty acids (FAs).<sup>10</sup> Other studies in human subjects have also found no relationship between the hepatic concentration of triglycerides and insulin resistance.<sup>11</sup> This suggests that other lipids, such as free FAs (FFAs) and free cholesterol, may be more important in terms of lipotoxicity.<sup>12</sup> This idea, referred to as the 'lipid partitioning theory,' suggests that the nature of the accumulated lipids rather than their quantity is important in the pathogenesis of NASH.

Saturated FAs (SFAs) have been found to be more effective in producing oxidative stress than unsaturated FAs (UFAs), due to the high SFA-oxidizing capacity of the liver. It is also thought that stearoyl-CoA desaturase 1 (SCD-1) plays a key role in controlling the ratio of UFAs to SFAs in the liver, and this could affect not only the control of diabetes mellitus and cholesterol levels but also NASH.<sup>13,14</sup> In addition to FAs, free cholesterol has been shown to increase susceptibility to lipotoxic effects. Therefore, in addition to the UFA: SFA ratio, the cellular distribution of free cholesterol should be considered as a factor influencing oxidative stress and lipotoxicity. However, according to Mari *et al.*,<sup>15</sup> if mitochondria are loaded with free cholesterol, FFAs increase the frequency of apoptosis.

Lipoic acid (LA) is a free radical scavenger that can regenerate endogenous antioxidants, such as vitamin E.<sup>16</sup> As it is soluble in water and lipid, water soluble antioxidants (e.g., vitamin C) are found in the cell cytoplasm, and fat soluble antioxidants (e.g., vitamin E) are found on the cell membrane. This means that

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LA can act both inside the cell and on the cell membrane and so provide dual protection. It has protective activity in both reduced and oxidized form,<sup>17</sup> and the reduced form, dihydrolipoic acid is more effective as an antioxidant. Animal foods (mainly in liver and muscle) have high LA contents (range, 0.55 to 2.36 ppm),<sup>17,18</sup> whereas plant foods contains very little (0.09 ppm) or no detectable LA.<sup>19</sup>

As an antioxidant, LA can be very effective against a variety of diseases. LA supplementation has been found to be beneficial in preventing diabetic neuropathy, and may also be effective against diseases such as liver cirrhosis, diabetic nephropathy, multiple sclerosis, and Alzheimer's disease.<sup>20</sup>

LA can reduce the accumulation of fat in the liver by inhibiting sterol regulatory element binding protein-1c and blocking transforming growth factor-beta.<sup>13,14</sup> Moreover, it has important effects on both apoptosis and proliferation.<sup>21</sup> However, its impact on lipid partitioning has not been studied. The aim of our experiments was to examine the effect of LA on free cholesterol distribution and types of FFA found in hepatocytes.

## MATERIALS AND METHODS

### 1. Cell culture and treatment

The human hepatoma cell line HepG2 was cultured under 5% CO<sub>2</sub> and 95% O<sub>2</sub> in RPMI 1640 medium (GIBCO, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 100 units/mL of penicillin and 100 mg/mL of streptomycin (control group). Lipotoxicity was induced by incubation with 300 μM palmitic acid (PA; Sigma-Aldrich, St. Louis, MO, USA) for 24 hours (PA group). For LA (Sigma-Aldrich) treatment, the cells were co-incubated with different concentration of LA (10, 100, and 1,000 μM) and 300 μM PA for 24 hours (PA+LA group).

### 2. Electron microscopy

Cells were pre-fixed at 0°C to 4°C in a 2.5% glutaraldehyde solution and washed with 0.1 M phosphate buffer. They were post-fixed in 1% of OsO<sub>4</sub> for 2 hours and washed with buffer. After ethanol dehydration, they were placed in propylene oxide and embedded in Epon, which was polymerized at 37°C for 12 hours, 45°C for 12 hours, and 60°C for 48 hours. The tissue was cut into 0.5 μm-thick sections, double stained with uranyl acetate and lead citrate, and observed by transmission electron microscopy (TEM, H-7100; Hitachi-High Technologies Co., Tokyo, Japan).

### 3. Real-time polymerase chain reaction (RT-PCR)

Total RNA was analyzed with RNA-Bee solution (Tel-Test, Friendswood, TX, USA). Complementary DNA was synthesized by the first-strand cDNA synthesis method. PCR mixtures contained 2 mL of cDNA, 5 mL of 10×PCR buffer solution (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 1.5 mM MgCl<sub>2</sub>), 4 mL of 2.5 mM dNTP, 20 pmol of primers (GAPDH forward primer;

CCA GGT GGT CTC CTC TGA CTT C, GAPDH reverse primer; Primers and probes were as follows: GTG GTC GTT GAG GGC AAT G, mitochondria forward primer; CCA GCG TCT CGC AAT GCT, mitochondria reverse primer; CTC CAT GCA TTT GGT ATT TTC G, mitochondria probe; FAM–TCG CGT GCA CAC CCC CCA–TAMRA, GAPDH probe; FAM–ACA GCG ACA CCC ACT CCT CCA CCT T–TAMRA.), and 1 unit of AmpliTaq Gold DNA polymerase (Roche, Applied Biosystems, Foster City, CA, USA), adjusted to a final concentration of 50 mL. Amplification cycles consisted of denaturation for 5 minutes at 94°C, then the mixtures underwent denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 5 minutes at 72°C. In total, 40 cycles were conducted. Relative mitochondrial copy number was calculated as  $2^{\Delta\text{CT}}$  ( $\Delta\text{CT}$  equals  $\text{Ct}_{\text{GAPDH}} - \text{Ct}_{\text{mtDNA}}$ ).

### 4. Gas chromatography

Collected cells were centrifuged for 10 minutes at 300 g, sonicated in 5 mL of 0.025 M Tris/0.3 M sucrose buffer (pH 7.6), and centrifuged for 20 minutes at 15,000 g. The pellet was removed and only the top layer of the solution was centrifuged at 190,000 g (ca. 650,000 rpm) for 1 hour at 4°C (Beckman rotor, TLC 100.3). The isolated cell membrane fraction was placed in a 2 mL vial with 250 μL of boron trifluoride methanol-benzene (B1252; Sigma-Aldrich), heated for 10 minutes at 100°C in a sand bath, cooled at room temperature, and mixed for 30 seconds with 250 μL of HPLC-reagent water and 250 μL of HPLC-grade hexane. It was then centrifuged for 3 minutes at 3,000 rpm. The hexane layer was removed and placed in a 12×32 gas chromatography vial with a glass insert, and analyzed by gas chromatography (Shimadzu 2010AF; Shimadzu Scientific Instrument, Kyoto, Japan) using a capillary column (SP2560; Supelco, Bellefonte, PA, USA). To analyze FA composition, we used a standard mixture (GLC-727; Nu-Check Prep, Elysian, MN, USA) and calculated the differences between batches. To ensure test accuracy, the same blood sample was used as control for each batch.

### 5. Confocal microscopy

HepG2 cells were fixed in 0.1 M phosphate buffer for 10 minutes with 3.7% paraformaldehyde and permeabilized in bovine serum albumin/phosphate buffered saline (PBS) buffer with 0.1% saponin. The cells were stained with 50 mg/mL of filipin for 2 hours, washed with PBS 0.0025% saponin, and treated with secondary antibodies for 45 minutes. All the staining procedures were conducted in a darkroom.

### 6. Reactive oxygen species (ROS)

Following administration of PA or the combination of PA+LA, ROS were measured after 5 hours. We used a commercial ROS detection kit (Invitrogen, Eugene, OR, USA) according to the manufacturer's recommendations. After removing the medium, 10 μM CM-H<sub>2</sub>DCFDA dissolved in 20 μL of phenol-red-

free medium was added to the wells; they were then incubated at 37°C and washed 3 times with 50  $\mu$ L PBS per well. Fluorescence was measured at excitation/emission (ex/em) wavelengths of 485/530 nm.

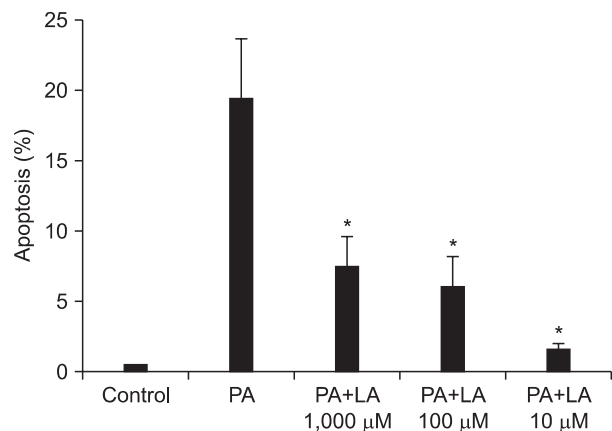
## 7. Data analysis

All data are expressed as mean $\pm$ standard deviation. Differences between groups were compared by analysis of variance (ANOVA), followed by the Kruskal-Wallis test to correct for multiple comparisons and the chi-square test. Differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

### 1. Inhibition of FFA-induced apoptosis by LA

TUNEL assays showed that the PA-treated cells had a

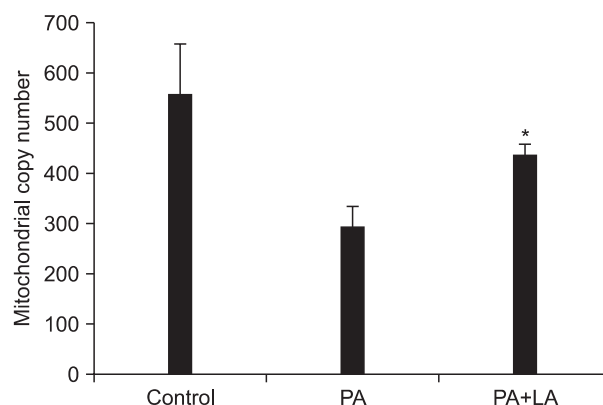


**Fig. 1.** Apoptosis induced by palmitic acid (PA) is decreased by lipoic acid (LA). Data are presented as mean $\pm$ SD ( $n=5$  in each group). Control, medium alone; PA, 300  $\mu$ M PA; PA+LA, 300  $\mu$ M PA with 10, 100, and 1,000  $\mu$ M LA. \* $p < 0.05$  for all data compared to the PA group with the Kruskal-Wallis test.

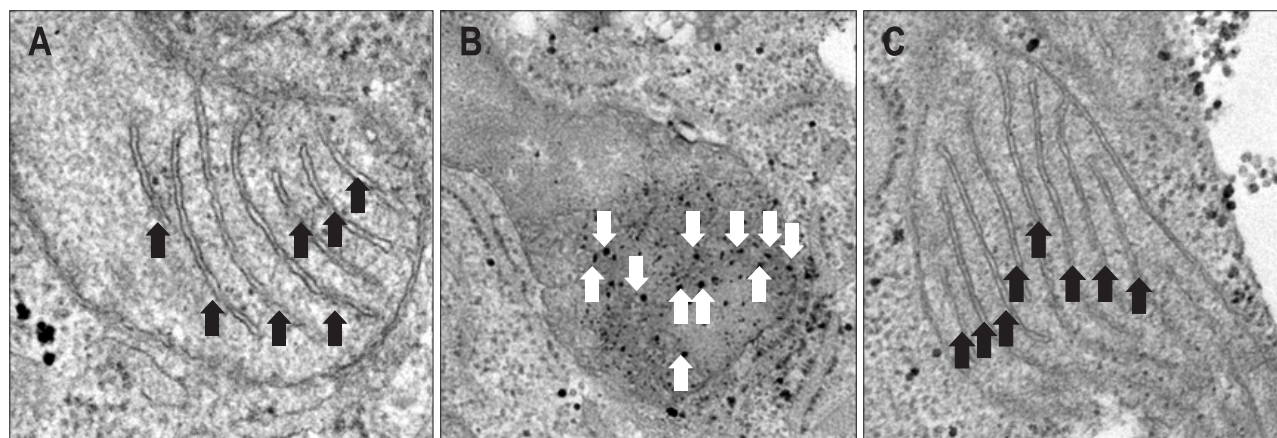
higher rate of apoptosis than the normal controls (control:PA, 0.5%:19.5%,  $p < 0.05$ ), and apoptosis was greatly attenuated in the PA+LA co-treatment group compare to PA group (PA:PA+LA 10  $\mu$ M; 19.5%:1.6%,  $p < 0.05$ ). As 10  $\mu$ M LA had the highest anti-apoptotic effect, we used it in all subsequent experiments (Fig. 1).

### 2. Effects of LA on mitochondrial morphology and DNA copy number

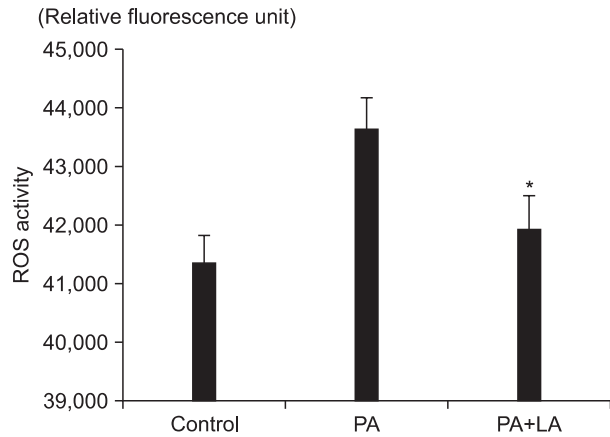
We compared changes in mitochondrial DNA (mDNA) copy number after exposure to PA and PA+LA by quantitative PCR. In the control group, copy number was  $553 \pm 33.8$ , in the PA-treated cells, it was  $291 \pm 14.55$  ( $p < 0.05$ ), and in the PA+LA cells it rose again to  $421 \pm 21.05$  ( $p < 0.05$ ) (Fig. 2). TEM showed that the mitochondria of PA-treated cells were enlarged, had a reduced number of cristae, and contained an abundant granular matrix and dense intramatrix granules. The PA+LA cells con-



**Fig. 2.** The mitochondrial DNA copy number is decreased by palmitic acid (PA) treatment and restored by lipoic acid (LA). Data are presented as mean $\pm$ SD ( $n=5$  in each group). Control, medium only; PA, 300  $\mu$ M PA; PA+LA, 300  $\mu$ M PA and 10  $\mu$ M LA. \* $p < 0.05$  by the Kruskal-Wallis test.



**Fig. 3.** Mitochondrial ultrastructure. (A) Transmission electron microscopy of normal mitochondria (controls). (B) Palmitic acid-treated mitochondria, with loss of cristae, abundant granular matrix, and dense intramatrix granules. (C) Normal ultrastructure after cotreatment with lipoic acid (black arrows, cristae; white arrows, intramatrix granules).



**Fig. 4.** Reactive oxygen species (ROS) are increased by palmitic acid (PA) treatment, and this increase is opposed by lipoic acid (LA). Intracellular ROS levels were measured at 12 hours. Data are presented as mean±SD (n=5 in each group). Control, medium only; PA, 300 μM PA; PA+LA, 300 μM PA and 10 μM LA. \*p<0.05 in the Kruskal-Wallis test.

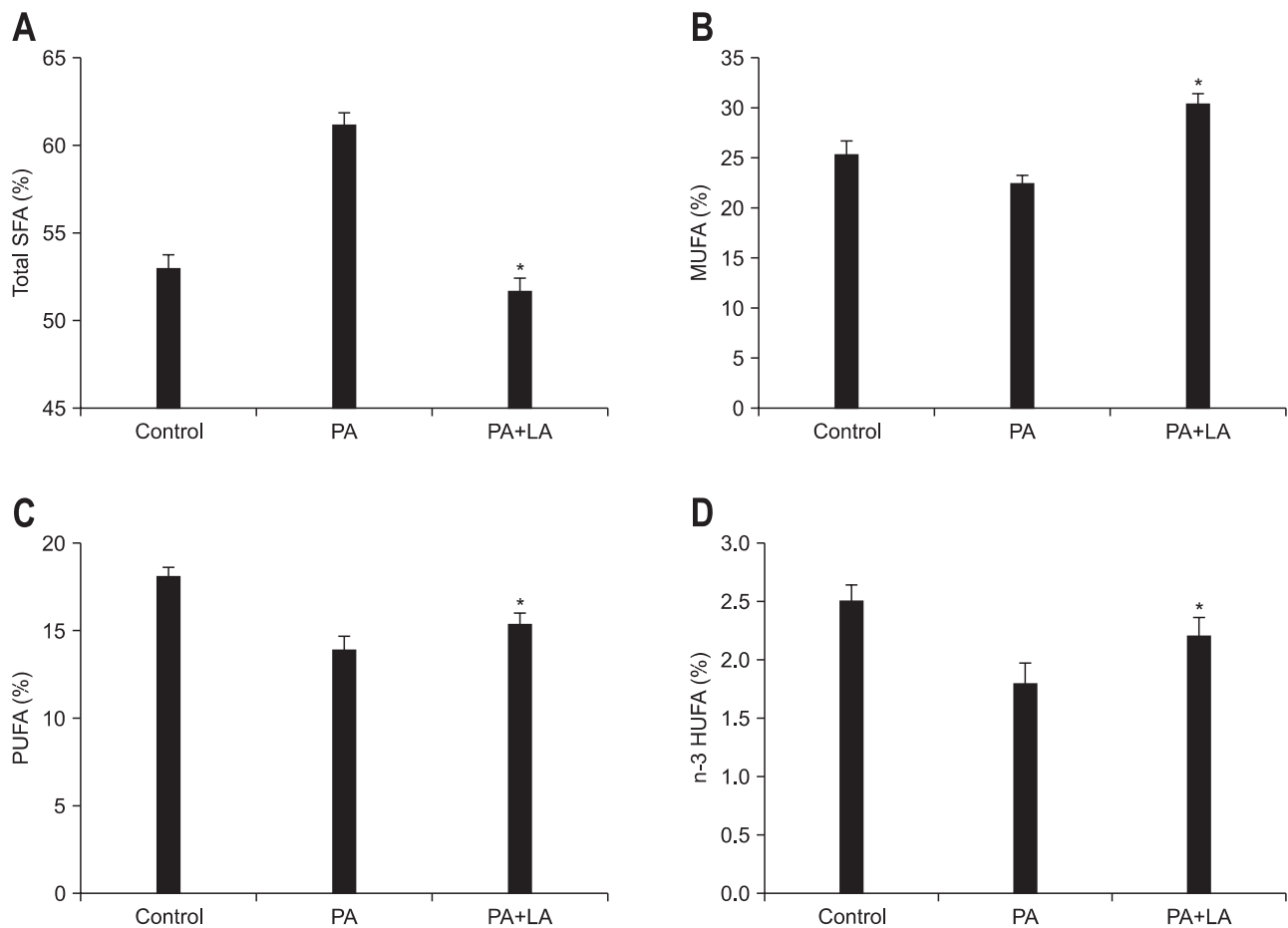
tained normal-looking cristae (Fig. 3).

**3. Intracellular ROS activity and mRNA expression of SCD-1**

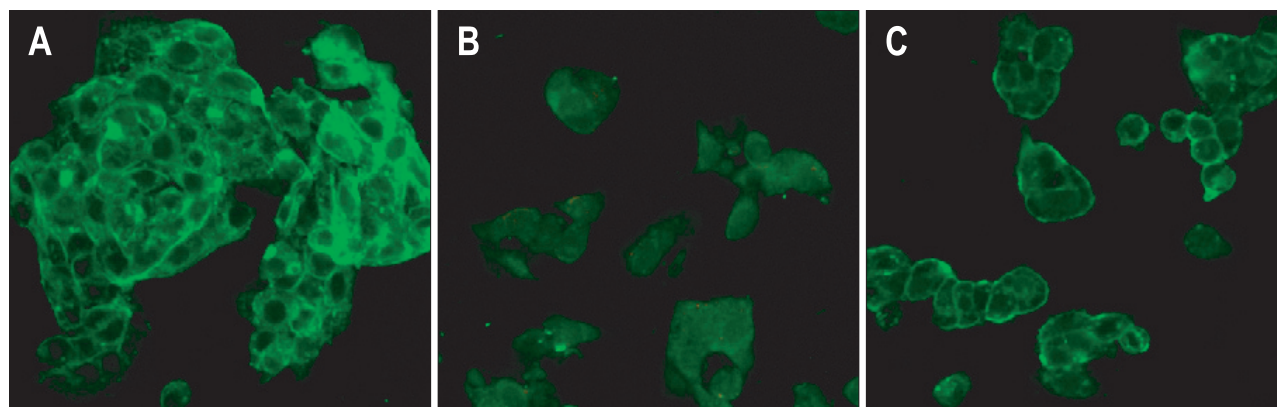
FFA treatment increased ROS compared to the control group (43,646 relative fluorescence unit [RFU] vs 41,382 RFU, p<0.05), and LA cotreatment counteracted this effect (43,646 RFU vs 41,935 RFU, p<0.05) (Fig. 4). SCD-1 mRNA expression increased 1.45 fold on treatment with 300 μM PA compare to control group. When LA was simultaneously treated, SCD-1 mRNA expression increased 2.44 fold compare to control group. But neither PA nor PA+LA group reached statistical significance.

**4. Lipid partitioning: gas chromatography**

Total percent SFA was 53.0% in the HepG2 cell membrane, this increased to 61.2% with PA pretreatment, and fell with LA cotreatment (61.2% vs 51.7%, p<0.05) (Fig. 5A). Both the mono-unsaturated FA (MUFA) and poly-unsaturated FA (PUFA) fractions were increased by LA treatment (Fig. 5B and C), as was the



**Fig. 5.** Lipid partitioning is changed by palmitic acid (PA) and lipoic acid (LA) administration. (A) The proportion of total saturated fatty acids (SFAs) is decreased by LA. (B) The proportion of mono-unsaturated FAs (MUFAs) is increased by LA. (C) The proportion of poly-unsaturated FAs (PUFAs) is increased by LA. (D) The proportion of n-3 HUFAs is increased by LA. Data are presented as mean±SD (n=5 in each group). Control, medium only; PA, 300 μM PA; PA+LA, 300 μM PA and 10 μM LA. HUFA, highly unsaturated fatty acids. \*p<0.05 in the Kruskal-Wallis test.



**Fig. 6.** Distribution of free cholesterol observed by confocal microscopy. (A) In control cells, free cholesterol is found in the cell membrane. (B) In palmitic acid (PA)-treated cells, it is found in the cell membrane and in the cytoplasm. (C) In PA plus lipoic acid-treated cells, it is found mainly in the cell membrane.

percent n-3 highly unsaturated FAs (2.2% vs 1.8%,  $p < 0.05$ ) (Fig. 5D).

### 5. Distribution of free cholesterol

In the control group, free cholesterol was mainly found in the cell membrane, while in the PA cells it was distributed throughout the cytoplasm as well as in the cell membrane. Cell membrane localization was largely restored in the PA+LA cells (Fig. 6).

## DISCUSSION

Our data showed that LA prevents FFA-induced hepatic lipotoxicity not only by scavenging ROS but also by changing intracellular lipid composition. LA is a powerful antioxidant, which increases the supply of nicotinamide adenine dinucleotide phosphate and nicotinamide adenine dinucleotide, and acts as a general scavenger of oxidized radicals. Its antioxidant effect is thought to contribute to improved outcomes in vascular endothelial disorders and peripheral neuropathy due to diabetes mellitus.<sup>22-29</sup> Recently, Min *et al.*<sup>30</sup> reported that LA reduced hepatic lipid accumulation and hepatic inflammation by lowering levels of cytochrome P450 2E1, endoplasmic reticulum stress, mitogen-activated protein kinase, and nuclear factor kappa-light-chain-enhancer of activated B cells. However, few studies have reported the effects of LA on NASH. In our study, we have shown that LA can prevent FFA-induced lipotoxicity by several mechanisms.

First, it is well known that mitochondria play a critical role in the development of NASH.<sup>31-33</sup> Although the exact mechanism is still unclear, LA acts as a co-factor of mitochondrial enzyme complexes, and promotes energy conversion via energy metabolism.<sup>20,34</sup> Valdecantos *et al.*<sup>35</sup> reported that LA increased the number and activity of mitochondria by increasing sirtuin levels. We found that LA prevented the decrease of mitochondrial copy number and morphological changes induced by FFAs. This

means that LA has mitochondrial protective effects in this lipotoxicity model. However we did not examine functional aspects of mitochondria including respiratory chain enzyme activities and beta oxidation activity. Additional comprehensive tests for mitochondrial function are required. Second, we showed that LA decreased SFA levels in the cell membrane, and increased levels of MUFAs, which may reduce oxidative stress and cell damage. A study by Celik *et al.*<sup>36</sup> reported that administration of LA increased the UFA:SFA ratio in brain tissue, thus decreasing oxidative stress. LA also increased the UFA:SFA ratio in the erythrocytes of diabetic rats.<sup>37</sup> However, few studies to date have investigated the direct impact of LA on SCD-1 activity. SCD-1 plays an important role in maintaining the balance between SFA and UFA in the cell.<sup>38</sup> We have examined SCD-1 mRNA expression by RT-PCR. Unfortunately there were no significant changes of SCD-1 mRNA expression in the LA group. Some other mechanism may therefore be responsible for the changes of intracellular FA composition. Finally, in addition to the UFA:SFA ratio, the distribution of free cholesterol is an important factor in apoptosis. Recent studies have shown that an increase of free cholesterol in mitochondria is toxic for neural and liver cells.<sup>15,38</sup> In our study we found that administration of LA led to a redistribution of FFAs from the cytosol to the cellular membrane.

In conclusion, LA inhibits PA-mediated lipotoxicity via mechanisms involving changes in the lipid composition of cell membranes and in the distribution of free cholesterol.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

## ACKNOWLEDGEMENTS

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