Lipoic acid entrains the hepatic circadian clock and lipid metabolic proteins that have been desynchronized with advanced age

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A B S T R A C T

It is well established that lipid metabolism is controlled, in part, by circadian clocks. However, circadian clocks lose temporal precision with age and correlates with elevated incidence in dyslipidemia and metabolic syndrome in older adults. Because our lab has shown that lipoic acid (LA) improves lipid homeostasis in aged animals, we hypothesized that LA affects the circadian clock to achieve these results. We fed 24 month old male F344 rats a diet supplemented with 0.2% (w/w) LA for 2 weeks prior to sacrifice and quantified hepatic circadian clock protein levels and clock-controlled lipid metabolic enzymes. LA treatment caused a significant phase-shift in the expression patterns of the circadian clock proteins Period (Per) 2, Brain and Muscle Arnt-Like1 (BMAL1), and Reverse Erythroblastosis virus (Rev-erb) β without altering the amplitude of protein levels during the light phase of the day. LA also significantly altered the oscillatory patterns of clock-controlled proteins associated with lipid metabolism. The level of peroxisome proliferator-activated receptor (PPAR) α was significantly increased and acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) were both significantly reduced, suggesting that the LA-supplemented aged animals are in a catabolic state. We conclude that LA remediates some of the dyslipidemic processes associated with advanced age, and this mechanism may be at least partially through entrainment of circadian clocks.

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1. Introduction

Aging increases the incidence and severity of metabolic disorders such as hyperlipidemia, insulinemia, inflammation and metabolic syndrome [1,2]. While the intrinsic factors that deregulate metabolic allostasis in the elderly are not completely understood, a link between disruption of circadian pacemakers and the incidence of age-associated metabolic disorders has been discerned. Both human and rodent models of aging show severe declines in temporal precision of expression for core clock genes that govern cell-autonomous transcriptional feed-back loops [3,4]. This loss of clock precision with age can have detrimental impacts on physiological processes including energy regulation, hormone secretion, cardiac function, and sleep-wake cycles. Thus, dysentrainment of circadian rhythms may lead to poor health and reduced life expectancy [5]. For the liver, age-associated changes in clock gene oscillations would adversely affect glucose and fatty acid metabolism, and also triglyceride and cholesterol synthesis through disruption of clock-associated transcription factors (e.g. PPARα, D site of albumin promoter binding protein, sterol regulatory element-binding protein (SREBP), hepatocyte nuclear factor 4) [6–8]. The influence of hepatic clock gene dampening to the aforementioned metabolic disorders is not trivial. The ablation of one or more core clock genes in transgenic animals results in obesity, dyslipidemia, hyperlipidemia, hyperglycemia, and hyperinsulinemia [9–11]. These profiles thus directly link hepatic clock disruption to metabolic distress generally evident in aging.

Lipoic acid is a naturally occurring dithiol compound that has been used in various clinical applications including chelating heavy metals and improving diabetes polyneuropathies [12,13]. LA may have beneficial applications for the treatment of hypertension,

improvement of cellular detoxification systems, and scavenging free radicals [14,15]. While the diverse functions of LA are becoming elucidated, the precise mechanism(s) and how LA mediates these diverse functions remain undefined. In exploring its pharmacological action we recently discovered that LA, when supplemented in the diet of rats, mediates two distinct, but intertwined, processes: fatty acid metabolism and circadian rhythm-dependent gene expression. Our lab previously demonstrated that supplementing rodents with LA for 2 weeks lowers gene transcript levels of circadian-regulated lipid anabolic enzymes, which otherwise significantly increase with age [16]. We also revealed that 5 weeks of LA supplementation improves liver and plasma triglyceride levels in the Zucker diabetic fatty rat [17]. However, the effects of dietary LA on circadian-regulated enzymes of lipid metabolism have not yet been monitored over a circadian cycle. Therefore, it is not currently known whether LA mediates its pharmacological action directly on the core feedback loops of the hepatic clock proteins, or downstream on clock-influenced lipid metabolizing genes.

To fill this critical gap-in-knowledge, the present work investigates how feeding LA to aged rats modulates the rhythmic expression of circadian and circadian-regulated proteins in the liver. We herein present the novel results that LA can be used to entrain clock proteins and clock-controlled lipid metabolic enzymes. Thus LA may prove to be an additional tool available to improve circadian clock dysfunctions, such as jet lag and delayed sleep phase syndrome, as well as age-associated metabolic dyslipidemia (see Fig. 1).

2. Methods

2.1. Animals and LA supplementation

Old (24 month) male Fischer 344 rats were obtained through the NIA, maintained in AALAC-approved housing on the Oregon State University campus, and kept on 12 h light/dark cycles. The rats were divided into two groups and fed the AIN-93M diet (Dyets) without or with 0.2% (wt/wt) LA (MakWood) for 2 weeks prior to sacrifice. Water was available *ad libitum*. To control for differences in amount of total food intake, animals on the control diet were pair-fed relative to the food intake of the LA-supplemented rats as previously described [16]. This supplementation scheme resulted in an approximate dose of 80 mg LA/kg body weight per day. Tissues were collected as previously described [16]. No more than 2 animals were sacrificed at a time to ensure adherence to the time points examined. All animal experiments were performed in accordance with National Institutes of Health guidelines and were approved by Oregon State University's Institutional Animal Care and Use Committee (approval #3751). Rats sacrificed for the zeitgeber time (ZT) 0 time-point (i.e., at the initiation of the light phase) were kept under dark conditions with dim red lamps used for visualization during the collection. Lights were turned on after the animals were sacrificed. Animals collected at ZT 12 (when lights were turned off) were maintained in full light conditions throughout the collection.

2.2. Western blotting

Tissues were processed with a glass-Teflon homogenizer in homogenization buffer (10 mM Tris, 100 mM NaCl, 1% Triton-X, 0.5% Igepal, 1 mM EGTA, 1 mM EDTA). Samples were solubilized in Laemmli loading buffer containing SDS and equal protein amounts were separated by standard SDS–PAGE procedures. All samples were normalized by total protein loaded, since total solubilized protein did not change over the time course of this study.
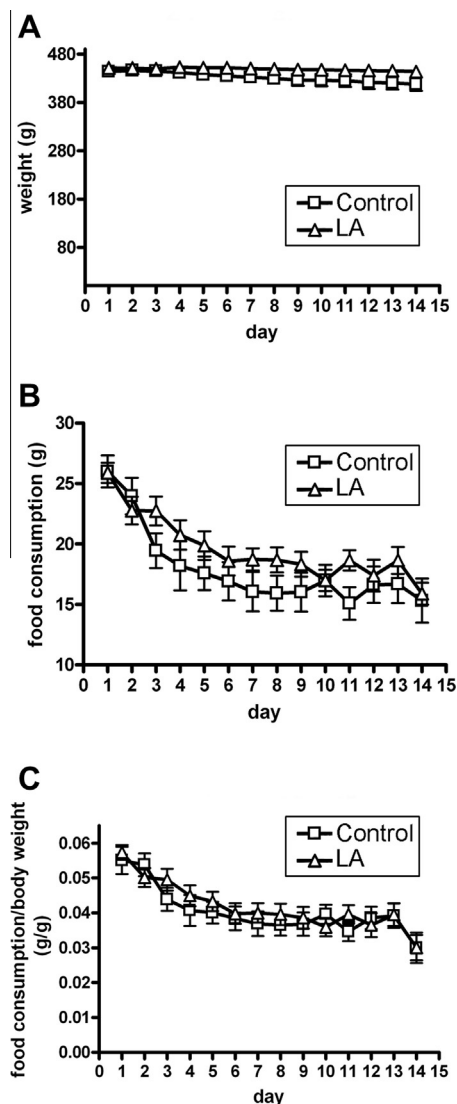

Fig. 1. The pair feeding protocol controlled for LA-induced changes in caloric intake and weight changes. Animals were paired to match body weights prior to beginning supplementation. Changes in body weight (A) and food consumption (B) were recorded daily. The proportion of food consumed relative to body weight was calculated daily for each animal (C).

Proteins were transferred to nitrocellulose membranes and were blocked in 1% BSA in TBS. Proteins were detected using chemiluminescence. Membranes were incubated with antibodies detecting BMAL1 (Abcam), Reverbβ (Aviva), Per2 (BD Transduction), PPARα (Proteintech), ACC (Cell Signaling), phospho-ACC (Cell Signaling), and FAS (Cell Signaling).

2.3. Corticosterone

ELISA kits were used according to manufacturer's instructions (Assay Designs) to quantify levels of corticosterone in the blood plasma.

3. Results and discussion

Advanced age leads to both loss of entrainment of clock gene expression and dyslipidemia. Additionally, previous work from our lab showed that LA altered expression of hepatic clock genes and level of enzymes associated with lipid metabolism in 24-month old rats [16,17]. These results thus suggest that LA may

function to entrain circadian cycles and therefore limit downstream pathophysiologies associated with hepatic clock dysfunction. The current study was thus designed to examine whether LA asserted its beneficial effects on age-related dyslipidemia via the hepatic clock in old rats. For this discernment, 24-month old rats were pair-fed with or without LA, and tissue samples were taken at 5 time points during the day (ZT 0–12) to examine the rhythmic nature of the circadian clock with age and how LA supplementation may affect hepatic clock function. This study focused on the protein oscillation during the light-portion of the day, which is when these nocturnal animals are typically inactive and when clock-associated lipid metabolism proteins should be increasing.

3.1. LA shifts general cycles of the circadian clock which otherwise become dys-entrained with age

To discern both the extent that age and LA affected general circadian cycles, we measured the levels of blood plasma corticosterone over the 12-h light period. Blood levels of this glucocorticoid hormone demonstrate circadian regulation with levels low while an animal is sleeping, rapidly rising as the animal awakes, and a trend of decreasing over the course of the animal's active period [18]. Additionally, circulating glucocorticoids can alter the circadian clocks in peripheral tissues [19]. Thus corticosterone measurements are typically used as a broad indication of circadian clock function and timing [20,21]. Results showed that the circadian pattern of corticosterone was significantly different from control diet fed rats (Fig. 2A; two-way ANOVA, $F = 3.89$ $p < 0.05$). This LA-induced shift in corticosterone patterns could not be associated with general changes in caloric intake [22] as the pair-feeding

protocol limited differences in calorie consumption between the feeding groups. Corticosterone in LA-supplemented animals increased from baseline and peaked in concentration at ZT12, whereas the pair-fed control animals displayed maximal corticosterone levels at ZT6 versus baseline. The pattern of corticosterone seen in LA-treated old rats corresponds to that generally described in the literature for *ad libitum*-fed young animals where the peak of plasma corticosterone typically occurs just as the animals are entering the active phase of their daily cycle [18]. Area under the curve (AUC) measurements for the ZT0-ZT12 timecourse in this study reveal that LA reduced total blood serum corticosterone levels by 20%. However, the maximum corticosterone level reached was not different between the two groups. Taken together, these results suggest that LA directly influences circadian cycles in old rats and leads to a pattern of corticosterone cycling that is more reminiscent of patterns typical in young animals.

3.2. LA-dependent changes to core clock proteins

Physiological rhythms are regulated by cellular clocks consisting of interlocking positive and negative transcription/translation feedback loops. The core circadian machinery is composed of a positive arm of gene transcription represented by the proteins BMAL and Circadian Locomotor Output Cycles Kaput (CLOCK). These proteins heterodimerize and bind to E-box transcription elements in the 5′ flanking regions of genes involved in cell cycle progression, energy utilization, detoxification, and lipid metabolism. BMAL and CLOCK also activate transcription of other proteins (e.g. PER, Cryptochrome, and Rev-erb) that serve as negative feedback loops to down-regulate transcription of BMAL and hence
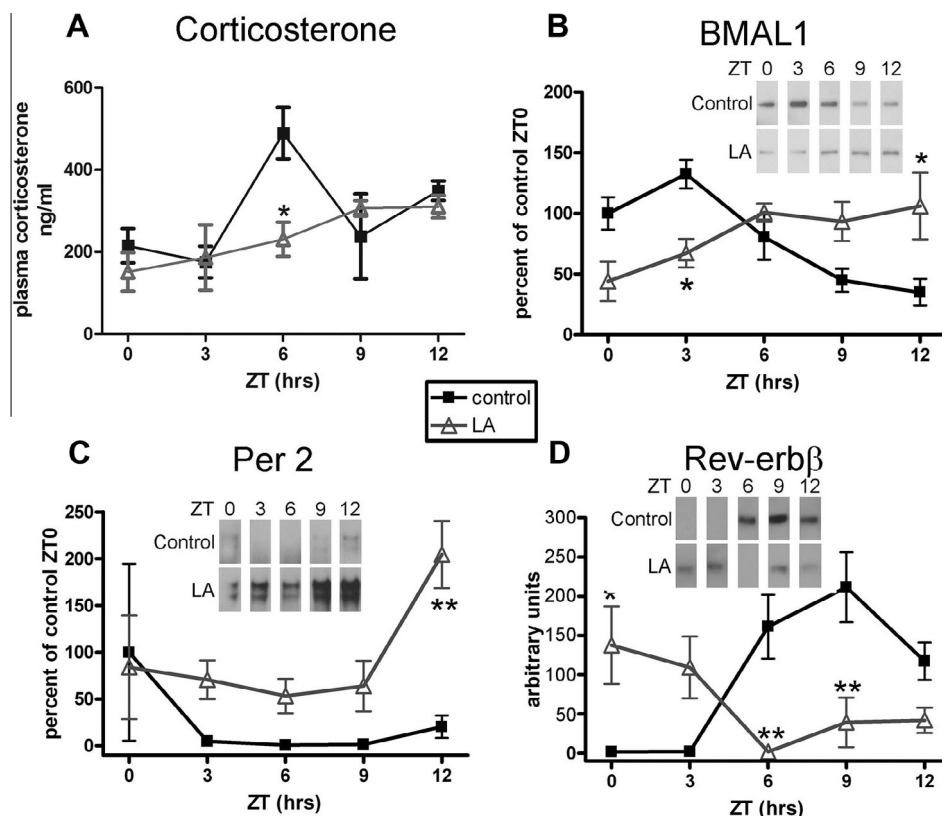

Fig. 2. LA alters the rhythmic expression patterns of core circadian proteins. LA alters the release pattern of corticosterone (A) in aged animals. Corticosterone levels in the serum were quantified at the indicated times (*$p < 0.05$, two-way ANOVA with Bonferroni post test). Protein levels of BMAL1 (B), Per2 (C), and Rev-erbβ (D) in the livers of LA supplemented and control animals were quantified by western blot detection (*$p < 0.05$, **$p < 0.01$, two-way ANOVA with Bonferroni post test). All control samples were run on a single gel. All LA samples were run on a single gel along with ZT0 control samples. Extraneous lanes have been removed for clarity. N = 4–5 animals/time point/treatment.

E-box-regulated genes. The complexity of both positive and negative feedback loops influencing both central clock function in the suprachiasmatic nucleus or in peripheral clocks, such as the liver, is rapidly increasing with regular identification of new interaction pathways [21,23].

To determine how LA influenced the hepatic clock, temporal changes in proteins associated with both the positive and negative arms of the core oscillators were monitored. Western blot analysis revealed that the timing of BMAL1, Per2, and Rev-erbβ oscillations were markedly altered by LA treatment, which reflected a general shift in phase of core clock cycling (Fig. 2). For example, BMAL1 levels in LA-fed old rats reaches a maximum 6–9 h after the peak evident in the control group (Fig. 2B). Furthermore, two-way ANOVA analysis showed that there is a significant interaction between the two independent variables, indicating that BMAL1 levels are significantly different between LA- and control-treated animals at different times of day ($F = 7.63$, $p < 0.001$). While there was no significant interaction between time and treatment on Per2 expression there was a significant effect of LA supplementation on Per2 protein levels. In this regard, LA-fed rats displayed heightened Per2 levels throughout the light phase where the maximum increase in Per2 was 12 h later than for control rats (Fig. 2C, $p < 0.01$). Finally, monitoring the levels of Rev-erbβ throughout the 12-h timecourse showed that its levels were maximal in LA-supplemented rats approximately 9 h prior to the peak seen in control, pair-fed rats (Fig. 2D). As with BMAL, there is a significant interaction between the time of day and supplementation (two-way ANOVA, $F = 10.97$, $p < 0.0001$). Summing these observations suggests that LA supplementation alters the expression patterns of multiple core loops of the hepatic circadian clock in aged animals to a pattern reminiscent of that observed in young rodents [24,25].

3.3. LA alters clock-dependent lipid metabolism

Our prior studies showed that LA attenuates gene expression of multiple metabolic enzymes including FAS, ACC, Spot 14, and Stearoyl-Coenzyme A desaturase 1 [16]. To discern whether LA mediates the changes in lipid metabolism in a circadian-dependent fashion, we measured LA-mediated changes to known effectors of lipid homeostasis that are directly connected to central hepatic clock oscillators. In this regard, one of the most important clock-controlled mediators is the transcription factor PPARα, which in turn regulates expression of a number of genes associated with fatty acid and cholesterol synthesis [23,26]. Supplementing the diets of old rats with LA resulted in changes in the oscillatory patterns of PPARα over the timecourse, with an expression peak at ZT 6 (Fig. 3). Moreover, there was a significant interaction between

time of day and supplementation (two-way ANOVA, $F = 16.28$, $p < 0.0001$), which suggested that LA was working through changes in function of the core hepatic clock feedback loops to alter PPARα patterns versus controls.

We further examined the effects of LA on lipid biogenesis by examining the levels of ACC and FAS during the light phase. We chose ACC and FAS because (i) age-related hepatic dyslipidemia partly stems from elevated levels of these enzymes [16] and (ii) their levels are governed by PPARα and SREBP-1c in a clock-controlled manner [27]. Additionally, it has been reported that enzyme levels of these proteins are low during the time of day when animals are inactive (i.e., the light phase for rats), which is the observation period of the present study [23,28–32]. Thus the levels and activities of ACC and FAS are useful markers for assessing clock-regulated lipid homeostasis, especially in aged animals.

Monitoring ACC levels from ZT0 to ZT12 revealed that LA-fed rats exhibited markedly altered ACC levels compared to pair-fed controls. AUC measurements showed that LA treatment resulted in hepatic ACC concentrations that were 90% lower than controls. The significant decrease in total ACC protein in LA-supplemented animals was most evident at ZT12 ($p < 0.0001$, Fig. 4A). Moreover, consistent with the association of circadian cycling previously shown by BMAL1 and PPARα, we observed a significant interaction between the time of day and LA supplementation (two-way ANOVA, $F = 3.89$, $p < 0.05$).

ACC activity is further regulated by multiple kinases, including by AMP-activated protein kinase (AMPK), which catalyzes the phosphorylation of the Ser79 residue and thereby inhibits ACC activity [33]. As LA induces AMPK activity in rodents [34], we hypothesized that LA-supplemented rats may also exhibit lower ACC activity in conjunction with attenuated ACC protein levels. However, by monitoring Ser79 phosphorylation status, little LA-induced changes in Ser79 phosphorylation state were observed over the timecourse of the study (Fig. 4B). As a result, the phospho-ACC/total ACC ratio was significantly affected by LA at ZT12 (two-way ANOVA, $F = 6.18$, $p < 0.001$, Fig. 4C). Taken together, these results show that LA primarily downregulates ACC activity via limiting its protein status, and the loss of ACC levels are not further markedly influenced by changes in phosphorylation state of Ser79.

LA supplementation resulted in a similar significant attenuation in FAS protein levels (two-way ANOVA, $F = 15.43$, $p < 0.001$, Fig. 4D) as was evident for ACC. As shown in Fig. 4D, FAS levels trended down in pair-fed controls and LA supplementation caused a significant decline in FAS abundance throughout the light phase (AUC 57.8% lower versus control).

Analyzing our results on LA-induced changes to FAS and ACC with respect to literature reports on their circadian activities suggest that the aged animals display significant dys-entrained circadian rhythms of these lipogenic enzymes [16,32]. In fact, the protein oscillations of FAS and ACC during the daylight hours in the LA treated old rats resembles that seen in young, untreated rats [32,35]. Thus it is possible that LA corrects age-associated changes in lipogenic pathways to levels typically seen in young animals. Further experiments will be necessary to confirm this possibility, but this concept would be in line with our prior revelations that LA corrects multiple age-associated changes in hepatic gene transcription, including *Acaca* and *Fasn* [16]. Furthermore, compounds that activate PPAR are known to inhibit ACC activity [36], and fibrate treatment *in vivo* and in hepatocytes reduces hepatic mRNA and enzyme activities of ACC, FAS, HMG-CoA synthase, HMG-CoA reductase, and SREBP2 [37]. Thus our results are consistent with methods known to have positive lipid-relevant health effects mediated by clock-controlled transcription factors.

Overall, the data shown are in keeping with our previous study showing LA strongly influenced transcript levels of clock genes,
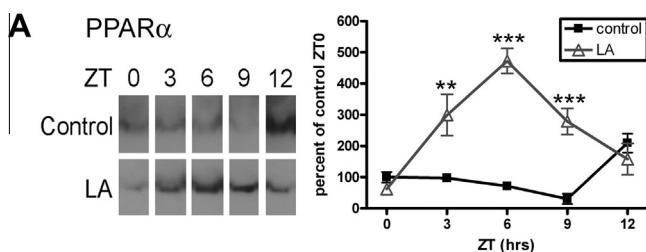

Fig. 3. LA enhances the expression of PPARα during the lights-on portion of the day. Proteins were extracted from liver tissue of LA supplemented and control animals. (**$p < 0.01$, ***$p < 0.001$, two-way ANOVA with Bonferroni post test). All control samples were run on a single gel. All LA samples were run on a single gel along with ZT0 control samples. Extraneous lanes have been removed for clarity. $N = 4$–5 animals/time point/treatment.
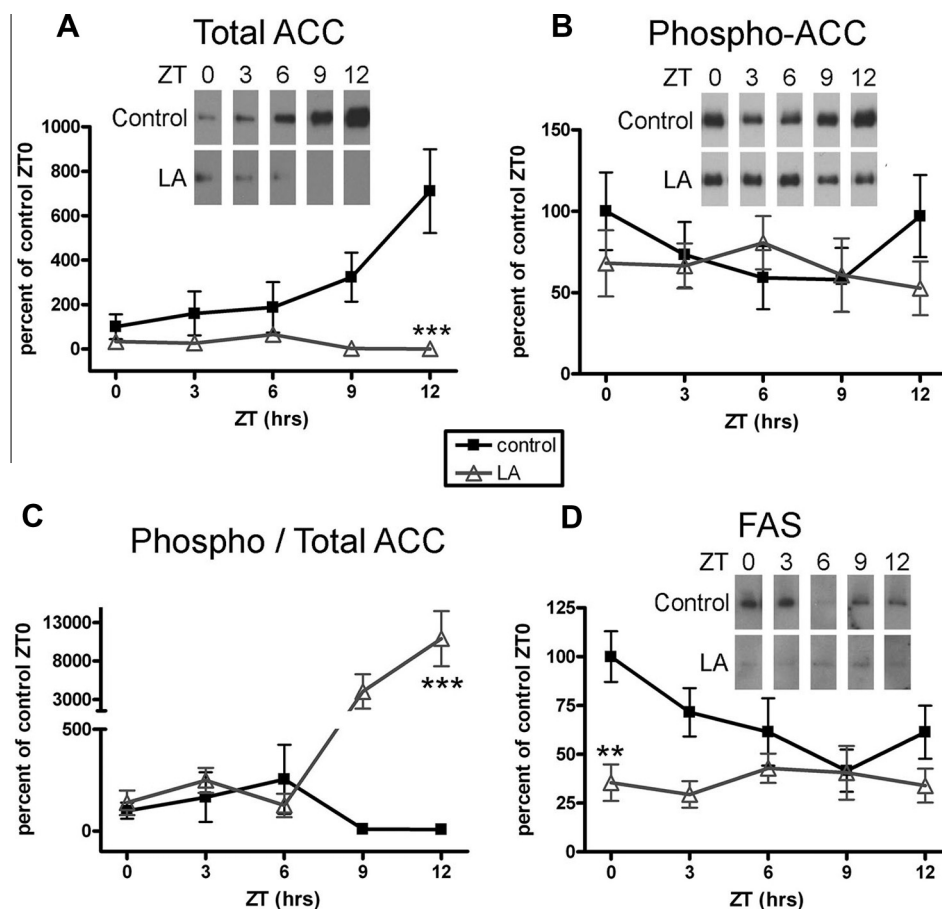
Fig. 4. LA reduces lipid anabolic enzymes during the lights-on portion of the day. Proteins were extracted from liver tissue of LA supplemented and control animals. Levels of total ACC (A), phosphorylated ACC (B), and the ratio of those values representing inactive ACC (C) are presented. Total FAS protein levels were also quantified by western blot detection (D). ($^{**}p < 0.01$, $^{***}p < 0.001$, two-way ANOVA with Bonferroni post test). All control samples were run on a single gel. All LA samples were run on a single gel along with ZT0 control samples. Extraneous lanes have been removed for clarity. $N = 4$–5 animals/time point/treatment.

clock-controlled transcription factors and lipid metabolism genes [16]. The present work significantly adds to our prior work as the previous study was not designed to monitor changes over a circadian period. Thus this study shows that LA alters the hepatic clock and the downstream clock-controlled lipid metabolic genes. Complete sampling of the 24 h day will be necessary to fully determine the extent of LA's action on the circadian clock.

These findings reveal the potential of the naturally occurring dithiol compound, LA, which is readily bioavailable [38], to correct circadian rhythms, which have become dysregulated with age. LA may thus be added to the pharmacopeia of compounds (e.g., melatonin) available to treat various circadian dysfunctions. Additional studies will be needed to determine the magnitude of circadian rhythm shifts afforded by different doses of LA. Furthermore, long term studies will be necessary to determine if the entraining effects are maintained with continued LA supplementation.

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