Published in final edited form as:

Mol Neurobiol. 2018 July ; 55(7): 6037–6049. doi:10.1007/s12035-017-0813-y.

Lipoic acid stimulates cAMP production in healthy control and secondary progressive MS subjects

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Abstract

Lipoic acid (LA) exhibits antioxidant and anti-inflammatory properties; supplementation reduces disease severity and T lymphocyte migration into the central nervous system in a murine model of multiple sclerosis (MS), and administration in secondary progressive MS (SPMS) subjects reduces brain atrophy compared to placebo. The mechanism of action (MOA) of LA's efficacy in suppression of MS pathology is incompletely understood. LA stimulates production of the immunomodulator cyclic AMP (cAMP) in vitro. To determine whether cAMP could be involved in the MOA of LA in vivo, we performed a clinical trial to examine whether LA stimulates cAMP production in healthy control and MS subjects, and whether there are differences in the bioavailability of LA between groups. We administered 1200 mg of oral LA to healthy control, relapsing remitting MS (RRMS) and SPMS subjects, and measured plasma LA and cAMP levels in peripheral blood mononuclear cells (PBMCs). There were no significant differences between the groups in pharmacokinetic (PK) parameters. Healthy and SPMS subjects had increased cAMP at 2 h and 4 h post LA treatment compared to baseline, while RRMS subjects showed decreases in cAMP. Additionally, plasma concentrations of prostaglandin E2 (PGE₂, a known cAMP stimulator) were significantly lower in female RRMS subjects compared to female HC and SPMS subjects 4 h after LA ingestion. These data indicate that cAMP could be part of the MOA of LA in SPMS, and that there is a divergent response to LA in RRMS subjects that may have implications in the efficacy of immunomodulatory drugs.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Compliance with Ethical Standards: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

This article does not contain any studies with animals performed by any of the authors.

This clinical trial, “Defining the Anti-inflammatory Role of Lipoic Acid in Multiple Sclerosis,” NCT00997438, is registered at <https://clinicaltrials.gov/ct2/show/record/NCT00997438>

Keywords

multiple sclerosis; thioctic acid; lipoic acid; cyclic AMP; pharmacokinetics; prostaglandin E2

Introduction

Lipoic acid (LA, also known as alpha-lipoic acid or thioctic acid) is an eight-carbon sulfur containing fatty acid that is synthesized from octanoic acid by lipoic acid synthase in most prokaryotic and eukaryotic microorganisms, plants and animals [1]. LA can also be absorbed from the diet, including natural food sources and over the counter nutritional supplements. LA exhibits antioxidant and anti-inflammatory properties and supplementation has been shown to suppress and reduce disease severity and T lymphocyte migration into the central nervous system (CNS) in the murine model of multiple sclerosis (MS), experimental autoimmune encephalitis (EAE) [2-4]. Mice treated with LA after the onset of clinical disease showed a rapid decrease in paralysis compared with controls and had significantly reduced demyelination and axonal injury. Furthermore, LA at a dose of 100 mg/kg/day completely prevented EAE when administered 7-10 days after immunization before the onset of clinical disease [2]. LA also inhibited T cell migration into the CNS and suppressed the expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) by CNS endothelial cells (ECs). ICAM-1 and VCAM-1 are involved in immune cell adhesion to endothelial cells (ECs) [5]. In a mouse focal cortical model of EAE, LA decreased CD4+ and galectin-3+ immune cell infiltration into the brain [6]. Efficacy of LA in preventing EAE development in rats in a dose dependent fashion was confirmed by Schreiber *et al.* [7]. This study also showed that LA decreased the migration of monocytes across the blood brain barrier (BBB), which is correlated with the clinical improvement seen in EAE.

Due to its success in treating EAE, studies have expanded to include human cells and clinical trials have begun in MS patients. An early pilot trial of oral LA in MS subjects conducted by Yadav *et al.* demonstrated that oral LA reduces serum matrix metalloproteinase 9 (MMP-9) and sICAM-1 levels [4]. These proteins are associated with inflammatory disease activity and have been targeted for therapy. In another small clinical trial, 1200 mg/day of LA for 12 weeks significantly improved total antioxidant capacity [8]. Most recently, Dr. Spain conducted a 2-year randomized, double-blind, placebo-controlled pilot trial and made the exciting discovery that daily LA ingestion produced a 68% reduction in brain atrophy compared to placebo over a 2 year period in people with secondary progressive MS [9].

Though data from EAE models and these trials indicate that LA may be effective as a treatment for MS, the mechanism of action in humans has not been fully addressed. We have discovered that LA stimulates production of the small molecule second messenger cyclic adenosine monophosphate (cAMP) in T-cell enriched peripheral blood mononuclear cells

(PBMcs) and in natural killer (NK) cells from healthy human donors *in vitro* [10]. cAMP is a potent inhibitor of T-cell activation and proliferation (reviewed in [11-13]). Increased levels of cAMP have been shown to inhibit the expression of pro-inflammatory cytokines such as interleukin (IL)-2, interferon (IFN)- γ and tumor necrosis factor (TNF)- α , and to stimulate the production of anti-inflammatory cytokines such as IL-10 [14]. Agents that elevate cAMP levels are also known to increase the barrier function of tight junctions in the BBB and peripheral vascular endothelial cells [15-17]. In this study, we designed a clinical trial to determine whether LA can stimulate cAMP production in subjects with MS (relapsing remitting and secondary progressive) and healthy controls, and whether there are differences in bioavailability of LA between groups. We also determined if cAMP can be used as a biomarker that would allow us to monitor the therapeutic efficacy of LA at earlier time points than the extended periods necessary to achieve clinical effects, such as measuring brain atrophy.

Materials and Methods

Subject recruitment and consenting

This study received approval from the VA Portland Healthcare System (VAPORHCS) and Oregon Health & Science University (OHSU) Institutional Review Boards prior to initiation (OHSU eIRB#5659). Healthy control (HC), relapsing remitting MS (RRMS), and secondary progressive MS (SPMS) subjects were recruited at these institutions via flyers, screening of potential candidates receiving care at the VAPORHCS and OHSU MS clinics, and word of mouth. Interested subjects were phone-screened for eligibility prior to scheduling an enrollment visit. All enrolled subjects gave informed consent in writing before study participation. To qualify, HC subjects needed to be an adult at least 18 years of age able to provide informed consent, and meet none of the following exclusion criteria: 1) self-reported current substance abuse (except tobacco), alcohol abuse/dependence or sobriety for less than 90 days, 2) pregnant or breast-feeding, 3) anemia as indicated by point-of-care (POC) hemoglobin <12, 4) history of bleeding disorders, 5) current anti-coagulant use such as Heparin, Coumadin, or daily aspirin, 6) weight less than 110 lbs., 7) other significant inflammatory diseases or health problems (e.g. active coronary heart disease, diabetes mellitus, other auto-immune disorder), or 8) active infection (as indicated by a fever). In addition to the above inclusion/exclusion criteria, patients enrolled as MS subjects needed to have a current diagnosis of relapsing remitting MS (RRMS), and were excluded for experiencing MS exacerbation(s) or receipt of systemically administered corticosteroids within 30 days of the blood draw.

Treatment, specimen collection and processing

At visit 1, subject eligibility screening and informed consent was performed; medical history questionnaire (including current medication and supplement listing) was completed, weight and vital signs were measured and recorded, and physical exam (if indicated based on medical history and/or vital signs) and anemia checks were performed. Additionally, for subjects with MS, Self-Administered Expanded Disability Status Scale (EDSS) Questionnaire was administered. Physical and neurological exams were also performed. Subjects were asked not to consume caffeine, and to stop taking supplements that may

interfere with the absorption of LA or the production of cAMP [i.e. non-steroidal anti-inflammatory drugs (NSAIDs), vitamins C and D, calcium, multivitamins, omegas/fish oil, etc.] until the completion of the study. The use of prescription medications was permitted. Subjects were also asked to abstain from alcohol for 24 h and fast for 12 h prior to visit 2. At visit 2, subjects of child bearing potential were given pregnancy tests, and all patients had peripheral IVs inserted. Patients received a standardized breakfast, followed by baseline blood draw (time 0). Subjects then ingested 1200 mg racemic LA by taking four 300 mg gelatin capsules (Vital Nutrients Middletown, CT USA) with water. Blood was collected again at 1 h, 2 h, 3 h, and 4 h after taking LA. Subjects returned to the clinic for visits 3 and 4 for 24 h and 48 h blood draws, completing subject involvement in the study.

At each time point, approximately 50 ml of blood was collected in 5 (10 ml) Ethylenediaminetetraacetic acid (EDTA) coated tubes (Thermo Fisher Scientific, Waltham, MA) and immediately processed. To obtain plasma for pharmacokinetic (PK) analyses, one tube was centrifuged (10 min at $1430 \times g$, 5°C). Separated plasma was aliquoted and frozen at -80°C . To obtain PBMCs, the remaining 4 EDTA tubes were combined and diluted with approximately 25 ml phosphate buffered saline (PBS). PBMCs were isolated via Ficoll (Thermo Fisher Scientific, Waltham, MA) gradient separation, washed three times in Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific, Waltham, MA), and separated into dry pellets (stored at -80°C) and cryopreserved cells [resuspended in RPMI+dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO) then cooled at a constant rate to -80°C prior to transfer to liquid nitrogen storage].

Trial profile

137 volunteers were phone-screened for eligibility; 69 subjects were deemed eligible and chose to schedule a screening and consenting visit (Visit 1) to enroll in the study (Figure 1). Seven subjects (3 HC, 2 RRMS and 2 SPMS) did not meet the inclusion/exclusion criteria based on medical questionnaire and physical/neurological examination, and were withdrawn from the study. Two additional subjects (1 RRMS and 1 SPMS) declined further participation for personal reasons. Sixty subjects took the study drug (Visit 2). Three subjects were withdrawn during this visit; one healthy control subject fainted at the 2 h blood draw, and 2 SPMS subjects had difficulty with blood draw. The remaining 57 subjects completed the study. Subject demographics are listed in Table 1.

Pharmacokinetic (PK) analysis using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

LA concentrations were measured in plasma samples at baseline and after ingestion of LA using LC-MS/MS at the OHSU Pharmacokinetics Core.

Materials—Lipoic acid (LA) was from Sigma-Aldrich (St. Louis, MO). The internal reference, racemic α -lipoic acid-1,2,5,6- $^{13}\text{C}_4$, was from Isotec a division of Sigma-Aldrich. The sample vials and silanized inserts were obtained from Sun Sri (a subdivision of Fisher Scientific, Rockwood, TN). MS grade methanol, acetonitrile and water were purchased from Burdick and Jackson (Muskegon, MI). Acetic acid was from J.T. Baker (Phillipsburg, NJ).

Preparation of Samples and Calibrators—Concentrated stocks of LA standard and the internal reference ($^{13}\text{C}_4\text{-LA}$) were prepared at 1mg/ml in methanol and stored at -80°C . A 50 μL aliquot of plasma was removed and treated with 150 μL of acetonitrile containing 10 ng/ml of $^{13}\text{C}_4\text{-LA}$. LA standards were prepared by spiking naïve plasma with 0 to 1000 ng/ml LA. The samples were vortexed and protein removed by centrifugation. The supernatant was filtered through a 0.22 μm Ultrafree[®] spin filter (EMD Millipore, Darmstadt, Germany) and 10 μL was injected for LC-MS/MS analysis. The sample concentrations were determined from the slope and intercept of standard curves of the area ratio of the analyte/internal standard versus the LA concentration prepared with each sample set. The lower limit of quantification was 1 ng/ml of LA. Processed plasma samples were analyzed using a 5500 QTRAP-hybrid/triple quadrupole linear ion trap mass spectrometer (SCIEX, Framingham, MA) with electrospray ionization (ESI) in negative mode. The mass spectrometer was interfaced to a Shimadzu (Columbia, MD) SIL-20AC XR auto-sampler followed by two LC-20AD XR LC pumps. The instrument was operated with the following settings: source voltage -4500 V, GS1 50, GS2 50, CUR 50, TEM 500 $^\circ\text{C}$ and CAD gas high. The MRM transitions monitored with a 75 ms dwell time were: LA, m/z 205 $\rightarrow$ 171; $^{13}\text{C}_4\text{-LA}$: m/z 209 $\rightarrow$ 175. Transitions were optimized by direct infusion of purified compounds. The gradient mobile phase was delivered at a flow rate of 0.3 ml/min and consisted of two solvents: 0.03% acetic acid in water (A) and 0.03% acetic acid methanol (B). The initial concentration of B was 40%, followed by an increase to 95% B by 2 min, held at 95% for 2 min, then decreased to 40% B in 0.1 min, followed by re-equilibration for 2.9 min for a total time of 7 min. Flow was directed to the source from 2.5 min to 3.9 min. The column was a Zorbax SB-C18, 2.1 $\times$ 50mm; 3.5 μ from Agilent (Santa Clare, CA) maintained at 35 $^\circ\text{C}$ using a Shimadzu CTO-20AC column oven. Data were acquired using Analyst 1.6.2 software and analyzed using Multiquant 3.0.1 software.

Enzyme-linked immunosorbent assays (ELISAs)

To measure cAMP content, PBMC pellets were resuspended in 100 μL PBS; 50 μL of suspension were added to 350 μL 0.1M HCl, mixed and boiled for 10-15 minutes to lyse cells for cAMP assay. The remaining 50 μL of cell suspension was saved and re-frozen at -80°C . cAMP colorimetric competitive immunoassays were performed in triplicate, according to manufacturer's instructions (Enzo Life Sciences Inc., Farmingdale, NY, USA). To normalize cAMP values to protein content, BCA assays were performed in triplicate according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA).

To measure plasma albumin, a DuoSet ELISA kit was purchased from R&D Systems (Minneapolis, MN), and assays were performed in duplicate according to manufacturer's protocol in half-volume plates.

To measure plasma TNF- α , a Ready-SET-Go ELISA kit was purchased from Affymetrix (now a division of Thermo Fisher), and assays were performed in half-volume plates according to manufacturer's protocol in duplicate, with overnight sample incubation at 4 $^\circ\text{C}$.

To measure prostaglandin E2 (PGE₂) concentrations in plasma, an ELISA kit was purchased from Enzo Life Sciences Inc. (Farmingdale, NY). Assays were performed according to manufacturer's protocols in duplicate.

For all ELISAs, plates were read using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Values for each donor were determined as an average of the replicates, which were generated from internal 4 parameter logistic standard curves using Softmax software (Molecular Devices). Some initial sample analyses yielded values above the standard curve. These samples were diluted further in PBS (cAMP assays) or assay buffer (TNF- α and PGE₂) until values were within range of the assay's detection.

Statistical analyses

Plasma LA pharmacokinetic data were analyzed by non-compartmental methods using PKSolver, a freely available add-in program for use with Microsoft Excel [18]. Area under the plasma concentration-time curve from time zero to the last time point (48 h, AUC_{0-last}) was calculated by the linear trapezoidal rule. Terminal elimination half-life ($t_{1/2}$) was calculated as $\ln 2/\lambda_z$, where λ_z is the terminal elimination rate constant, determined from regression analysis of the concentration-time points in the terminal elimination phase. Oral clearance (CL/f) was calculated as oral dose over/AUC_{0- ∞} , where AUC_{0- ∞} is AUC_{0-last} plus the terminal area. Finally, volume of distribution (V_z/f) was calculated as CL/ λ_z . Subject groups were tested for significant differences in PK parameters with Student's t-tests (HC v RRMS and HC v SPMS) and one-way ANOVAs.

Median cAMP levels and interquartile range were calculated at each time point (baseline, 2 and 4 h) and by subject group (HC, RRMS, and SPMS). The distribution of cAMP levels were assessed graphically and were shown to be heavily right-tailed. Thus, cAMP was log-transformed prior to further analysis. In order to account for the repeated measurements within patients and over time, a general estimating equation (GEE) was used to estimate the change (slope) in cAMP overall (baseline to hour 4) and between each consecutive time points (baseline to hour 2, hour 2 to hour 4). Linear combinations were used to estimate the difference in cAMP change (difference in slopes or difference-in-difference) between RRMS, SPMS and HC subjects. Reported are the estimated slopes and 95% confidence intervals as well as the p-values for the difference in slope between subject groups. Since cAMP was log-transformed, the slopes are a ratio of two time points and are interpreted as 'for each unit change in time, median cAMP levels change by a factor of X.'

For albumin, TNF- α and PGE₂, values for each donor were determined as an average of the replicates, which were generated from internal 4 parameter logistic standard curves using Softmax software (Molecular Devices). These values were used to determine the arithmetic mean, plus or minus the standard error of the mean, of each condition. Mean values were then tested for significant differences within and between groups in Microsoft Excel 2010 using oneway ANOVAs and Student's t-tests. Further analyses [median, potential significance of median differences, interquartile range (IQR), and outliers] and graphing were performed using BoxPlotR: a free web-tool for generation of box plots.

Results

LA pharmacokinetics (PK)

In a previous study by Yadav *et al.* assessing the pharmacokinetics of LA, MS subjects receiving 1200 mg of racemic LA had peak median serum levels of 4800 ng/ml, with $T_{\max}$ between 1–4 h [4]. In a follow up study to test different formulations of LA, mean serum $C_{\max}$ in MS subjects taking 1200 mg of LA from Vital Nutrients was 9980 ng/ml, with a mean $T_{\max}$ of 1.19 h, and half-life of 0.67 h [19]. These values differed significantly from previous PK studies using healthy subjects, raising the possibility that MS alters LA bioavailability [20–24]. Thus, to determine whether MS subjects process LA differently than healthy controls, we performed comparative PK analyses of LA levels using plasma from HC, RRMS and SPMS subjects who took a single 1200 mg oral dose of LA (Vital Nutrients) with food. This replicated previous conditions in which Yadav *et al.* demonstrated that a comparable dose in mice was highly effective at suppressing EAE [19].

At the time of our analyses, plasma samples from one HC and one SPMS subject were not available, leaving 19 HC, 21 RRMS, and 15 SPMS subjects for PK studies (parameters outlined in Table 2). Figure 2 shows the plasma LA concentration-time curves for HC, RRMS, and SPMS subjects (mean values, $\pm$ standard error). In all subjects, LA levels were below the minimum detection level at baseline. After ingestion, peak median LA plasma concentrations of 1986 ± 302 ng/ml (HC), 1895 ± 305 ng/ml (RRMS), and 2110 ± 351 ng/ml (SPMS) were reached at $T_{\max}$ 1.8 ± 0.2 h, 1.81 ± 0.2 h and 1.53 ± 0.2 h, respectively. Wide inter-patient variability was observed in all parameters (see reported ranges in Table 2). There are no significant differences between subject groups (HC, RRMS and SPMS) in any of the parameters examined, including half-life, $T_{\max}$, $C_{\max}$, AUC_{0-} , volume of distribution, and oral clearance.

cAMP

Useable PBMCs were not obtained from one HC and 2 RRMS patients, leaving 19 HC, 19 RRMS, and 16 SPMS subjects for cAMP analyses. Assays of cAMP levels from our early samples, combined with data from our previous studies suggested that the time of greatest interest for cAMP detection was 2 h and 4 h post-LA administration; we thus focused our resources on these time points [25]. Supplemental Fig. 1 depicts cAMP data from all patients and mean cAMP level at each time point from each subject group. Figure 3 depicts these data on a more limited scale, “zoomed” to better show the cAMP levels of the majority of individual donors, and also includes mean data points. Table 3 displays the median cAMP level for each group at each time point. Model estimated changes in cAMP levels overall and between consecutive time points are displayed in Table 4. cAMP levels in RRMS patients decreased by a factor of 0.76 from baseline to hour 2 (95% confidence interval [CI]: 0.62, 0.94) and increased slightly by a factor of 1.03 at hour 4 (95% CI: 0.83, 1.26). In comparison, cAMP levels in SPMS patients increased by a factor of 1.28 from baseline to hour 2 (95% CI: 1.03, 1.6), then decreased by a factor of 0.94 at hour 4 (95% CI: 0.75, 1.17). cAMP levels in HC increased by a factor of 1.11 from baseline to hour 2 (95% CI: 0.9, 1.37) and continued to increase by a factor of 1.02 at hour 4 (95% CI: 0.83, 1.26). There

were no significant correlations between LA $AUC_{0-\infty}$, (or C_{max}) and either peak cAMP levels or changes in cAMP (Supplemental Fig. 2 and data not shown).

Overall change in cAMP differed significantly between RRMS patients (0.78, 95% CI: 0.64, 0.97) and HC (1.11, 95% CI: 0.9, 1.37) (p-value=0.0204; Table 4). Overall change also significantly differed between RRMS patients and SPMS patients (1.20, 95% CI: 0.96, 1.5) (p-value<0.001). For both comparisons, the significant difference in change was largely driven by the baseline to hour 2 time period, in which the RRMS group had a decrease in cAMP levels while the SPMS and HC groups had increasing cAMP levels. SPMS and HC did not differ (p-value=0.6168). These data are supported by individual T_{max} values; though the percentage of subjects reaching (LA) T_{max} at each of the measured time points was fairly similar in all three groups (Supplemental Fig 3A), most HC and SPMS subjects had peak cAMP levels at 2 or 4 hours post-LA (77.8% and 75%, respectively), while the majority of RRMS subjects (61.1%) had peak cAMP levels at the baseline measurement (Supplemental Fig. 3B).

Albumin, TNF- α and PGE₂

To check for gross differences in plasma protein content within or between subject groups, we analyzed albumin levels by ELISA. This analysis revealed no significant differences in albumin concentrations between HC, RRMS or SPMS subjects at any time point tested (Supplemental Table 1). Results for all of our groups were slightly higher than what is considered to be within normal range (35-50 mg/ml). We believe this is likely due to the use of an ELISA kit that is optimized for cell culture, rather than clinical samples. However, we cannot exclude the possibility that LA increases plasma albumin levels.

We also assayed plasma for the presence of TNF- α , an inflammatory cytokine that is tied to the pathogenesis of MS [26]. Though there was a trend toward lower median levels of TNF- α in RRMS plasma, this difference was not significant, and mean values across all three groups were very similar (Supplemental Fig. 4a). Additionally, lipoic acid appeared to have absolutely no effect on plasma TNF- α values at the time points examined (2 and 4 h), as variation in concentrations between baseline and 4 h from the majority of individual donors was extremely minimal (Supplemental Fig. 4b). Although not our original intention, this stability serves as an internal control suggesting that differences seen between time points in other parameters assayed are real, and not artifacts of sample collection variation.

We have previously demonstrated that LA stimulates cAMP production via G-protein coupled receptors (GPCRs), including the PGE₂ receptors EP2 and EP4 [25,27,28]. Because we saw differences in cAMP response to oral LA between subject groups, we also wanted to examine PGE₂ production. Unfortunately, PBMC samples from a number of our subjects were exhausted in cAMP assays, so the only available samples for PGE analyses were plasma aliquots. However, assaying PGE₂ levels in plasma is an appropriate functional measurement since the excreted PGE₂ is the mediator of various signaling events. When analyzing all donors, there were no significant differences in PGE₂ concentrations between subject groups, nor between time points within subject groups (Fig. 4, top panel).

Because there are known sex differences in MS (disease onset, incidence, progression and type) [29], we performed all analyses for this study both in aggregate, and also breaking the data apart by sex. For most of the data, no significant differences emerged (data not shown). However, when we examined PGE₂ concentrations by sex, a significant difference was seen in females, but not males; mirroring cAMP data, HC and SPMS subject groups did not differ, but female RRMS subjects had levels of PGE₂ that were significantly lower than both HC and SPMS (female) subjects at 4 h post-oral LA ingestion (Fig. 4, middle panel).

In addition to analyzing all data by sex, we also looked for correlations between each of our tested parameters (LA, cAMP, albumin, TNF- α and PGE₂) with each of the others, and with disease-modifying medication status (Table 1). There were no significant correlations (data not shown).

Discussion

PK analyses

This is the first study to directly compare the bioavailability of oral LA and its effect on cAMP levels in healthy control, RRMS and SPMS subjects, and demonstrates for the first time that MS does not appear to alter the bioavailability of oral LA. This study revealed lower $C_{\max}$ and AUC_{0- ∞} , and higher $T_{\max}$, half-life, volume of distribution and oral clearance when compared to a small cohort of MS subjects in a previous pilot trial, or compared to healthy controls in other studies. There are many potential reasons for the observed differences, including the different formulations of LA and whether LA was taken with or without food [19,30,31]. Another very likely reason for the differences is that our measurements of LA were from plasma samples, which often give significantly different values than metabolites measured in serum [19,31]. For example, in a study that used LC-MS/MS to quantify 33 antidepressant drugs spiked into whole blood, plasma and serum, there was wide variability in recovery and matrix effects between the drugs, and this variability was not consistently tied to matrix type – i.e. while one drug may have highest recovery in plasma, and lowest in serum, other drugs showed opposite trends, while many others were best recovered from whole blood. However, it was demonstrated in this study that plasma samples yielded accurate and precise quantification for the highest number of drugs tested [32]. Additional variables that could be responsible for our findings include the larger sample size included in this study, and the inclusion of relatively more male subjects, coupled with the high variability between subjects seen in all PK studies of LA [4,19-23,30].

cAMP as a mediator of LA MOA

LA has been shown to be therapeutic in EAE (the rodent model of MS), and our co-authors recently published data showing that LA treatment reduces brain atrophy in SPMS subjects compared to placebo control [9]. However, the clinical MOA of LA has yet to be described. Abnormal regulation of the inflammatory response is an important component of MS, and LA has been shown to be anti-inflammatory in vitro [33]. We have previously demonstrated that these anti-inflammatory properties are mediated in part by the cAMP signaling cascade [25,27,28]. We also reported preliminary evidence that oral administration of different formulations of LA supplements elevated cAMP levels in PBMCs from a small, mixed

population of MS subjects 4 h after ingestion [25]. Together, these data led us to hypothesize that the MOA of LA in vivo is dependent on stimulation of cAMP, resulting in suppression of neuroinflammatory processes in MS. The primary purpose of this study was to begin to test this hypothesis by performing a clinical trial examining cAMP response to LA treatment in HC, RRMS and SPMS subjects. As expected, PBMCs from HC and SPMS subjects showed elevated cAMP levels at 2 h and 4 h post-LA compared to baseline. Somewhat surprisingly, RRMS subjects had decreased levels of cAMP at 2 h and 4 h post-LA, though there were no significant differences in plasma LA concentrations. This differed from our previous finding that LA treatment increased cAMP levels. The discrepancy is likely due to the differences in the sample population; the previous results were obtained from a mixed population of both RRMS and SPMS subjects grouped together in the analyses [25]. Given the wide variability between subjects in their PK response to LA, a larger study to confirm our findings would be beneficial.

PGE₂, LA and cAMP

The reason(s) for the divergent cAMP response in RRMS vs. SPMS subjects are not immediately clear given our finding that there are no differences in the pharmacokinetics of LA between the two cohorts. However, our work with another agent that stimulates cAMP production (PGE₂) may provide some clues and avenues for exploration. The role of PGE₂ in both healthy and disease states is complex, as it elicits both pro- and anti-inflammatory effects. This is likely due in part to its multiple receptors, two of which (EP2 and EP4) affect increases in cAMP, while splice variants of a third (EP3) inhibit cAMP production [34]. We have previously demonstrated that LA stimulates cAMP production by binding to and activating the PGE₂ (EP2/EP4) receptors, and have further shown that LA/PGE₂ co-stimulation has a synergistic effect on cAMP production in PBMCs and NK cells from healthy subjects [27]. However, in vitro evidence in non-immune cells indicates that LA can inhibit PGE₂ production (see Fig. 5 for a schematic summary of known LA, PGE₂, EP receptor and cAMP interactions [35,36]). Further complicating the story, PGE₂ signaling has a well demonstrated, but complex and ill-defined role in EAE and MS (discussed in detail in [37-40]). In normal inflammatory processes, human macrophages transiently produce high levels of PGE₂. MS patients however, have elevated basal levels of PGE₂ in their PBMCs and cerebrospinal fluid (CSF), and cells from MS patients have been shown to make more PGE₂ than healthy controls when stimulated [41]. These data appear to support an upregulation in PGE₂-dependent processes in MS patients, but this supposition is challenged by a report demonstrating that monocytes from MS patients are less sensitive to PGE-stimulating reagents and are less sensitive to PGE-mediated increases in cAMP compared to healthy control monocytes. In summation, the current state of knowledge of PGE₂ signaling in general, and its specific role in MS, make it very difficult to predict the net effect of LA on cAMP production in vivo, but it is clear that to gain any understanding of the MOA of LA, it is essential to examine PGE₂ production in a clinical setting.

PGE₂ mediates cellular effects via excretion, thus we analyzed its concentration in plasma. Our analyses revealed no significant differences between the three cohorts at baseline. These data seem at odds with previous reports indicating elevated basal PGE₂ in MS subjects, but those studies were performed in mixed MS populations, including RRMS subjects in both

relapse and remission, and in both primary and secondary progressive MS subjects [41,42], making direct comparisons to our data difficult. Post-LA administration, significantly lower PGE₂ levels were observed in female RRMS subjects 4 h after LA was taken compared to both HC and SPMS (female) subjects, paralleling our cAMP data, where RRMS, but not SPMS subjects exhibited significant differences from HC subjects. One previous study showed that monocytes from a small cohort of patients with chronic progressive MS (a group which includes both primary and secondary progressive MS) produced more PGE₂ in response to stimulation with complement than in those from RRMS patients [42]; this raises the possibility that abnormal PGE₂ response is part of the mechanism responsible for the differences seen here, i.e. the lower PGE₂ response in female RRMS subjects leads to less synergistic stimulation of cAMP production in the presence of LA compared to HC and SPMS subjects.

Though the mechanisms discussed above offer some insights, they do not directly address the fact that female RRMS PGE₂ concentrations are not simply lower than HC and SPMS values, but actually appear to be driven down compared to baseline after LA administration. A possible explanation could lie in altered EP receptor expression, which can be up or down-regulated in response to external stimuli, including inflammation. For example, up-regulation of EP2 and EP4 receptors has been detected in neurons following inflammatory stimulus [43]. In contrast, LPS has been shown to down-regulate EP4 expression in peritoneal macrophages, and very interestingly, this reduction appears to be dependent on PGE₂ and cAMP, suggesting a negative feedback mechanism, whereby high PGE₂/cAMP levels cause down-regulation of the EP4 receptor in activated macrophages [44]. This raises the possibility that MS subjects, who have been shown to have elevated levels of PGE₂ in relapsing and chronic disease states, may also have altered EP receptor expression as a result. Of relevance to our data, one hypothesis is that the altered ratio of EP2/4 receptors (which favor cAMP production, and are activated by both PGE₂ and LA) to EP3 receptors (which favor cAMP inhibition) could yield opposing results to the same treatment. These divergent immunomodulatory responses to LA between HC, SPMS and RRMS subjects highlight the gaps in mechanistic understanding of the pathogenesis of different MS subtypes and its potential treatments, underscoring the need for continuing laboratory studies. An important next step in future studies on PGE₂ in MS patients is to compare EP receptor expression between subject groups, to examine whether differential expression correlates with cAMP and PGE₂ concentrations in individual subjects, and to determine whether LA treatment alters receptor expression and/or cAMP/PGE₂ response over time.

Sex-dependent differential PGE₂ response

Prostaglandins are involved in sex hormone signaling, and sex-dependent differential PGE₂ production, response and receptor expression has previously been reported [45-47], so differences between male and female PGE₂ levels are not unexpected. This provides a possible explanation for our result showing significantly lower PGE₂ concentrations in female, but not male RRMS subjects, compared to HC and SPMS. A recent study in rodent models supports this data, as female mice and rats with acute inflammation produced lower levels of PGE₂ than male rodents, with the most significant differences observed at 4 h post-inflammatory stimulus. These results were mirrored in complementary in vitro experiments

using human neutrophils, which demonstrated that cells from females produced significantly less PGE₂ than cells from males 4 h post-LPS stimulation [48]. It is therefore conceivable that sex could play a role in response to treatments that involve prostaglandin production, including LA.

Significance of changes in cAMP

The main effector of cAMP signaling is cAMP-dependent protein kinase (PKA), which is a ubiquitous, broad specificity kinase involved in the regulation of a diverse array of cellular events, including immunomodulation. The ubiquity of cAMP/PKA expression means that tight spatiotemporal regulation of its signaling is essential. Targeting of PKA activity is mediated by binding to a family of proteins called A-kinase anchoring proteins (AKAPs). Tethering of PKA to specific compartments by AKAPs allows PKA to be immediately available to phosphorylate localized substrates response to cAMP activation. AKAPs have been shown to simultaneously bind to PKA and other signal transduction molecules such as calmodulin, phosphodiesterases, phosphatases and other kinases. The co-anchoring of PKA and phosphodiesterases (PDEs) is especially important for localizing the effects of cAMP - while cAMP activates PKA, PDEs catalyze the hydrolysis of cAMP, providing signal termination (reviewed in [49]). For this reason, studies of cAMP signaling often require the use of pan-PDE inhibitor 3-Isobutyl-1-methylxanthine (IBMX) in order to identify changes in cAMP levels that would otherwise be too transient to detect [50]. The mechanistic implications of the relatively small changes seen in cAMP in this study are not known, but given the tightly regulated and targeted nature of cAMP signaling, and the fact that we did not inhibit PDEs in our analyses, the small changes we detected in overall cellular levels of cAMP may reflect an outsized effect on specific function; thus we believe that this study provides support for the hypothesis that cAMP is involved in the MOA of LA in SPMS patients. In future studies, it would be of interest to perform fluorescence resonance energy transfer (FRET) analyses with LA to more specifically track its effects on cAMP/PKA within immune cells.

TNF- α

Though TNF- α is known to be important in MS pathology, variable results from studies suggest it is not a good biomarker for disease; some reports show significant differences in TNF- α concentrations in CSF, PBMCs and/or plasma between healthy controls and MS subjects, while others do not. Additionally, some have reported correlations between increased TNF- α and relapse in RRMS, but again, the consistency and significance of the correlation is questionable [26,51-53]. Counterintuitively, TNF- α blockers have recently been associated with demyelination, even in patients with no prior history of MS [54]. In light of these conflicting reports, our finding that there are no significant differences in baseline TNF- α concentrations between HC and MS subjects that are in remission is not surprising. The complete lack of effect of LA on TNF- α concentrations in our study was somewhat unexpected, given that a variety of cell culture experiments have shown that pre-treatment with LA blocks inflammatory TNF- α production [55]. Several possibilities exist to explain the lack of effect. First, the effects may be cell type specific, and therefore may not be evident in a gross bodily measure such as plasma concentrations. Alternatively, the effects of LA on TNF- α may be indirect and downstream of its primary mechanism,

meaning that any suppression may take time to show up in bloodwork. Previous studies that appeared to show suppressed TNF- α concentration with LA treatment in conditions other than MS showed significance at 2 weeks or more of LA administration [56-58]. Further analyses after longer LA administration times would therefore be required to determine its effects on TNF- α in MS patients, though the importance of these analyses is questionable given the indications that TNF- α is not a reliable MS biomarker.

Conclusions

This study establishes for the first time that the bioavailability of LA in RRMS and SPMS patients is similar to HC subjects, indicating that the disease state does not alter the LA bioavailability. Additionally, we show that oral LA alters cAMP levels in vivo, stimulating an increase in HC and SPMS subjects, which significantly differed from the decrease seen in RRMS. Finally, we demonstrate that plasma PGE₂ concentrations are significantly lower in female RRMS subjects compared to HC and SPMS female subjects 4 h after LA ingestion. These data suggest a novel mechanistic difference between the MS subtypes and that cAMP is a candidate as a biomarker to determine therapeutic efficacy in SPMS, but not RRMS patients. Whether this indicates that LA will lack efficacy in the treatment of RRMS is not known; MS pathology is a result of both immune dysregulation and oxidative stress-induced damage, and since LA is an antioxidant (as well as an immunomodulator), it is possible that it could be therapeutic in RRMS via a different MOA compared to SPMS. Further laboratory and larger clinical studies will need to be conducted to fully elucidate the role of LA, PGE₂ and cAMP in both healthy and disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by a Clinical Science Research and Development Service Career Development Award and a Biomedical Laboratory Research and Development Service Merit Review (1I01BX001793), both from the United States Department of Veterans Affairs. The statistical analyses in this publication were supported by The Portland VA Research Foundation and by the Oregon Clinical and Translational Institute (OCTRI) National Institutes of Health (NIH) grant UL1TR000128 from the National Center for Advancing Translational Sciences (NCATS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Funding and Acknowledgements: This work was supported by a Clinical Science Research and Development Service Career Development Award and a Biomedical Laboratory Research and Development Service Merit Review (1I01BX001793), both from the United States Department of Veterans Affairs. The statistical analyses in this publication were supported by The Portland VA Research foundation and by the Oregon Clinical and Translational Institute (OCTRI) National Institutes of Health (NIH) grant UL1TR000128 from the National Center for Advancing Translational Sciences (NCATS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

We thank Dennis Koop at the OHSU Bioanalytical Shared Resource/Pharmacokinetics Core for measuring plasma LA concentrations. We also thank OCTRI, which provided clinical coordination, nursing services, and blood processing.

References

1. Morikawa T, Yasuno R, Wada H. Do mammalian cells synthesize lipoic acid? Identification of a mouse cDNA encoding a lipoic acid synthase located in mitochondria. *FEBS Lett.* 2001; 498(1):16–21. [PubMed: 11389890]
2. Marracci GH, Jones RE, McKeon GP, Bourdette DN. Alpha lipoic acid inhibits T cell migration into the spinal cord and suppresses and treats experimental autoimmune encephalomyelitis. *J Neuroimmunol.* 2002; 131(1-2):104–114. [PubMed: 12458042]
3. Wang KC, Tsai CP, Lee CL, Chen SY, Lin GJ, Yen MH, Sytwu HK, Chen SJ. alpha-Lipoic acid enhances endogenous peroxisome-proliferator-activated receptor-gamma to ameliorate experimental autoimmune encephalomyelitis in mice. *Clin Sci (Lond).* 2013; 125(7):329–340. DOI: 10.1042/CS20120560 [PubMed: 23550596]
4. Yadav V, Marracci G, Lovera J, Woodward W, Bogardus K, Marquardt W, Shinto L, Morris C, Bourdette D. Lipoic acid in multiple sclerosis: a pilot study. *Mult Scler.* 2005; 11(2):159–165. [PubMed: 15794388]
5. Chaudhary P, Marracci GH, Bourdette DN. Lipoic acid inhibits expression of ICAM-1 and VCAM-1 by CNS endothelial cells and T cell migration into the spinal cord in experimental autoimmune encephalomyelitis. *J Neuroimmunol.* 2006; 175(1-2):87–96. [PubMed: 16644024]
6. Chaudhary P, Marracci G, Galipeau D, Pocius E, Morris B, Bourdette D. Lipoic acid reduces inflammation in a mouse focal cortical experimental autoimmune encephalomyelitis model. *J Neuroimmunol.* 2015; 289:68–74. DOI: 10.1016/j.jneuroim.2015.10.011 [PubMed: 26616873]
7. Schreibelt G, Musters RJ, Reijerkerk A, de Groot LR, van der Pol SM, Hendriks EM, Dopp ED, Dijkstra CD, Drukarch B, de Vries HE. Lipoic acid affects cellular migration into the central nervous system and stabilizes blood-brain barrier integrity. *J Immunol.* 2006; 177(4):2630–2637. [PubMed: 16888025]
8. Khalili M, Egtesadi S, Mirshafiey A, Eskandari G, Sanoobar M, Sahraian MA, Motevalian A, Norouzi A, Moftakhar S, Azimi A. Effect of lipoic acid consumption on oxidative stress among multiple sclerosis patients: a randomized controlled clinical trial. *Nutr Neurosci.* 2014; 17(1):16–20. DOI: 10.1179/1476830513Y.0000000060 [PubMed: 23485514]
9. Spain R, Powers K, Murchison C, Heriza E, Wings K, Yadav V, Cameron M, Kim E, Horak F, Simon J, Bourdette D. Lipoic acid in secondary progressive MS: A randomized controlled pilot trial. *Neurol Neuroimmunol Neuroinflamm.* 2017; 4(5):e374.doi: 10.1212/NXI.0000000000000374 [PubMed: 28680916]
10. Schillace RV, Pisenti N, Pattamanuch N, Galligan S, Marracci GH, Bourdette DN, Carr DW. Lipoic acid stimulates cAMP production in T lymphocytes and NK cells. *Biochem Biophys Res Commun.* 2007; 354(1):259–264. doi:S0006-291X(06)02872-5 [pii] 10.1016/j.bbrc.2006.12.195. [PubMed: 17210133]
11. Kammer GM. The adenylate cyclase-cAMP-protein kinase A pathway and regulation of the immune response. *Immunol Today.* 1988; 9(7-8):222–229. [PubMed: 2855581]
12. Schillace RV, Carr DW. The role of protein kinase A and A-kinase anchoring proteins in modulating T-cell activation: progress and future directions. *Crit Rev Immunol.* 2006; 26(2):113–131. doi:1f4a494c5e662938,57266a947475235b [pii]. [PubMed: 16700649]
13. Torgersen KM, Vang T, Abrahamsen H, Yaqub S, Tasken K. Molecular mechanisms for protein kinase A-mediated modulation of immune function. *Cell Signal.* 2002; 14(1):1–9. [PubMed: 11747983]
14. Zidek Z. Adenosine - cyclic AMP pathways and cytokine expression. *Eur Cytokine Netw.* 1999; 10(3):319–328. [PubMed: 10477388]
15. Huber JD, Witt KA, Hom S, Egleton RD, Mark KS, Davis TP. Inflammatory pain alters blood-brain barrier permeability and tight junctional protein expression. *Am J Physiol Heart Circ Physiol.* 2001; 280(3):H1241–1248. [PubMed: 11179069]
16. Karczewski J, Groot J. Molecular physiology and pathophysiology of tight junctions III. Tight junction regulation by intracellular messengers: differences in response within and between epithelia. *Am J Physiol Gastrointest Liver Physiol.* 2000; 279(4):G660–665. [PubMed: 11005751]

17. Stelzner TJ, Weil JV, O'Brien RF. Role of cyclic adenosine monophosphate in the induction of endothelial barrier properties. *J Cell Physiol.* 1989; 139(1):157–166. [PubMed: 2540209]
18. Zhang Y, Huo M, Zhou J, Xie S. PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Comput Methods Programs Biomed.* 2010; 99(3):306–314. DOI: 10.1016/j.cmpb.2010.01.007 [PubMed: 20176408]
19. Yadav V, Marracci GH, Munar MY, Cherala G, Stuber LE, Alvarez L, Shinto L, Koop DR, Bourdette DN. Pharmacokinetic study of lipoic acid in multiple sclerosis: comparing mice and human pharmacokinetic parameters. *Mult Scler.* 2010; 16(4):387–397. DOI: 10.1177/1352458509359722 [PubMed: 20150394]
20. Amenta F, Traini E, Tomassoni D, Mignini F. Pharmacokinetics of different formulations of thioctic (alpha-lipoic) acid in healthy volunteers. *Clin Exp Hypertens.* 2008; 30(8):767–775. DOI: 10.1080/10641960802563568 [PubMed: 19021026]
21. Teichert J, Kern J, Tritschler HJ, Ulrich H, Preiss R. Investigations on the pharmacokinetics of alpha-lipoic acid in healthy volunteers. *Int J Clin Pharmacol Ther.* 1998; 36(12):625–628. [PubMed: 9876998]
22. Teichert J, Hermann R, Ruus P, Preiss R. Plasma kinetics, metabolism, and urinary excretion of alpha-lipoic acid following oral administration in healthy volunteers. *J Clin Pharmacol.* 2003; 43(11):1257–1267. DOI: 10.1177/0091270003258654 [PubMed: 14551180]
23. Keith DJ, Butler JA, Bemer B, Dixon B, Johnson S, Garrard M, Sudakin DL, Christensen JM, Pereira C, Hagen TM. Age and gender dependent bioavailability of R- and R, S-alpha-lipoic acid: a pilot study. *Pharmacol Res.* 2012; 66(3):199–206. DOI: 10.1016/j.phrs.2012.05.002 [PubMed: 22609537]
24. Hermann R, Mungo J, Cnota PJ, Ziegler D. Enantiomer-selective pharmacokinetics, oral bioavailability, and sex effects of various alpha-lipoic acid dosage forms. *Clin Pharmacol.* 2014; 6:195–204. DOI: 10.2147/CPAA.S71574 [PubMed: 25506250]
25. Salinthon S, Yadav V, Schillace RV, Bourdette DN, Carr DW. Lipoic acid attenuates inflammation via cAMP and protein kinase A signaling. *PLoS One.* 2010; 5(9)doi: 10.1371/journal.pone.0013058
26. van Oosten BW, Barkhof F, Scholten PE, von Blomberg BM, Ader HJ, Polman CH. Increased production of tumor necrosis factor alpha, and not of interferon gamma, preceding disease activity in patients with multiple sclerosis. *Arch Neurol.* 1998; 55(6):793–798. [PubMed: 9626770]
27. Salinthon S, Schillace RV, Tsang C, Regan JW, Bourdette DN, Carr DW. Lipoic acid stimulates cAMP production via G protein-coupled receptor-dependent and -independent mechanisms. *J Nutr Biochem.* 2011; 22(7):681–690. DOI: 10.1016/j.jnutbio.2010.05.008 [PubMed: 21036588]
28. Salinthon S, Schillace RV, Marracci GH, Bourdette DN, Carr DW. Lipoic acid stimulates cAMP production via the EP2 and EP4 prostanoid receptors and inhibits IFN gamma synthesis and cellular cytotoxicity in NK cells. *J Neuroimmunol.* 2008; 199(1-2):46–55. DOI: 10.1016/j.jneuroim.2008.05.003 [PubMed: 18562016]
29. Harbo HF, Gold R, Tintore M. Sex and gender issues in multiple sclerosis. *Ther Adv Neurol Disord.* 2013; 6(4):237–248. DOI: 10.1177/1756285613488434 [PubMed: 23858327]
30. Gleiter CH, Schug BS, Hermann R, Elze M, Blume HH, Gundert-Remy U. Influence of food intake on the bioavailability of thioctic acid enantiomers. *Eur J Clin Pharmacol.* 1996; 50(6):513–514. [PubMed: 8858282]
31. Yu Z, Kastenmuller G, He Y, Belcredi P, Moller G, Prehn C, Mendes J, Wahl S, Roemisch-Margl W, Ceglarek U, Polonikov A, Dahmen N, Prokisch H, Xie L, Li Y, Wichmann HE, Peters A, Kronenberg F, Suhre K, Adamski J, Illig T, Wang-Sattler R. Differences between human plasma and serum metabolite profiles. *PLoS One.* 2011; 6(7):e21230.doi: 10.1371/journal.pone.0021230 [PubMed: 21760889]
32. Montenarh D, Wernet MP, Hopf M, Maurer HH, Schmidt PH, Ewald AH. Quantification of 33 antidepressants by LC-MS/MS--comparative validation in whole blood, plasma, and serum. *Anal Bioanal Chem.* 2014; 406(24):5939–5953. DOI: 10.1007/s00216-014-8019-x [PubMed: 25023973]

33. Smith AR, Shenvi SV, Widlansky M, Suh JH, Hagen TM. Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. *Curr Med Chem*. 2004; 11(9):1135–1146. [PubMed: 15134511]
34. Markovic T, Jakopin Z, Dolenc MS, Mlinaric-Rascan I. Structural features of subtype-selective EP receptor modulators. *Drug Discov Today*. 2017; 22(1):57–71. doi:S1359-6446(16)30290-2 [pii] 10.1016/j.drudis.2016.08.003. [PubMed: 27506873]
35. Li G, Fu J, Zhao Y, Ji K, Luan T, Zang B. Alpha-lipoic acid exerts anti-inflammatory effects on lipopolysaccharide-stimulated rat mesangial cells via inhibition of nuclear factor kappa B (NF-kappaB) signaling pathway. *Inflammation*. 2015; 38(2):510–519. DOI: 10.1007/s10753-014-9957-3 [PubMed: 24962643]
36. Fernandez-Galilea M, Perez-Matute P, Prieto-Hontoria PL, Martinez JA, Moreno-Aliaga MJ. Effects of lipoic acid on lipolysis in 3T3-L1 adipocytes. *J Lipid Res*. 2012; 53(11):2296–2306. DOI: 10.1194/jlr.M027086 [PubMed: 22941773]
37. Brudvik KW, Tasken K. Modulation of T cell immune functions by the prostaglandin E(2) - cAMP pathway in chronic inflammatory states. *Br J Pharmacol*. 2012; 166(2):411–419. DOI: 10.1111/j.1476-5381.2011.01800.x [PubMed: 22141738]
38. Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, Sugimoto Y, Narumiya S. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med*. 2009; 15(6):633–640. DOI: 10.1038/nm.1968 [PubMed: 19465928]
39. Esaki Y, Li Y, Sakata D, Yao C, Segi-Nishida E, Matsuoka T, Fukuda K, Narumiya S. Dual roles of PGE2-EP4 signaling in mouse experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A*. 2010; 107(27):12233–12238. DOI: 10.1073/pnas.0915112107 [PubMed: 20566843]
40. Grigoriadis N, van Pesch V. A basic overview of multiple sclerosis immunopathology. *Eur J Neurol*. 2015; 22(2):3–13. DOI: 10.1111/ene.12798
41. Merrill JE, Gerner RH, Myers LW, Ellison GW. Regulation of natural killer cell cytotoxicity by prostaglandin E in the peripheral blood and cerebrospinal fluid of patients with multiple sclerosis and other neurological diseases. Part 1. Association between amount of prostaglandin produced, natural killer, and endogenous interferon. *J Neuroimmunol*. 1983; 4(3):223–237. [PubMed: 6189855]
42. Aberg JA, Demers LM, Romano PJ, Tenser RB. Prostaglandin production in chronic progressive multiple sclerosis. *J Clin Lab Anal*. 1990; 4(4):246–250. [PubMed: 2391581]
43. Zhang J, Rivest S. Distribution, regulation and colocalization of the genes encoding the EP2- and EP4-PGE2 receptors in the rat brain and neuronal responses to systemic inflammation. *Eur J Neurosci*. 1999; 11(8):2651–2668. doi:ejn682 [pii]. [PubMed: 10457163]
44. Ikegami R, Sugimoto Y, Segi E, Katsuyama M, Karahashi H, Amano F, Maruyama T, Yamane H, Tsuchiya S, Ichikawa A. The expression of prostaglandin E receptors EP2 and EP4 and their different regulation by lipopolysaccharide in C3H/HeN peritoneal macrophages. *J Immunol*. 2001; 166(7):4689–4696. [PubMed: 11254729]
45. Stapleton PP, Strong VE, Freeman TA, Winter J, Yan Z, Daly JM. Gender affects macrophage cytokine and prostaglandin E2 production and PGE2 receptor expression after trauma. *J Surg Res*. 2004; 122(1):1–7. doi:S0022-4804(04)00197-0 [pii] 10.1016/j.jss.2004.04.020. [PubMed: 15522307]
46. Hoffman JF, Wright CL, McCarthy MM. A Critical Period in Purkinje Cell Development Is Mediated by Local Estradiol Synthesis, Disrupted by Inflammation, and Has Enduring Consequences Only for Males. *J Neurosci*. 2016; 36(39):10039–10049. DOI: 10.1523/JNEUROSCI.1262-16.2016 [PubMed: 27683901]
47. Konopka CK, Glanzner WG, Rigo ML, Rovani MT, Comim FV, Goncalves PB, Morais EN, Antoniazzi AQ, Mello CF, Cruz IB. Responsivity to PGE2 labor induction involves concomitant differential prostaglandin E receptor gene expression in cervix and myometrium. *Genet Mol Res*. 2015; 14(3):10877–10887. DOI: 10.4238/2015.September.9.25 [PubMed: 26400315]
48. Pace S, Rossi A, Krauth V, Dehm F, Troisi F, Bilancia R, Weinigel C, Rummler S, Werz O, Sautebin L. Sex differences in prostaglandin biosynthesis in neutrophils during acute inflammation. *Sci Rep*. 2017; 7(1):3759. doi: 10.1038/s41598-017-03696-8 [PubMed: 28630405]

49. Wong W, Scott JD. AKAP signalling complexes: focal points in space and time. *Nat Rev Mol Cell Biol.* 2004; 5(12):959–970. [PubMed: 15573134]
50. Boularan C, Gales C. Cardiac cAMP: production, hydrolysis, modulation and detection. *Front Pharmacol.* 2015; 6:203.doi: 10.3389/fphar.2015.00203 [PubMed: 26483685]
51. Chofflon M, Juillard C, Juillard P, Gauthier G, Grau GE. Tumor necrosis factor alpha production as a possible predictor of relapse in patients with multiple sclerosis. *Eur Cytokine Netw.* 1992; 3(6): 523–531. [PubMed: 1296800]
52. Tejera-Alhambra M, Casrouge A, de Andres C, Seyfferth A, Ramos-Medina R, Alonso B, Vega J, Fernandez-Paredes L, Albert ML, Sanchez-Ramon S. Plasma biomarkers discriminate clinical forms of multiple sclerosis. *PLoS One.* 2015; 10(6):e0128952.doi: 10.1371/journal.pone.0128952 [PubMed: 26039252]
53. Domingues RB, Fernandes GBP, Leite F, Tilbery CP, Thomaz RB, Silva GS, Manguiera CLP, Soares CAS. The cerebrospinal fluid in multiple sclerosis: far beyond the bands. *Einstein (Sao Paulo).* 2017; 15(1):100–104. doi:S1679-45082017000100100 [pii] 10.1590/S1679-45082017RW3706. [PubMed: 28444098]
54. Kemanetzoglou E, Andreadou E. CNS Demyelination with TNF-alpha Blockers. *Curr Neurol Neurosci Rep.* 2017; 17(4):36.doi: 10.1007/s11910-017-0742-1 [PubMed: 28337644]
55. Maczurek A, Hager K, Kenkies M, Sharman M, Martins R, Engel J, Carlson DA, Munch G. Lipoic acid as an anti-inflammatory and neuroprotective treatment for Alzheimer's disease. *Adv Drug Deliv Rev.* 2008; 60(13-14):1463–1470. DOI: 10.1016/j.addr.2008.04.015 [PubMed: 18655815]
56. Zhang Y, Han P, Wu N, He B, Lu Y, Li S, Liu Y, Zhao S, Liu L, Li Y. Amelioration of lipid abnormalities by alpha-lipoic acid through antioxidative and anti-inflammatory effects. *Obesity (Silver Spring).* 2011; 19(8):1647–1653. DOI: 10.1038/oby.2011.121 [PubMed: 21593803]
57. Nasole E, Nicoletti C, Yang ZJ, Girelli A, Rubini A, Giuffreda F, Di Tano A, Camporesi E, Bosco G. Effects of alpha lipoic acid and its R+ enantiomer supplemented to hyperbaric oxygen therapy on interleukin-6, TNF-alpha and EGF production in chronic leg wound healing. *J Enzyme Inhib Med Chem.* 2014; 29(2):297–302. DOI: 10.3109/14756366.2012.759951 [PubMed: 23360079]
58. Sardu C, Santulli G, Santamaria M, Barbieri M, Sacra C, Paolisso P, D'Amico F, Testa N, Caporaso I, Paolisso G, Marfella R, Rizzo MR. Effects of Alpha Lipoic Acid on Multiple Cytokines and Biomarkers and Recurrence of Atrial Fibrillation Within 1 Year of Catheter Ablation. *Am J Cardiol.* 2017; 119(9):1382–1386. doi:S0002-9149(17)30139-X [pii] 10.1016/j.amjcard.2017.01.040. [PubMed: 28258730]

Fig. 1. Study flow diagram

N=number of subjects in each group

Fig. 2. Plasma lipoic acid concentration-time profiles

Data points are mean plasma LA concentrations for healthy control (HC, N=19), relapsing remitting (RRMS, N=21), and secondary progressive (SPMS, N=15) subjects. Standard error is indicated by bars

Fig. 3. Cyclic adenosine monophosphate (cAMP) response of peripheral blood mononuclear cells (PBMCs) at three time points relative to lipoic acid treatment

Bold symbols are the estimated mean cAMP level at each time point and the lines represent the estimated change in cAMP between time points for each subject group: healthy control (HC, N=19), relapsing remitting (RRMS, N=19), and secondary progressive (SPMS, N=16). Mean cAMP levels and estimated changes in cAMP were estimated from general estimating equations. The range of the y-axis has been truncated in order to display changes in cAMP over time. Full graph is available as Supplemental Figure 1

Fig. 4. Plasma prostaglandin E2 (PGE₂) concentrations

PGE₂ enzyme-linked immunosorbent assays (ELISAs, Enzo Life Sciences) were performed on plasma from the same healthy control (HC, N=19), relapsing remitting (RRMS, N=21), and secondary progressive (SPMS, N=15) subjects as in Fig. 2 according to manufacturer's instructions. Results were analyzed in aggregate (top panel), as well as by sex (middle panel females, bottom panel males). Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; crosses represent sample means. These graphs were generated by BoxPlotR: a web-tool for

generation of box plots. * indicates significant differences ($p < 0.05$) between 4 h samples from HC and RRMS donors and between SPMS and RRMS donors. # indicates an outlier value not pictured on the chart due to the chosen graph scale: 4738.9 pg/ml PGE₂ for a baseline value of one SPMS subject

Fig. 5. Model of in vitro LA, PGE₂, and cAMP interactions

In this figure, arrow heads indicate stimulation, and circles indicate inhibition. A. LA and PGE₂ can each bind to and activate EP2/EP4 receptors, which in turn stimulates production of cAMP. Dual stimulation has a synergistic effect on cAMP production (dashed arrows). Conversely, in certain cell types, LA has been shown to suppress PGE₂ production, which has a negative effect on cAMP production via EP2/EP4 receptors. B. PGE₂ binds to and activates EP3 receptors, which inhibit cAMP production. In this case, LA suppression of PGE₂ would result in higher cAMP levels, since less activation of EP3 receptors by PGE₂ would lead to a reduction in inhibition of cAMP production by EP3. The nature of the LA/EP3 receptor relationship is unknown

Table 1

Subject demographics.

	Healthy Control	Relapsing remitting	Secondary Progressive
Number in group	20	21	16
Gender, M/F	8/12	7/14	6/10
Age (years)			
Median	49.5	51	57.5
Range	26-65	29-64	39-69
Race			
Caucasian	19	20	16
Non-Caucasian	1	1	0
Taking disease-modifying therapy	NA	14 ^a	5 ^b

^a Interferon beta-1a: 7 subjects, glatiramer acetate: 3 subjects, fingolimod: 2 subjects, natalizumab: 2 subjects

^b Interferon beta-1a: 2 subjects, glatiramer acetate: 3 subjects

Table 2

Lipoic acid pharmacokinetic parameters after a 1200 mg oral dose.

PK parameters	Healthy Control N=19	Relapsing remitting N=21	Secondary progressive N=15
Half-life (h)	4.76 ± 0.44 ^a	4.02 ± 0.19	4.11 ± 0.17
	3.57-10.9 ^b	0.48-4.88	2.41-5.13
	4.01 ^c	4.12	4.04
T _{max} (h)	1.79 ± 0.21	1.81 ± 0.2	1.53 ± 0.19
	1-4	1-4	1-3
	2	2	1
C _{max} (ng/mL)	1985.74 ± 301.74	1894.52 ± 304.98	2110.4 ± 350.55
	685-6220	500-5230	704-4870
	1700	1380	1750
AUC _{0-∞} (ng/ml*h)	7736.19 ± 1502.14	5338.74 ± 496.63	6100.15 ± 844.28
	2571.34-30585.91	1311.35-9905.21	1900.45-15111.9
	5815.11	5320.16	6045.38
Volume of distribution (mg)/(ng/ml)	1.75 ± 0.36	1.76 ± 0.29	1.57 ± 0.27
	0.21-6.82	0.1-6.44	0.41-4.25
	1.19	1.33	1.16
Oral clearance (mg)/(ng/ml)/h	0.23 ± 0.03	0.29 ± 0.04	0.26 ± 0.04
	0.04-0.47	0.12-0.92	0.08-0.63
	0.21	0.23	0.2

^aMean ± standard error,^brange,^cmedian.

Table 3

Median cAMP levels (interquartile range) at each time point by subject group.

	Baseline	Hour 2	Hour 4
Control	91.9 (64.6, 123.1) ^a	96.6 (75.1, 129.2)	103.5 (63, 149.6)
RRMS	98.2 (78.7, 131.8)	75.8 (56.8, 113)	79.1 (59.9, 108.2)
SPMS	67.7 (42.3, 101.6)	75.9 (46, 101.5)	72.6 (56.8, 90.1)

^a values are pmol cAMP/mg protein.

Table 4

Estimated *change* in cAMP overall and between consecutive time points by subject group.^a

Overall: Baseline to Hour 4				
	cAMP ₄ /cAMP ₀ (95% CI)			p-value ^b
RRMS vs. Control	0.78 (0.64, 0.97)	vs	1.11 (0.9, 1.37)	.0204
SPMS vs. Control	1.20 (0.96, 1.5)	vs	1.11 (0.9, 1.37)	.6168
RRMS vs. SPMS	0.78 (0.64, 0.97)	vs	1.20 (0.96, 1.5)	<.001
Baseline to Hour 2				
	cAMP ₂ /cAMP ₀ (95% CI)			p-value ^b
RRMS vs. Control	0.76 (0.62, 0.94)	vs	1.09 (0.89, 1.34)	.0175
SPMS vs. Control	1.28 (1.03, 1.6)	vs	1.09 (0.89, 1.34)	.2854
RRMS vs. SPMS	0.76 (0.62, 0.94)	vs	1.28 (1.03, 1.6)	<.001
Hour 2 to Hour 4				
	cAMP ₄ /cAMP ₂ (95% CI)			p-value ^b
RRMS vs. Control	1.03 (0.83, 1.26)	vs	1.02 (0.83, 1.26)	.9870
SPMS vs. Control	0.94 (0.75, 1.17)	vs	1.02 (0.83, 1.26)	.5795
RRMS vs. SPMS	1.03 (0.83, 1.26)	vs	0.94 (0.75, 1.17)	.5653

^a cAMP was log-transformed to achieve normality; thus the difference between two time points within a group, or *change*, is a ratio of the two time points and is interpreted as 'for each unit change in time, median cAMP levels increase by a factor of X'. For instance, cAMP increased by a factor of 1.11 or 11% from pre-treatment to hour 4 in controls.

^b p-value is for the difference in change between two groups or the difference-in-differences.