ONLINE SUPPLEMENT

Conjugated Linoleic Acid Modulates Clinical Responses to Oral Nitrite and Nitrate

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Short Title: Fatty Acids Modulate Responses to Nitrogen Oxides

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Recruitment of subjects. Subjects were randomized by The University of Pittsburgh Medical Center Investigational Drug Pharmacy Service. Subjects were recruited from local advertisements, the University of Pittsburgh Research Participant Registry and the ClinicalTrials.gov website (NCT01681836). Subjects' exclusion criteria included: 1) positive urine pregnancy test; 2) recent addition or change in dosing of birth control (pills, shot or intrauterine device); 3) concurrent use of medications affecting glucose or lipid metabolism; 4) current use of BP medications regardless of BP control; 5) current use of PDE5 inhibitors or organic nitrates; 6) not stable on treatments for the prior three months or not planning to remain on current dose of medications for birth control, etc.; 7) chronic mental health or medical conditions including diabetes, obesity syndromes, liver or kidney disease; 8) active smoker. Subjects were asked to avoid strenuous activity and mouthwash 24-hr prior to and throughout each 24-hr PK study, based on possible effects on BP, the generation of various secondary oxides of nitrogen (NOx) and reduction of the oral commensal bacteria. No subjects in the trials took antacids or non-steroidal anti-inflammatory drugs that could impact gastric pH or platelet function prior to study entry, throughout the 24-hr PK studies or during the washout periods. Subjects were also asked to follow a low NO_3^-/NO_2^- diet in both trials in addition to a low cLA diet in Trial 2 until their 24-hr PK assessment was complete.

Blood pressure methods. Single automatic blood pressures using a Dinamap DPC200X (GE Medical Systems Information Technology, Milwaukee, WI) and an appropriately sized cuff based on subject's upper arm circumference were performed by one of two trained medical staff throughout the study visits. Subjects were in the supine position with legs uncrossed and sitting upright in a hospital bed in a quiet room throughout the study visits. The blood pressure cuff was placed on the arm opposite the IV and both arms remained in a relaxed position at the subject's side.

Drug formulation. Total Lean™ cLA capsules (1 g) were purchased from General Nutrition Company (Pittsburgh, PA). The capsules were made from natural safflower oil and are a mixture of two isomers: cis-9, trans-11 and trans-10, cis-12 cLA, present in a 50:50 ratio. The ^{15}N isotopes, NO_3^- and NO_2^-, were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). The $Na^{15}NO_3$ and $Na^{15}NO_2$ capsules were formulated by the NIH Pharmaceutical Development Section. Each isotope was milled separately to provide uniformity in particle size with the needed excipients to ensure uniform powder mixture content and controlled disintegration and dissolution characteristics. NO_2^- was mechanically milled in a mini blender, passed through a mesh screen and then microcrystalline cellulose, pregelatinized starch, sodium starch glycolate, crospovidone and colloidal silicon dioxide were added sequentially. The mixture was milled again in a blender and passed several times through a mesh screen producing an approximate particle size of 425 micrometers. NO_3^- was mixed with all the same excipients used for NO_2^-, mechanically milled and passed through a mesh screen producing an approximate particle size of 250 micrometers. Uniformity of capsule strength was affected by use of a mini cap-100 capsule filler making full fill capsules of each salt form. The quality and stability of the capsules were measured by stability-indicating high performance liquid chromatography assays. All operations were performed in a good manufacturing practice pharmaceutical manufacturing facility with chemical analysis performed on site.

Drug Dose Selection: $Na^{15}NO_3$ and $Na^{15}NO_2$: Based on the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives Acceptable Daily Intake (ADI) for sodium nitrate and nitrite[1], recent modeling of the fruit and vegetable consumption patterns of The Dietary Approaches to Stop Hypertension diet[2], and the recently observed human and rodent effects of NO_3^- and NO_2^- on BP[3-5], the study investigators hypothesized a starting safe and likely therapeutically effective dose of $Na^{15}NO_3$ was 1,000 mg (11.8 mmol) and of $Na^{15}NO_2$ was 20 mg (0.29 mmol). The $Na^{15}NO_3$ and $Na^{15}NO_2$ dose selections were equivalent to 2.1 times the ADI. cLA: Published studies have supplemented cLA at doses ranging 0.7-6.8 g/day in healthy and overweight subjects for up to 1 year with little to no adverse side effects[6]. Given that mean estimated daily cLA intake is up to 2.6 g per day[7, 8] and

commercial cLA comes as 1g capsules, our study team estimated that a safe, readily available and therapeutically effective dose of cLA was 3 g.

Plasma NO_3^-, NO_2^- and RS-NO measurements. Blood was collected from an intravenous catheter into a plastic heparinized syringe containing 5 μL of heparin (5,000 units/mL) per mL of whole blood to limit NO_2^- contamination and to prevent clotting. Blood was immediately added to eppendorf tubes with a whole blood NO_2^- stabilizing solution containing 0.8 M ferricyanide (an oxidizing agent), 10 mM N-ethylmaleimide (NEM; a thiol alkylating agent), and 1% NP-40 (a cytolytic agent to provide access of the ferricyanide and NEM to the red cell contents) in a 5:1 dilution (vol:vol; whole blood-stop solution) and then flash frozen. A separate aliquot of blood was immediately centrifuged (15,000 x *g* for 2 minutes) to separate the plasma, as previously described[9]. All samples were frozen at the bedside and stored at -80˚C. Samples were thawed and analyzed for total (^{15}N + ^{14}N) NO_2^- and RS-NO concentrations using triiodide-based reductive chemiluminescence using Nitric Oxide Analyzer (Sievers) in the presence and absence of acidified sulphanilamide and mercuric chloride, and total (^{15}N + ^{14}N) NO_3^- measured by vanadium chloride based reductive chemiluminescence, as previously described[10, 11].

Red blood cell ^{15}NO-Hb measurement. Blood was collected into a plastic heparinized syringe (as above) and immediately centrifuged to isolate the red blood cell (RBC) pellet. Pellet samples were frozen at the bedside and stored at -80˚C. RBC pellets were analyzed at baseline, 0.5 and 1 hr for ^{15}NO-Hb by EPR spectroscopy. RBC pellets were thawed and mixed 1:1 with a solution of 40 mM inositol hexaphosphate (IHP) in order to stabilize the penta-coordinated α-hemoglobin chain responsible for NO-Hb formation used to characterize the hyperfine EPR features that distinguish ^{14}NO-Hb from ^{15}NO-Hb[12-14]. IHP was prepared by dissolving inositol hexaphosphate in PBS and titrating the solution with NaOH to pH 7.2. The samples were frozen in crystal EPR tubes in liquid nitrogen. EPR spectra were collected as previously reported[15, 16] on a Bruker EMX spectrometer operating at 9.4 GHz, 5-G modulation, 10.1-milliwatt power, 328-ms time constant, and 164-s scan over 600 G at 110 K. Each sample was scanned 8 times and averaged and then this was repeated 8 times so that each final scan is an average of 64 average scans. Basis spectra for known concentrations of ^{15}NO-Hb were prepared by mixing deoxyhemoglobin with excess $^{15}NO_2^-$ in a solution containing 20 mM IHP. Basis spectra for ^{14}NO-Hb pentacoordinate alphanitrosyl, hexacoordinate alphanitrosyl and betanitrosyl Hb were obtained from previous preparations and previously described[17]. The sample spectra were fit to these basis spectra by a least-squares regression, and the concentrations of ^{15}NO-Hb were determined from the resulting double integrals. EPR spectra from collected blood samples were fit to betanitrosyl, hexacoordinate alphanitrosyl, and either ^{14}NO or ^{15}NO pentacoordinate alphanitosyl Hb or both of these pentacoordinate species. ^{15}NO-Hb concentrations less than 1 μM were considered below the detection threshold of this method.

Plasma $^{15}NO_2$-cLA measurement. Blood was drawn into a sodium heparin tube and centrifuged to obtain plasma. Plasma was collected and stored in aliquots and frozen within 1 hr to avoid instability of $^{15}NO_2$-cLA in aqueous environments. Plasma aliquots were thawed and lipids were extracted in triplicate for quantitative stable isotope dilution LC-MS/MS analysis using a triple quadrupole mass spectrometer in negative ion mode (API 5000, Applied Biosystems, Framingham, MA) as previously reported[18, 19]. Sample extracts were separated with a C18 reversed phase column. Multiple reaction monitoring (MRM) transitions were used for detection of $^{14}NO_2$-cLA, $^{15}NO_2$-cLA and the internal standard, [$^{13}C_{18}$]-NO_2-OA. Quantification of NO_2-cLA levels was determined using a calibration curve with synthetic NO_2-cLA standard and [$^{13}C_{18}$]-NO_2-OA.

Platelet activation. Fasting blood was collected at time 0, 6 and 24 hr after cLA and ^{15}N study drug dosing. Platelet activation was examined in whole blood at baseline, 6 and 24 hr after each study drug dosing. This was accomplished by adding 2 antibodies to the prepped samples, anti-CD41, a platelet specific antibody, and anti-CD62 antibody, a marker of activated platelets, and thrombin as the positive control. The cells were fixed with 1% paraformaldehyde and the percentage of platelets positive for CD41

(Becton Dickinson), representing activated fibrinogen (GPIIb/IIIa) receptors specific to platelets, or CD62-P (Becton Dickinson), representing the surface expression of P-selectin, a marker of platelet α-granule release with activation, was quantified by flow cytometry.

Supplemental materials references

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Table S1. Trial 1 participant's characteristics

Parameter	n = 10
Age (years)	30.4 ± 2.1
Sex (Male/Female)	5/5
Race (Caucasian/Asian)	9/1
Weight (kg)	75 ± 4
Height (cm)	173 ± 4
BMI (kg/M^2)	25 ± 1
Hemoglobin (g/dL)	13.5 ± 0.4
Hematocrit (%)	39.8 ± 1
Creatinine (mg/dL)	0.8 ± 0.1
Washout period (days)	7.1 ± 0.6
Baseline hemodynamics	
SBP (mmHg)	122 ± 2
DBP (mmHg)	75 ± 2
MAP (mmHg)	91 ± 2

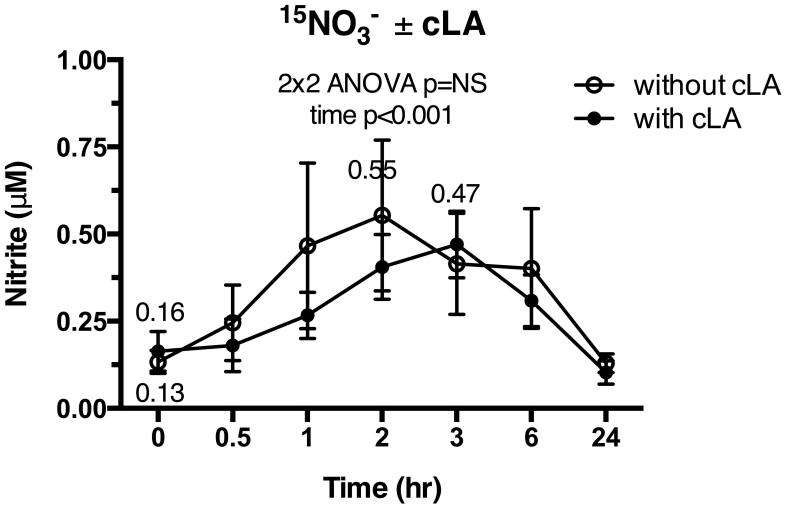

Figure S1: Plasma NO_2^- without and with cLA. When plasma NO_2^- concentrations were examined over time following $^{15}NO_3^-$ treatment alone (open circles) compared to $^{15}NO_3^-$ with cLA (closed circles), no significant differences in plasma NO_2^- concentrations were obsesrved. 2x2 repeated measures ANOVA with time as the within-subject effect and trial drug (without vs. with cLA) as the between subject effect was used to compare the endpoint measure between Trial 1 and Trial 2.

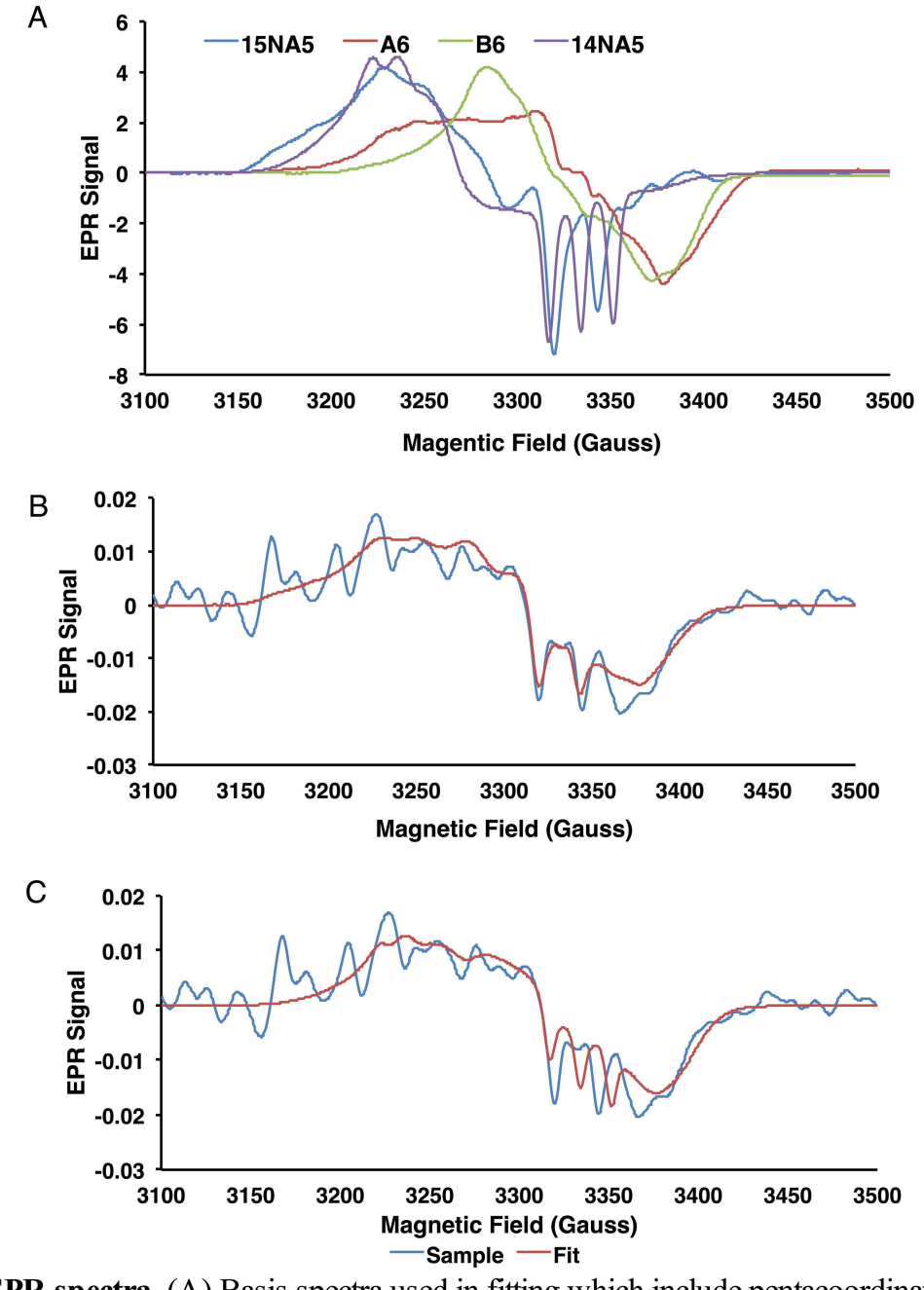

Figure S2: Spectral deconvolution of EPR spectra. (A) Basis spectra used in fitting which include pentacoordinate alphanitrosyl ^{14}NO-Hb (14NA5), pentacoordinate alphanitrosyl ^{15}NO-Hb (15NA5), hexacoordinate alphanitrosyl NO-Hb (A6), and betanitrosyl NO-Hb (B6). (B) Representative fit using all four basis spectra. Fit is 38% 15NA5, 4% 14NA5, 20% A6, and 38% B6. The sum of the squares of differences between the fit and the data were 0.00538 which is only slightly better than the fit shown in Fig 3C that does not include ^{14}NO-Hb or 14NA5 (which is 0.00541). (C) Representative fit not including ^{15}NO-Hb or 15NA5. Fit is 35% 14NA5, 27% A6, and 38% B6. The sum of the squares of differences between the fit and the data was 0.0083, considerably worse than when 15NA5 is included in the fit, and the fit clearly fails at the regions of the hyperfine structure.

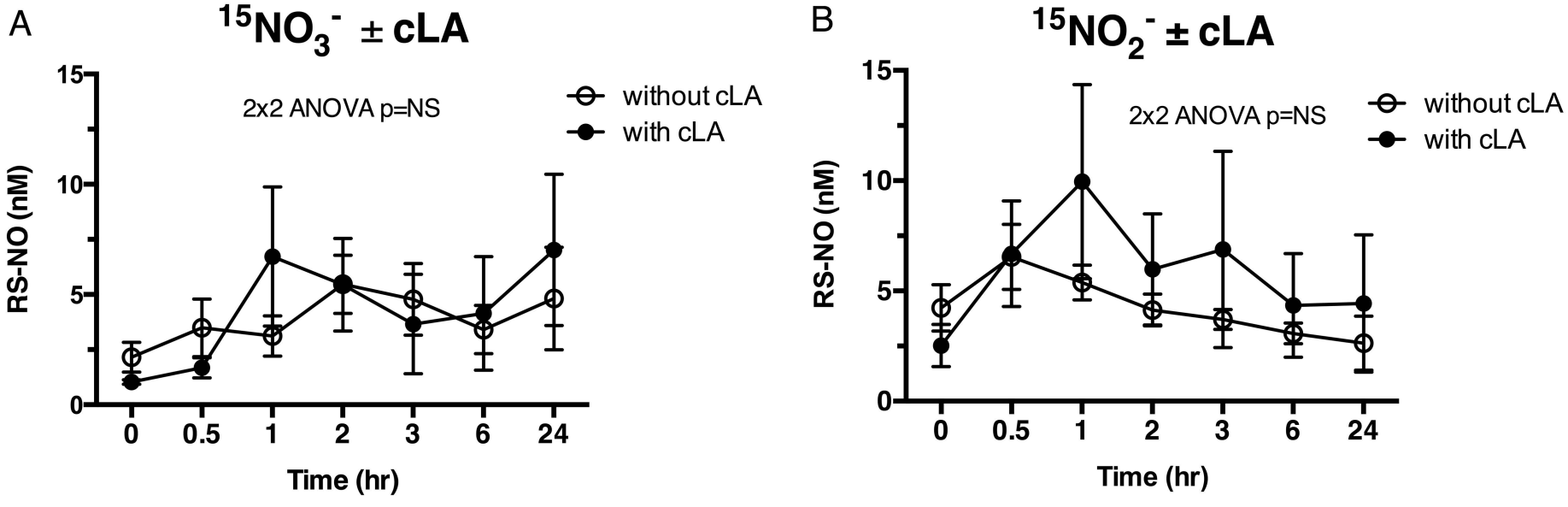

Figure S3: RS-NO formation without and with cLA. No differences in RS-NO concentrations were observed over time between the two treatments with $^{15}NO_3^-$ (A) or $^{15}NO_2^-$ (B) alone (open circles) vs. $^{15}NO_3^-$ or $^{15}NO_2^-$ in concert with cLA (closed circles). 2x2 repeated measures ANOVA with time as the within-subject effect and trial drugs(s) (without vs. with cLA) as the between subject effect was used to compare the endpoint measure between Trial 1 and Trial 2.

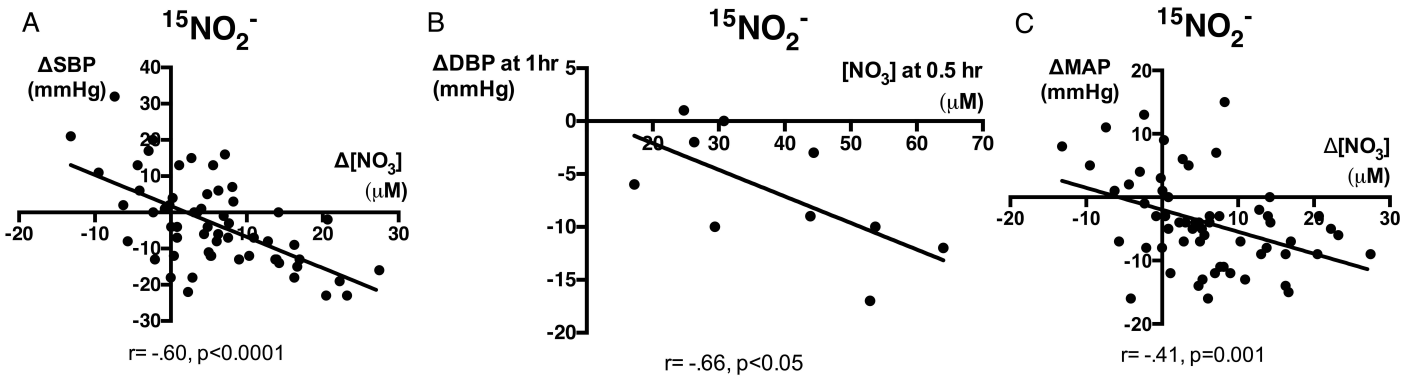

Figure S4: Correlations of change in BP with plasma NO_3^- concentrations. With $^{15}NO_2^-$ administration, the greater the reduction in SBP (A) and MAP (C), the greater the increase in $^{15}NO_3^-$ concentrations, and the greater the reduction in DBP at 1 hr, the greater the $^{15}NO_3^-$ concentration at 0.5 hr (B). Pearson correlation (r) was performed for BP measures.

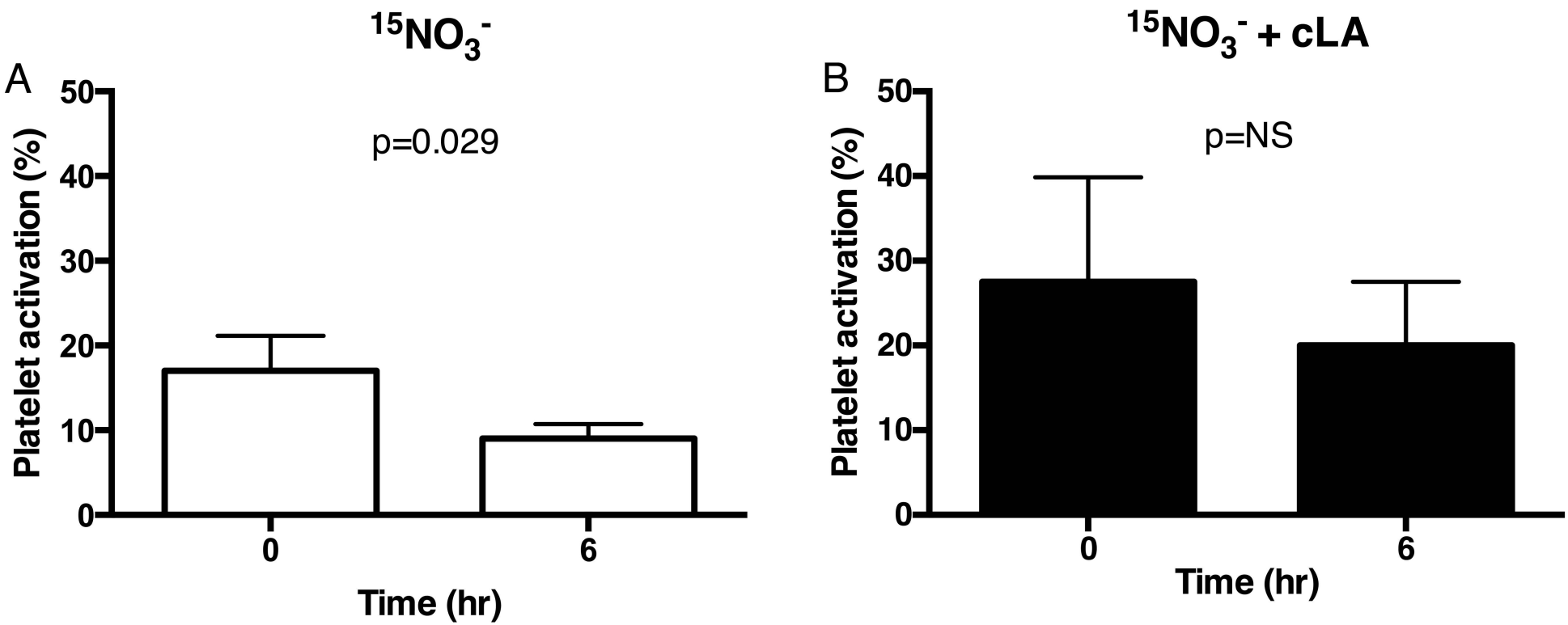

Figure S5: Platelet activation effect with $^{15}NO_3^-$ dosing without and with cLA.
Following $^{15}NO_3^-$ dosing in all 10 subjects from Trial 1, significant inhibition of platelet activation was seen at 6 hr (A). With co-administration of $^{15}NO_3^-$ and cLA in Trial 2, there was no significant inhibition of platelet activation in whole blood at 6 hr (B). Paired t-tests were used to compare baseline and 6 hr platelet activation responses in Trials 1 and 2.

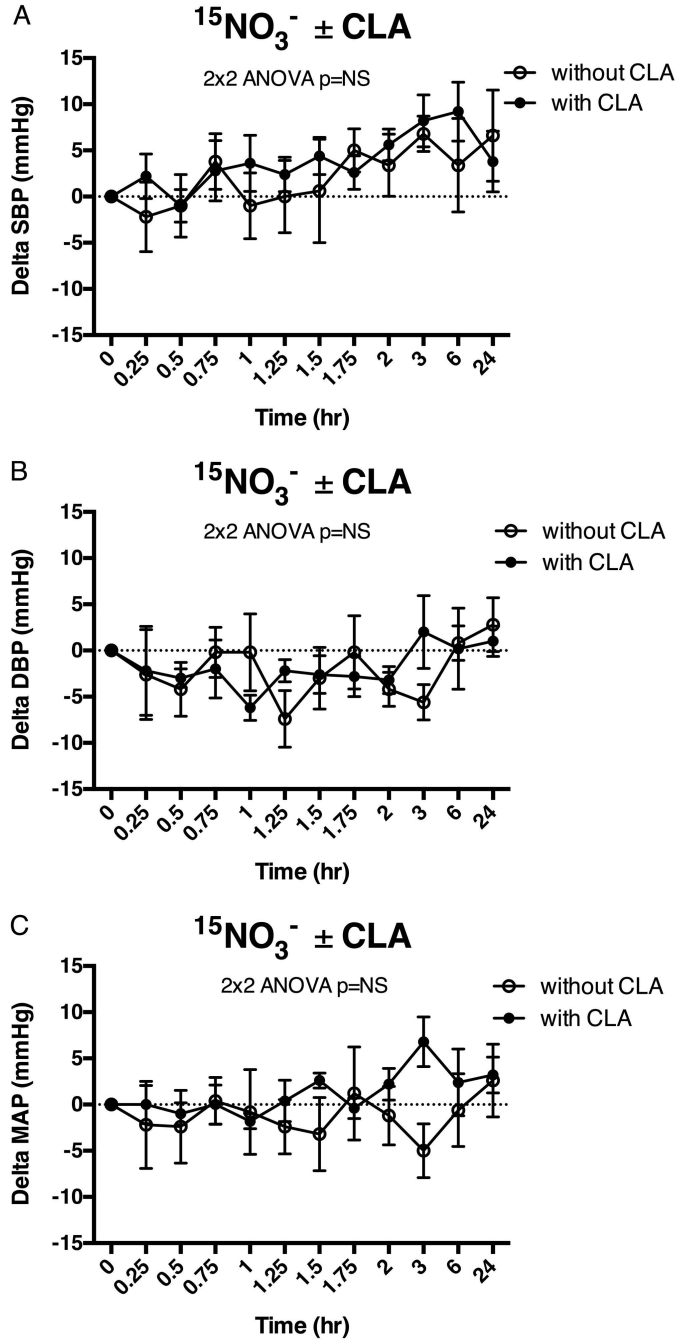

Figure S6: Physiological effects and NO_3^- signaling without and with cLA. In Trial 1, oral $^{15}NO_3^-$ dosing induced a small reduction in DBP at 3 hr compared to baseline (B, open circles) with no significant change in SBP and MAP (A and C, open circles). Following $^{15}NO_3^-$ + cLA dosing, there were no significant changes in SBP, DBP and MAP (A, B, and C, closed circles). 2x2 repeated measures ANOVA with time as the within-subject effect and trial drug(s) (without vs. with cLA) as the between subject effect was used to compare the endpoint measures between Trial 1 and Trial 2 in A-C.