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# Conjugated Linoleic Acid Modulates Clinical Responses to Oral Nitrite and Nitrate

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## Abstract

Dietary nitrate ($NO_3^-$) and nitrite ($NO_2^-$) support nitric oxide ($^{\cdot}NO$) generation and downstream vascular signaling responses. These nitrogen oxides also generate secondary nitrosating and nitrating species that react with low molecular weight thiols, heme centers, proteins and

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unsaturated fatty acids. To explore the kinetics of $NO_3^-$ and $NO_2^-$ metabolism and the impact of dietary lipid on nitrogen oxide metabolism and cardiovascular responses, the stable isotopes $Na^{15}NO_3$ and $Na^{15}NO_2$ were orally administered in the presence or absence of conjugated linoleic acid (cLA). The reduction of $^{15}NO_2^-$ to $^{15}NO$ was indicated by electron paramagnetic resonance spectroscopy detection of hyperfine splitting patterns reflecting $^{15}NO$-deoxyhemoglobin complexes. This formation of $^{15}NO$ also translated to decreased systolic and mean arterial blood pressures and inhibition of platelet function. Upon concurrent administration of cLA, there was a significant increase in plasma cLA nitration products 9- and 12-$^{15}NO_2$-cLA. Co-administration of cLA with $^{15}NO_2^-$ also impacted the pharmacokinetics and physiological effects of $^{15}NO_2^-$, with cLA administration suppressing plasma $NO_3^-$ and $NO_2^-$ levels, decreasing $^{15}NO$-deoxyhemoglobin formation, $NO_2^-$ inhibition of platelet activation, and the vasodilatory actions of $NO_2^-$, while enhancing the formation of 9- and 12-$^{15}NO_2$-cLA. These results indicate that the biochemical reactions and physiologic responses to oral $^{15}NO_3^-$ and $^{15}NO_2^-$ are significantly impacted by dietary constituents such as unsaturated lipids. This can explain the variable responses to $NO_3^-$ and $NO_2^-$ supplementation in clinical trials and reveals dietary strategies for promoting the generation of pleiotropic nitrogen oxide-derived lipid signaling mediators.

### Keywords

nitrate; nitrite; nitric oxide; conjugated linoleic acid; pharmacokinetics; blood pressure; platelet activation

## Introduction

Inorganic nitrite ($NO_2^-$) undergoes reactions that yield vasodilatory products that contribute to blood pressure regulation and hypoxic signaling[1, 2]. The entero-salivary microbiome-mediated reduction of $NO_3^-$ to $NO_2^-$ and $^{\cdot}NO$ in mammals also promotes vascular relaxation and other pleiotropic pharmacologic responses[3–6]. These reactions of $NO_3^-$/$NO_2^-$/$^{\cdot}NO$ both complement L-arginine/$^{\cdot}NO$ synthase/$^{\cdot}NO$/cGMP signaling and expand the spectrum of nitrogen oxide intermediates that instigate signaling responses beyond the activation of guanylate cyclase. This new understanding contrasts with the previous perspective that tissue $NO_3^-$ and $NO_2^-$ represented potentially toxic and relatively stable $^{\cdot}NO$ oxidation products.

There are an abundance of metabolic and inflammatory reactions that $NO_3^-$ and $NO_2^-$ undergo that can differentially impact downstream signaling responses[1, 2, 4] (Figure 1A). While $NO_3^-$ is formed upon the oxidation of $^{\cdot}NO$ by oxyhemoglobin, much greater endogenous $NO_3^-$ levels are achieved upon the ingestion of dietary $NO_3^-$ sources, including leafy green vegetables, roots (e.g., beets) and fruits[7]. Once consumed, $NO_3^-$ is readily reduced to $NO_2^-$ by commensal oral bacterial nitrate reductase activities[8]. While the majority of $NO_3^-$ is reduced in the oral cavity by commensal bacteria, xanthine oxidoreductase can reduce $NO_3^-$ to $NO_2^-$[9]. Up to 25% of plasma $NO_3^-$ can be stored and concentrated by the salivary glands, thus providing a facile route for entero-salivary recycling[10–13]. Also acquired from both endogenous $^{\cdot}NO$ oxidation reactions and dietary sources, the more intrinsically-reactive $NO_2^-$ can undergo multiple reactions such as

protonation to nitrous acid ($HNO_2$) in the low pH environments of the GI tract, inflammatory foci and the mitochondrial intermembrane compartment. Nitrite is also absorbed into the circulation and tissues where it can react with a) deoxyhemoglobin and other heme proteins, as well as molybdopterin-containing enzymes to form $^{\cdot}NO$ [14, 15,16, 17] or b) heme peroxidases to yield the nitrating species nitrogen dioxide ($^{\cdot}NO_2$)[18]. During intermediary metabolism and inflammatory responses, partially reduced oxygen species such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) and lipid peroxyl radicals will also undergo a variety of reactions with $^{\cdot}NO$ and $NO_2^-$ to accelerate rates of production of nitrosating and nitrating species[19.] $^{\cdot}$Nitric oxide, $NO_2^-$ and $HNO_2$ will also undergo a variety of reactions that lead to the generation of symmetric and asymmetric dinitrogen trioxide (ONONO and OONNO)[20,21]. These unstable intermediates rapidly decay to yield secondary nitrosating (addition of $^{\cdot}NO$) and nitrating (addition of $^{\cdot}NO_2$) species, as well as $^{\cdot}NO$. These and other redox reactions are responsible for propagating downstream signaling and pathogenic responses that are a consequence of oxidative, nitrosative and nitrative post-translational protein modification (PTM) reactions.

Human studies and animal models reveal that up to 15 day courses of inorganic and dietary $NO_3^-$ and $NO_2^-$ administration induce robust responses that include decreased blood pressure (BP)[1, 2, 11, 22], hypoxic vasodilation[1, 23, 24], modulation of mitochondrial function under hypoxic or exercise stress[25, 26], prevention of endothelial dysfunction[27] and inhibition of platelet aggregation[28, 29]. It remains uncertain as to whether these effects are mediated by $^{\cdot}NO$, secondary nitrating and nitrosating products or a combination thereof that will induce functionally-significant PTMs such as thiol S-nitrosation or thiol alkylation[30, 31].

One class of of nitrogen oxide metabolites are electrophilic fatty acid nitroalkene derivatives ($NO_2$-FA)[32], formed by radical addition reactions of $^{\cdot}NO_2$ with alkenyl carbons of unsaturated fatty acids. Once formed, these lipid electrophiles rapidly react with the nucleophilic amino acids Cys, and to a lesser extent His, residues via reversible Michael addition[33]. Endogenously present at low nM concentrations in healthy human plasma and urine, the rates and extents of production of $NO_2$-FA can be increased by an array of metabolic and inflammatory-related nitration reactions (Fig. 1C)[34]. Upon the PTM of functionally-significant hyperreactive Cys residues in critical enzymes and transcriptional regulatory proteins, $NO_2$-FA influence gene expression and inflammatory responses[35]. For example, specific pro-inflammatory and blood pressure regulation enzymes are directly inhibited by $NO_2$-FA, including xanthine oxidoreductase, cyclooxygenase-2 and soluble epoxide hydrolase (sEH)[36–38]. Notably, $NO_2$-FA mediate pleiotropic signaling actions: this includes the PTM of p65 inhibit nuclear factor-κB to inhibit pro-inflammatory cytokine expression, the activation of heat shock factor-1 dependent heat shock protein expression, the partial agonism of peroxisome proliferator-activating receptor-γ and the activation of nuclear factor (erythroid-derived) 2-regulated anti-inflammatory gene expression[39–43].

The most prevalent endogenous $NO_2$-FA, nitro-conjugated linoleic acid ($NO_2$-cLA), is detected as both the free acid, complex lipid-esterified and as protein-adducts[34, 44]. Conjugated linoleic acid (predominantly 18:2, cis-9, trans-11) is a dietary polyunsaturated fatty acid prevalent in dairy products, meats and plants. Endogenous generation of cLA in humans occurs by the isomerization of bis-allylic linoleic acid (LA) to a conjugated diene

and by the desaturation of oleic acid to cLA, with both reactions catalyzed by the gut microbiome[45]. Importantly, the external flanking carbons of the conjugated diene of cLA are 3–4 orders of magnitude more reactive than bis-allylic LA[44]. This promotes facile addition reactions of radical species such as $^{\cdot}NO_2$ to yield $NO_2$-cLA. It will always be a daunting challenge to dissect which products of nitrogen oxide metabolism, such as $^{\cdot}NO$, metal-NO complexes, S-nitrosothiols (RS-NO) or $NO_2$-FA, are proximally responsible for modulating the physiological actions of nitrogen oxides in humans, such as the regulation of inflammatory responses, BP regulation and platelet function.

We hypothesized that ingested $NO_3^-$ is reduced to the redox-active metabolite $NO_2^-$ *in vivo,* which is then metabolized to both $^{\cdot}NO$ and $NO_2$-cLA. Since $^{\cdot}NO$ and $NO_2$-cLA are both signaling molecules known to modulate BP and platelet function[3, 38, 46–50], we evaluated nitrogen oxide levels and clinical responses upon consumption of oral $^{15}NO_3^-$ and $^{15}NO_2^-$ by healthy adults, with and without cLA supplementation. The use of FDA Investigational New Drug (IND)-approved (IND #115926) oral formulations of $^{15}NO_3^-$ and $^{15}NO_2^-$ allowed for specific metabolite tracking *in vivo,* and demonstrated that $^{15}NO$-Hb and $^{15}NO_2$-cLA formation primarily occurred following $^{15}NO_2^-$ consumption, and that the physiological responses and nitrogen oxide product profiles were strongly influenced by the concomitant presence of cLA.

## Material and Methods

### Study design: Two pharmacokinetic (PK) studies of $^{15}NO_3^-$ and $^{15}NO_2^-$ metabolism were conducted, without (Trial 1) and with cLA (Trial 2) supplementation

In Trial 1, ten subjects were enrolled. To understand the precise mechanism of how the metabolites signal and exert their effects, these subjects were invited to return to participate in a modified PK study, Trial 2, to serve as a direct paired control. Five subjects completed Trial 2. Subjects were randomized in both trials as shown in Figure 1B. All study subjects were ages 18–60 years and had a normal BP defined as systolic BP≤130 and diastolic BP≤85 mmHg. The study was approved by the University of Pittsburgh Institutional Review Board and the U.S. Food and Drug Administration for use of these INDs. Prior to performing any of the research study procedures or interventions, subjects provided written informed consent and procedures were followed in accordance with institutional guidelines.

The selected drug doses were 1 g $Na^{15}NO_3^-$ (11.8 mmol) and 20 mg $Na^{15}NO_2^-$ (0.29 mmol) with and without 3 g cLA. With each drug dose, plasma samples were collected for PK analysis of $NO_3^-$ and $NO_2^-$ and methemoglobin (MetHb) was assessed using non-invasive co-oximetry (Masimo Corp., Irvine, CA), at times 0 (baseline or trough prior to study drugs), 0.5, 1, 2, 3, 6 and 24 hr post-drug administration. Prior to the start of each PK study visit, subjects fasted for 10–12 hr. BP and mean arterial blood pressure (MAP) were measured for 30–45 min to ensure subjects were at a steady state prior to the time 0 MAP and administration of the study drugs. BP, MAP, respiratory rate and heart rate monitoring were measured every 15 min during the first 2 hr, then at 3, 6, and 24 hr post-drug administration.

To track $NO_2^-$ metabolism *in vivo* (Figure 1C), PK evaluations were utilized to examine total ($^{15}N$ and $^{14}N$) plasma $NO_3^-$, $NO_2^-$ and RS-NO (S-nitrosothiols) by gas phase reductive ozone-based chemiluminescence detection. $^{15}NO$ was differentiated from endogenous $^{14}NO$ in blood using electron paramagnetic resonance spectroscopy (EPR) via the formation of the $^{15}NO^{\cdot}$ ligand to deoxyhemoglobin ($^{15}NO$-Hb). This iron-nitrosyl paramagnetic species has a distinctive hyperfine absorbance measured by EPR which produces a doublet for NO-Hb labeled with $^{15}N$. To elucidate the overall metabolic fate of $^{15}NO_3^-$ and $^{15}NO_2^-$ without and with cLA, plasma and urinary $^{15}NO_2$-cLA was differentiated from endogenous $^{14}NO_2$-cLA using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described[44]. A detailed description of the urinary measures is published separately[34]. Platelet activity was monitored at time 0, 6 and 24 hr after $^{15}N$ drug with and without cLA. Comprehensive methods are described in Supplemental Material.

### Statistical analysis

Detailed statistical analyses for each data set are included in the individual figure legends. Where differences existed between trial drug treatments, post-hoc multiple comparisons were performed using Bonferroni correction. Measurements shown represent mean ± SEM. A p value <0.05 was considered significant.

## Results

### $^{15}NO_3^-$/$^{15}NO_2^-$ pharmacokinetics with and without cLA

Ten healthy adult volunteers (baseline characteristics in Table S1) who fulfilled the inclusion/exclusion criteria were randomized into one of two subject cohorts of 5 subjects to receive a single dose of each study drug, oral $Na^{15}NO_3^-$ (1 g) and $Na^{15}NO_2^-$ (20 mg) in random order (Figure 1B). PK visits were separated by a 3–7 day washout period to ensure washout of nitrogen oxides and metabolites. Total plasma $NO_3^-$ concentrations were significantly increased after $^{15}NO_3^-$ dosing (peak level of 769 ± 38 μM, $p<0.001$, Figure 2A). Upon dosing with $^{15}NO_2^-$, total plasma $NO_3^-$ concentrations rose over 3 hr compared to baseline ($p<0.001$, Figure 2B). When the same subjects received oral $^{15}NO_3^-$, total plasma $NO_2^-$ concentrations increased over 6 hr compared to baseline ($p<0.01$, Figure 2C). In contrast, when subjects received oral $^{15}NO_2^-$, total plasma $NO_2^-$ concentrations rapidly peaked at 0.5 hr (5.5 ± 0.7 μM, $p<0.001$, Figure 2D) and returned to basal concentrations after 3 hr.

Five of the 10 healthy volunteers who completed Trial 1 returned for Trial 2 and were randomized to receive a single dose of each study drug, $Na^{15}NO_3^-$ (1 g) and $Na^{15}NO_2^-$ (20 mg), plus 3 g cLA, in random order, separated by a 3–7 day washout period so that metabolic responses could be compared to those from Trial 1 (Figure 1B). Total plasma $NO_3^-$ concentrations were lower following $^{15}NO_3^-$ + cLA at 1 hr (473.2 ± 72.4 μM, Figure 2E closed circles) compared to $^{15}NO_3^-$ supplementation (743.1 ± 72.2 μM, $p<0.01$, Figure 2E open circles). Peak plasma $NO_3^-$ concentration occurred at 2 hr for $^{15}NO_3^-$ + cLA administration and 1 hr for $^{15}NO_3^-$ alone. No significant differences in plasma $NO_2^-$ concentrations were observed for $^{15}NO_3^-$ treatment alone vs. $^{15}NO_3^-$ + cLA (Figure S1). While total plasma $NO_2^-$ concentrations increased over time after $^{15}NO_2^-$ + cLA ($p=0.001$),

a trend towards lower total plasma $NO_2^-$ concentrations was seen at 0.5 and 1 hr (Figure 2F closed circles) when compared to $^{15}NO_2^-$ alone (p=0.059, Figure 2F open circles). Peak plasma $NO_2^-$ concentration was 3.0 ± 0.5 μM for $^{15}NO_2^-$ + cLA and 4.6 ± 1.0 μM for $^{15}NO_2^-$ at 0.5 hr.

## Formation of MetHb and $^{15}$NO-Hb

Further metabolism of $^{15}NO_2^-$ was also reflected by MetHb, $^{15}$NO-Hb, plasma RS-NO and $^{15}NO_2$-cLA generation (Figure 1C). Following $^{15}NO_3^-$ administration, no significant increase in MetHb was observed (Figure 3A). Following $^{15}NO_2^-$ dosing, a small but significant increase in MetHb occurred from baseline over 1 hr (baseline 1.2 ± 0.1%, peak at 1 hr 1.6 ± 0.1%, p<0.001, Figure 3B) and returned to baseline by 3 hr. cLA supplementation did not impact MetHb levels (not shown). The metabolism of $^{15}NO_2^-$ to $^{\cdot 15}$NO was measured by the formation of $^{15}$NO-Hb in red blood cells sedimented from venipuncture blood samples. A representative EPR spectra (red) shows the hyperfine splitting characteristic of $^{15}$NO-Hb from one subject (raw data in blue, Figure 3C). There was no evidence for the formation of $^{14}$NO-Hb (Figure S2B). $^{15}$NO-Hb formation was only detected between 0.5 and 1 hr after $^{15}NO_2^-$ supplementation (Figure 3D). $^{15}$NO-Hb derivatives were not detectable after $^{15}NO_2^-$ + cLA and $^{15}NO_3^-$ + cLA administrations (not shown).

## Formation of RS-NO

Representative plasma RS-NO traces are shown at baseline and for peak concentrations of RS-NO at 0.5 hr after $^{15}NO_2^-$ dosing, followed by an additional time point after $^{15}NO_2^-$ treatment (Figure 4A). $^{15}NO_2^-$ consumption significantly increased plasma RS-NO concentrations (baseline 3.2 ± 1.0 nM, peak concentration of 6.1 ± 1.0 nM at 0.5 hr, p<0.05, Figure 4B) and returned to basal concentrations after 3 hr. In contrast, after $^{15}NO_3^-$ dosing, there was no significant impact on RS-NO concentration (Figure S3A open circles). There was also no impact of cLA supplementation on plasma RS-NO concentrations after dosing in combination with either $^{15}NO_3^-$ or $^{15}NO_2^-$ compared to $^{15}NO_3^-$ or $^{15}NO_2^-$ alone (Figure S3A and B).

## Formation of $NO_2$-cLA

HPLC-MS/MS analysis of the structural regioisomers, β-oxidation metabolites, Michael addition products and pharmacokinetics of $^{15}NO_3^-$ and $^{15}NO_2^-$-induced fatty acid nitration in Trials 1 and 2 has been reported[34]. Endogenous plasma $^{14}NO_2$-cLA concentrations averaged 1.58 ± 0.2 nM after $^{15}NO_3^-$ and $^{15}NO_2^-$ and rose significantly to 2.84 ± 0.24 nM upon cLA supplementation. Mean plasma $^{15}NO_2$-cLA concentrations were maximal at 24 hr after $^{15}NO_3^-$ + cLA (3.06 ± 1.79 nM), and $^{15}NO_2^-$ + cLA supplementation resulted in maximal $^{15}NO_2$-cLA concentrations that were sustained between 1–6 hr (6.61 ± 4.84 nM to 4.86 ± 2.39 nM). These $^{15}NO_2^-$ related levels paralleled the levels of free plasma cLA. These data support that free cLA concentrations are limiting for fatty acid nitration. A representative LC-ESI-MS/MS chromatogram of one volunteer's plasma lipid extract (Figure 5) shows the $^{15}NO_2$-cLA regioisomers generated upon dosing with $^{15}NO_2^-$ + cLA[34].

### Physiologic responses to $^{15}NO_3^-$ and $^{15}NO_2^-$ supplementation with and without cLA supplementation

In Trial 1, oral $^{15}NO_2^-$ dosing induced vasodilation, decreasing systolic (Δ−12.1±3.3 mmHg, p<0.001), diastolic (Δ−8.1±2.0 mmHg, p=0.002) and mean arterial pressure (Δ −10.4±1.2 mmHg, p=0.001) (Figure 6A and B open circles) with no differences in heart rate (data not shown). In this $^{15}NO_2^-$ dosed cohort, the reduction in SBP and MAP correlated with the change in plasma $NO_3^-$ concentration (Figure S4A and C). Also, the greater the reduction in DBP at 1 hr, the greater the rise in total $NO_3^-$ concentration at 0.5 hr (Figure S4B). Notably, the significant decreases in SBP and MAP that persisted for up to 2 hr after subjects were treated with $^{15}NO_2^-$ were abolished upon co-administration of cLA in Trial 2 (Figure 6A and B closed circles).

Inhibition of platelet activation in whole blood was observed 6 hr following $^{15}NO_2^-$ dosing (baseline 33.2 ± 7.1%, 6 hr post-dosing 7.8 ± 1.6%, p=0.02, Figure 6C open bars), an effect that was eliminated when subjects were dosed with $^{15}NO_2^-$ + cLA, (Figure 6C dark bars). Following $^{15}NO_3^-$ dosing in all 10 subjects who completed Trial 1, there was significant inhibition of platelet activation 6 hr after $^{15}NO_3^-$ administration (17.0 ± 4.1%, 6 hr post-dosing 9.0 ± 1.7%, p=0.029, Figure S5A). With co-administration of $^{15}NO_3^-$ + cLA in Trial 2, there was no significant inhibition of platelet activation in whole blood at 6 hr (27.5 ± 12.3%, 6 hr post-dosing 20.0 ± 7.5%, Figure S5B).

In subjects given oral $^{15}NO_3^-$, plasma $^{15}NO_2^-$ concentrations were significantly increased from 1–2 hr post dosing (Figure S1, open circles), with a maximum of 1.40 μM reached. In Trial 1, oral $^{15}NO_3^-$ dosing induced a small reduction in DBP at 3 hr compared to baseline (ΔDBP = −6.3±1.2 mmHg, p=0.003, Figure S6B open circles) with no significant change in SBP and MAP (Figure S6A and C open circles) or heart rate (data not shown). No significant correlations were noted between changes in SBP, DBP or MAP and absolute $NO_3^-$ or $NO_2^-$ concentrations after $^{15}NO_3^-$ administration (not shown). Following $^{15}NO_3^-$ + cLA dosing, there were no significant changes in SBP, DBP and MAP (Figure S6A, B and C closed circles) or heart rate (data not shown).

## Discussion

There are a panoply of physiological reactions that nitrogen oxides can undergo, all uniquely influenced by factors such as changes in pH, oxygen tension, $CO_2/H_2CO_3$ levels, rates of production of reactive inflammatory mediators and redox-reactive metalloprotein levels. The relative significance of these mitigating factors will change as basal metabolism transitions to metabolic stress and inflammatory responses. This will critically impact the specific reactions that occur between $^{\cdot}NO/NO_2^-$, molecular oxygen, partially reduced oxygen species and metal centers. In the context of $^{\cdot}NO$ signaling, the physiological outcomes of these reactions are manifested by the diversion of $^{\cdot}NO$ away from canonical guanylate cyclase activation and cGMP-dependent signaling responses to the generation of a spectrum of highly reactive and transient secondary oxidizing, nitrosating and nitrating products (Figure 1A)[51]. At high concentrations, these reactive products may be pathogenic, but under physiological conditions these chemically-reactive intermediates a) represent a metastable $^{\cdot}NO$ reserve that still signals via guanylate cyclase activation and cGMP-dependent

mechanisms and b) react with and modify the structure and function of cell targets (e.g. unsaturated fatty acids, proteins). These latter reactions instigate an array of PTMs and non-cGMP-dependent signaling responses[31]. In particular, the nucleophilic amino acid Cys confers proteins with a sensitivity for reduction-oxidation (redox)-induced PTMs that include Cys oxidation, glutathionylation, S-nitrosation, and alkylation upon Michael addition by electrophilic species[52]. These PTMs intimately link metabolic and inflammatory status with changes in cell and organ function, since many enzymes, receptors and transcriptional regulatory proteins that regulate metabolism and inflammation are endowed with functionally-significant hyperreactive Cys moieties. Herein, we report that oral co-administration of 3 g of the polyunsaturated fatty acid cLA with $^{15}NO_2^-$ significantly redirects the vasodilatory and platelet inhibitory actions of $NO_2^-$ to alternative pathways.

Nitrite and its reduction product $^{\cdot}NO$ are typically viewed to induce vasodilation via cGMP-dependent mechanisms[1, 2]. In the human brachial artery, forearm blood flow increases after $NO_2^-$ infusion, in concert with a simultaneous rise in $^{\cdot}NO$ formation[1]. Abundant clinical studies have demonstrated significant reductions in BP in healthy adults following both single ingestion[11, 53], repetitive 15 day[22, 54, 55] courses of $NO_3^-$ rich foods[56] or consumption of a traditional Japanese diet[57], another rich source of dietary $NO_3^-$. Various dosages of pure $NO_3^-$ and $NO_2^-$ preparations also significantly reduced BP in healthy adults[53,58,59]. These human studies have largely been limited to testing single or short-term doses of $NO_3^-$ or $NO_2^-$, with no evaluation of downstream metabolites. Animal models also recapitulate canonical vasodilatory and BP lowering effects of $^{\cdot}NO$ stemming from $NO_2^-$ and $NO_3^-$[60, 61]. There are no statistically significant differences between RS-NO concentrations with $^{15}NO_2$ alone vs. $^{15}NO_2$ + cLA, with peak concentrations of $^{15}$NO-Hb being ~150 times greater than peak concentrations of RS-NO. Thus, these human $NO_2^-$ PK data collectively demonstrate elevated plasma $^{15}NO_2^-$ concentrations (Figure 2D) and $^{15}NO_2^-$ reduction to $^{15}NO^{\cdot}$ (Figure 3D) *in vivo* as the most likely mediators of the simultaneous systolic and mean arterial blood pressure reductions upon $^{15}NO_2$ administration (Figure 6A and B, open circles). Oral administration of $^{15}NO_2$ in concert with cLA blunted hemodynamic responses (Figure 6A and B, closed circles), with no detectable formation of $^{15}$NO-Hb adducts. Our lack of blood pressure response with cLA addition does not indicate that our nitrite drug dose is not vasodilating, but that we are reducing the nitrite concentrations enough to reduce the blood pressure lowering extreme. A reduction in blood pressure requires a systemic increase in blood flow (or a decrease in systemic vascular resistance - SVR) that cannot be compensated by an increase in stroke volume and heart rate (MAP - CVP = CO x SVR). We have previously measured vasodilation in the human forearm at nitrite concentrations as low as 150–200 nM (0.1–0.2 uM)[1].

Oral administration of single doses of $^{15}NO_3^-$ and $^{15}NO_2^-$ gave detectable increases in plasma nitrogen oxide concentrations, with each nitrogen oxide displaying unique pharmacokinetic and metabolite profiles. Moreover, co-administration of cLA significantly affected these parameters. Oral $^{15}NO_3^-$ reached its highest plasma $NO_3^-$ concentration 1 hr after dosing and yielded a peak concentration of plasma $NO_2^-$ 2–6 hr after dosing (Figure 2A and C). This concentration of plasma $NO_2^-$ with oral $^{15}NO_3^-$ was not sufficient to increase MetHb or to yield detectable red blood cell $^{15}$NO-Hb levels (Figure 3A and D) or to induce (Figure S6A and C) or sustain (Figure S6B) reductions in BP parameters. Dosing of

$^{15}NO_3^-$ in concert with cLA suppressed peak plasma $NO_3^-$ levels by ~30% (Figure 2E closed circles). For oral $^{15}NO_2^-$, the highest plasma concentration of $NO_2^-$ was measured 0.5 hr after dosing (Figure 2D), giving a modest but significant increase in plasma $NO_3^-$ metabolite concentrations 2–3 hr later (Figure 2B). Notably, the co-administration of cLA with $^{15}NO_2^-$ also suppressed peak plasma $NO_2^-$ concentrations by ~30% (Figure 2F closed circles).

Nitrite reacts with oxyHb to form $NO_3^-$ and MetHb, and with deoxyhemoglobin to ultimately form NO-Hb[16]. Upon $NO_2^-$ protonation to $HNO_2$ and reaction with $^{\cdot}NO$, both symmetric (ONONO) and asymmetric (OONNO) dinitrogen trioxide intermediates are generated, serving as proximal mediators of biomolecule nitrosation and nitration[20, 62]. After $^{15}NO_2^-$ administration, evidence of all of these reactions was noted, with a significant 25% increase in MetHb levels, the formation of ~1 μM paramagnetic $^{15}NO$-Hb species and a 3 nM increase in mean plasma protein RS-NO levels 0.5 hr post-dosing (Figures 3B and D, 4A and B). With cLA supplementation, mean plasma $NO_2$-cLA concentration increased by ~5 nM[34]. Only a few studies have reported changes in plasma $^{\cdot}NO$ concentrations at baseline and following dietary $NO_3^-$ or $NO_2^-$ supplementation. For example, after a single 5.6 mmol dose of $NO_3^-$ rich beetroot juice[53], plasma cGMP, an indicator of $^{\cdot}NO$ generation, increased within 3 hr. Following a single 24 mmol dose of $KNO_3$, plasma cGMP increased within 3 hr and remained elevated through 24 hr[53].

Following entero-salivary reduction of $NO_3^-$ to $NO_2^-$, $NO_2^-$ is readily protonated (pKa=3.4) to nitrous acid ($HNO_2$) in the acidic gastric compartment. This promotes a “redirection” of nitrite chemistry where $HNO_2$ in turn gives rise to secondary $N_2O_3$ species that mediate nitrosation, nitration and oxidation of susceptible targets. These reactions can be blunted by administering proton pump inhibitors such as esomeprazole, thus inhibiting gastric acid secretion and $NO_2^-$ protonation to $HNO_2$, – a pH response that typifies the pharmacologic action of PPIs[63]. Esomeprazole also inhibited the blood pressure-lowering effects of nitrite. The more basic conditions in the stomach after esomeprazole administration limited the protonation of nitrite to $HNO_2$, a species that undergoes both dismutation and nitric oxide reactions to yield the nitrosating and nitrating products symmetric and asymmetric dinitrogen trioxide (ONONO and ONNOO)[20]. The pathways leading to $NO_2$-cLA formation first require entero-salivary reduction of $NO_3^-$ to $NO_2^-$. In the present study, where nitrite and nitrate were orally consumed in the presence of cLA, stomach pH would either remain the same or be lowered by the acidic cLA (pKa = 4.0). The suppression of detectable plasma $NO_3^-$ and $NO_2^-$ by ~30% upon cLA co-administration indicated that the greater availability and reactivity of cLA favored its nitration, thus supporting the generation of $NO_2$-cLA derivatives rather than the accumulation of $NO_3^-$ and $NO_2^-$ in plasma and presumably other tissue compartments. Our present report supports the concept that the addition of cLA and its facile nitration by nitrite-derived species is a consequence of these acid-catalyzed reactions. Our data indicates that this concomitantly leads to an attenuation of NO-forming reactions that would otherwise result in the vasodilation and inhibition of platelet function that was observed when nitrite alone was administered. There may also have been an impact of cLA on gut nitrogen oxide absorption or microbial $NO_3^-$ reduction. The small increase in plasma RS-NO derivatives observed in the $^{15}NO_2^-$ treated cohort in Trial 1 at 0.5 hr post-administration (Figure 4B) was not significantly different compared to Trial 2 with addition

of cLA (Figure S3B). Dosing of volunteers with $^{15}NO_3^-$ with cLA had no impact on plasma RS-NO levels (Figure S3A).

In healthy, hypertensive and high BMI subjects, cLA supplementation (3–6.8g/day, 5–26 wk), compared to control or placebo displays no significant impact on BP in humans[64,65,66,67]. Herein, we observed that the decreased BP induced by $NO_2^-$ was abrogated by concomitant cLA administration (Figure 6AB). In addition to promoting a shift in nitrosative and nitrative chemistries, cLA might also be modulating $NO_2^-$ responses by impacting endogenous $^{\cdot}NO$ production[68,69, 70]. The present data do not diminish the significance of heme proteins such as deoxyHb in mediating reactions of nitrite that lead to changes in vascular function. Rather, our results further affirm the impact that other biomolecules such as conjugated diene-containing fatty acids that readily undergo radical addition reactions, can have on nitrogen oxide reaction pathways and downstream signaling responses. This is manifested by the fact that Fe-NO Hb levels in nitrite-treated subjects are decreased to undetectable levels upon nitrite + cLA administration (EPR analysis has a limit of quantitation of ~500 nM for Fe-NO complexes). These findings indicate that cLA supplementation and other dietary constituents have the ability to modulate the biochemical reactions of $NO_2^-$, the trafficking of downstream nitrogen oxide metabolites and the physiological concentrations of this pluripotent signaling mediator. Moreover, a more chronic exposure to elevated cLA and fat in the diet has the potential to also influence the entero-salivary microbiome and its impact on nitrogen oxide metabolism[71].

Along with vasodilatory actions, $^{\cdot}NO$ formed by the reduction of $NO_2^-$ inhibits platelet function, including platelet adhesion to the endothelium[47] and platelet aggregation[72]. In contrast, partially reduced oxygen species such as $O_2^{\cdot-}$ and $H_2O_2$ propagate platelet activation[73, 74]. The mechanism by which $^{\cdot}NO$ affects platelet activity is by activating cGMP-dependent signaling. While dietary $NO_3^-$ sources (beetroot juice or $KNO_3^-$) reduce platelet activation or aggregation[11, 29, 75, 76], to date only *in vitro* studies of human platelet function have addressed the effects of $NO_2^-$ sources on platelet activation or aggregation[28, 29, 77, 78]. Herein, the inhibition of platelet activation occurred in whole blood for 6 hr following $^{15}NO_2^-$ dosing, with the inhibition of platelet activation still evident well beyond the physiologic plasma $NO_2^-$ peak and the time of $^{15}$NO-Hb detection. Previous *in vitro* studies revealed that two synthetic nitro-fatty acids, nitro-linoleic acid and nitro-arachidonic acid (at high concentrations in buffered saline), inhibited platelet aggregation and activation, respectively[79, 80]. In the more clinically-relevant study herein, $^{15}NO_2^-$ administration *in vivo* decreased platelet activation in whole blood at 6 hr. The whole blood basal platelet activation levels of subjects were moderately increased, which are attributed to an artifact of the venipuncture sampling. There may be a threshold plasma nitrite concentration where sufficient $^{\cdot}NO$ formation subsequently reduces platelet activation with $^{15}NO_2^-$ dosing, whereas oral $^{15}NO_2^-$ and $^{15}NO_3^-$ in combination with cLA diverted nitrogen oxide metabolites to support the formation of $^{15}NO_2$-cLA, thus consistently attenuating $NO_2^-$ and $NO_3^-$ inhibition of platelet activation.

### Study Limitations and Strengths

The data presented here cannot exclude dietary cLA contribution during Trial 1 and dietary $NO_3^-$ and $NO_2^-$ contributions also influencing baseline plasma $NO_3^-$ and $NO_2^-$ concentrations in both trials. Additionally, no formal power calculations were performed as these were exploratory Phase I studies. While this study may be underpowered to detect an effect of $^{15}NO_3^-$ on BP, given that there were significant reductions in all BP parameters with $^{15}NO_2^-$, the abolishment of the $^{15}NO_2^-$ BP effect with the addition of cLA can be assumed to be due to the impact of cLA and not insufficient power. Traditional human $NO_3^-$ intakes of 40–100 mg/day[81, 82], $NO_2^-$ intakes of 0–20 mg/day[83] and cLA intakes of 150–200 mg/day[84] are quite low, but daily intakes of up to 2.6 g of cLA are reported in the literature. Therefore, it may be difficult to achieve all three of these intakes simultaneously through diet on a daily basis, so as to be comparable to those provided in capsule formulations herein. However, our goal with cLA supplementation was to define whether the biochemical reactions and physiologic responses to oral $^{15}NO_3^-$ and $^{15}NO_2^-$ were modulated by cLA. Our investigations are strengthened by utilizing paired PK studies in the same subjects so direct comparisons could be made using $^{15}N$ sodium $NO_3^-$ and $NO_2^-$ with and without cLA supplementation to track $^{\cdot}NO$ metabolism and the source of $^{\cdot}NO$ and $NO_2$-cLA species formation *in vivo* with each drug.

### Perspectives

The oral administration of cLA altered the metabolic fate of oral $^{15}NO_3^-$ and $^{15}NO_2^-$ and modulated the $^{\cdot}NO$-signaling and vasodilatory properties of these nitrogen oxides. The cGMP-dependent signaling actions of $NO_3^-$ and $NO_2^-$ can be further transformed by digestive reactions, the entero-salivary microbiome and metalloprotein-catalyzed reactions to yield $^{\cdot}NO$ and additional nitrogen oxides that elicit redox signaling responses via nitration, nitrosation and oxidation reactions. One would expect that the spectrum of nitrogen oxide products formed will depend on diet, metabolic and inflammatory status, acidic microenvironments and $NO_3^-$-reducing salivary bacteria. The concurrent administration of cLA in the diet decreased peak plasma $NO_3^-$ and $NO_2^-$ concentrations, enhanced the formation of $^{15}NO_2$-cLA, and decreased detectable $^{\cdot}NO$ concentrations in the vascular compartment, thus effectively diverting nitrosative reactions to nitration events. This diversion of nitrosative reactions to nitrative chemistries resulted in the increased generation of $NO_2$-FA, with concomitant attenuation of the inhibition of platelet activation and abrogation of the vasodilatory properties of $NO_2^-$. This affirms that the metabolic and the physiological responses to oral $NO_3^-$ and $NO_2^-$ can be significantly modulated by the composition of the diet, particularly with meat and dairy products that are rich in cLA.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Novelty and Significance

**What is new?**

We developed oral capsule formulations of sodium nitrate ($NO_3^-$) and nitrite ($NO_2^-$) with the stable [$^{15}N$] isotope allowing for specific metabolite tracking *in vivo* and to investigate the human vascular responses.

**What is relevant?**

- Oral $^{15}NO_2^-$ consumption resulted in $^{\cdot}NO$ formation, vasodilation and acute inhibition of platelet activation
- Conjugated linoleic acid (cLA) with oral $^{15}NO_3^-/NO_2^-$ diverted metabolic products to $NO_2$-cLA
- Addition of dietary cLA with oral $^{15}NO_2^-$ decreased $^{\cdot}NO$ formation and vasodilation and attenuated the acute inhibition of platelet activation

**Summary**

Concurrent cLA in the diet altered the metabolic fate of oral $^{15}NO_3^-$ and $NO_2^-$ with decreased plasma $NO_3^-/NO_2^-$ levels, enhanced formation of $NO_2$-FA species and decreased $^{\cdot}NO$ formation. This diversion of the downstream reactions of $NO_3^-$ and $NO_2^-$ to $NO_2$-cLA attenuated the inhibition of platelet activation and abrogated the vasodilatory properties of $NO_2^-$.

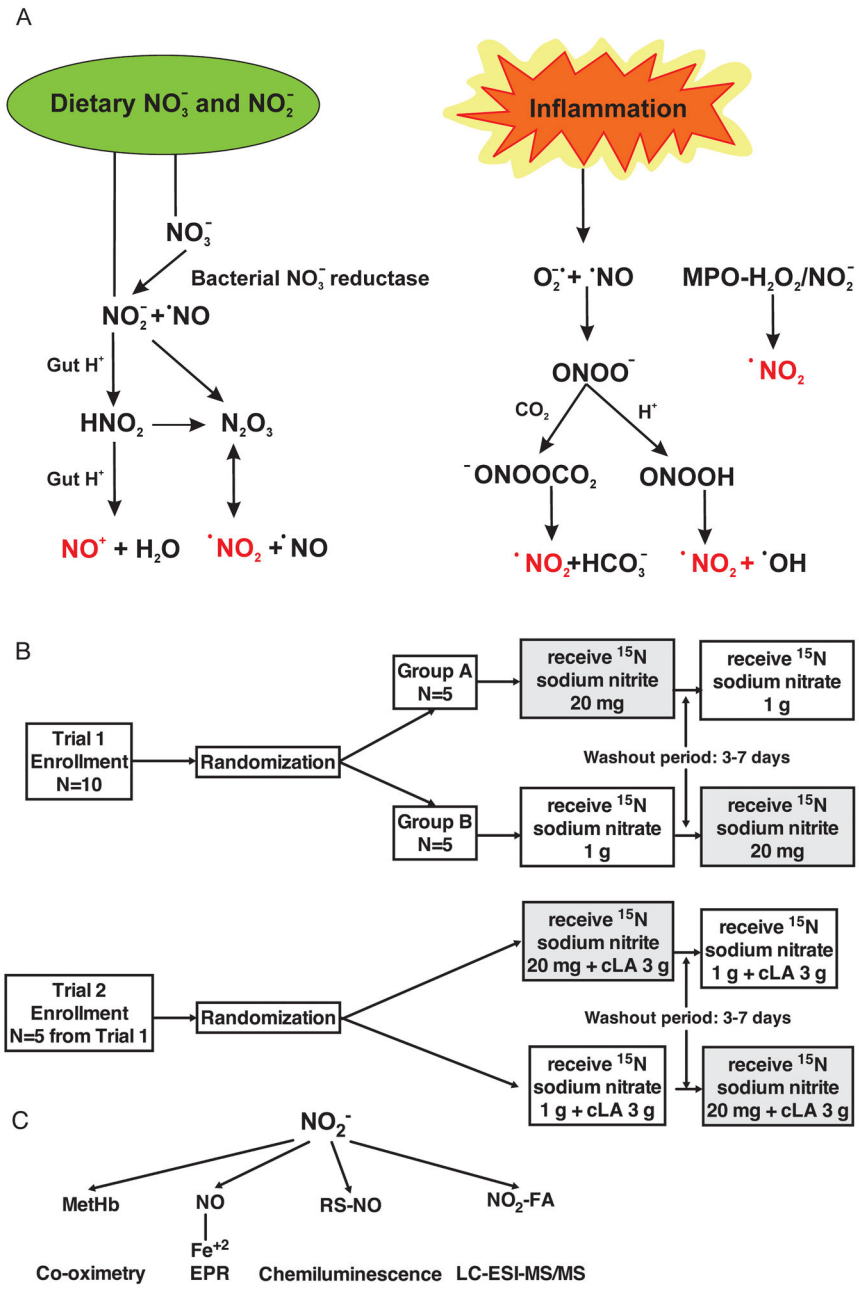


**Figure 1. Trial schemes and $NO_2^-$ signaling pathways**

(A) Multiple metabolic and inflammatory reactions yield nitrogen dioxide. Nitration ($^{\cdot}NO_2$) and nitrosation ($NO^+$) reactions produce an array of bioactive nitrogen oxide products, including $NO_2$-FA and S-nitrosothiol derivatives that have incompletely-characterized biological activities. (B) To understand the metabolism of single doses of heavy nitrogen labeled sodium $NO_3^-$ and $NO_2^-$ and to characterize their signaling pathways and physiologic effects, cross-over drug design studies were conducted. Ten healthy volunteers were randomized into one of two subject cohorts to receive a single dose of each study drug, oral $^{15}N$-labeled sodium $NO_3^-$ and $NO_2^-$, in random order, separated by a washout period.

Five of the 10 healthy volunteers who completed Trial 1 returned and were randomized to receive a single dose of each drug, oral $^{15}$N-labeled sodium $NO_3^-$ and $NO_2^-$ plus conjugated linoleic acid (cLA), in random order, separated by a washout period in Trial 2. (C) To track $NO_2^-$ metabolism *in vivo*, methemoglobin (MetHb), $^{15}$NO bound to the heme of hemoglobin ($^{15}$NO-Hb), RS-NO and $^{15}NO_2$-fatty acid (FA) formation were examined.

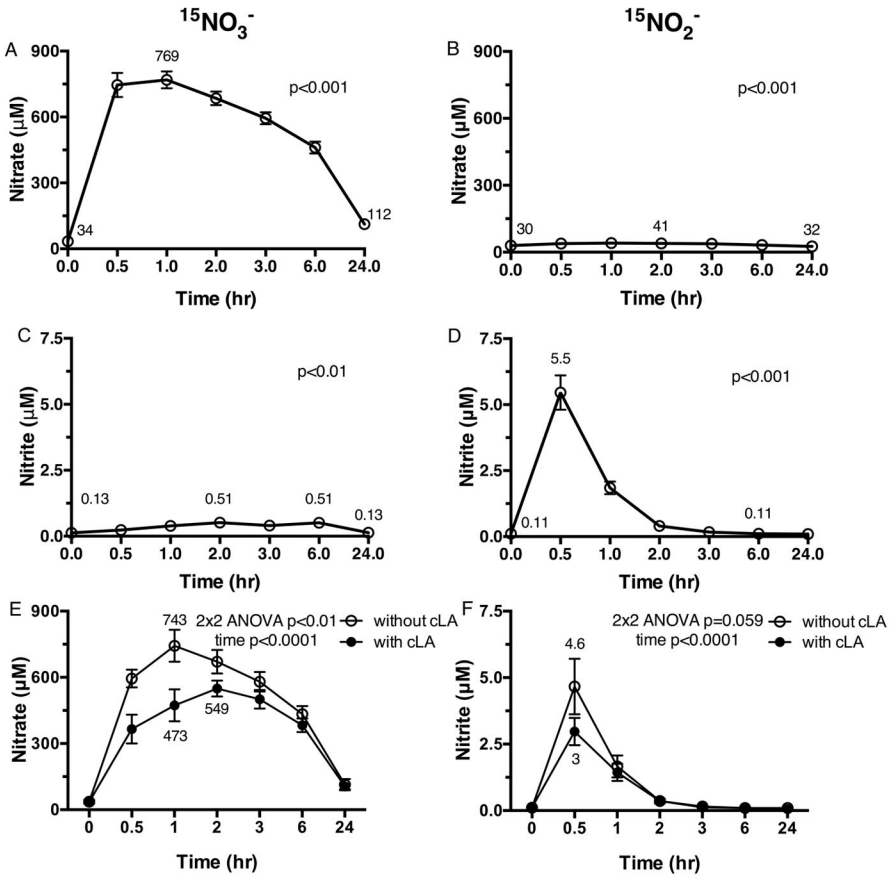


**Figure 2. $^{15}NO_3^-$ and $^{15}NO_2^-$ PK without and with cLA**

(A) Following oral $^{15}NO_3^-$, plasma $NO_3^-$ concentrations increase at all time points compared to baseline. (B) Following oral $^{15}NO_2^-$, plasma $NO_3^-$ concentrations rise through 3 hr compared to baseline. (C) Following oral $^{15}NO_3^-$, plasma $NO_2^-$ concentrations increase through 6 hr compared to baseline. (D) Following oral $^{15}NO_2^-$, plasma $NO_2^-$ concentrations increase at 0.5 hr and return to baseline after 6 hr. (E) When plasma $NO_3^-$ concentrations were examined following oral $^{15}NO_3^-$ treatment alone compared to $^{15}NO_3^-$ with cLA, lower plasma $NO_3^-$ concentrations were achieved with $^{15}NO_3^-$ plus cLA. (F) A trend towards lower plasma $NO_2^-$ concentrations was seen through 1 hr with $^{15}NO_2^-$ plus cLA compared to $^{15}NO_2^-$ alone. Repeated measures ANOVA with time as the within-subject effect was used to evaluate response to drug treatment for the endpoint measures in A–D. 2×2 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 E–F.

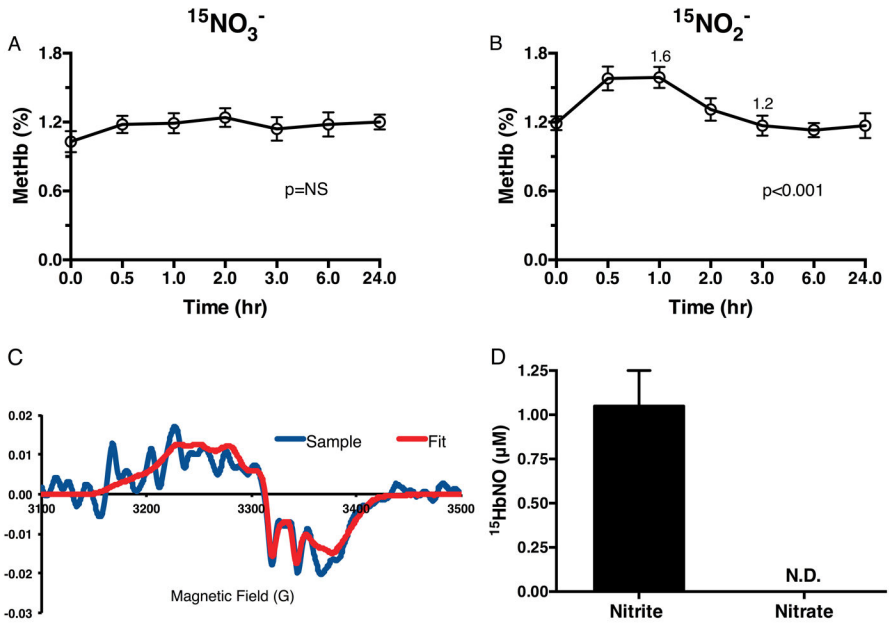


**Figure 3. $NO_2^-$ signaling and methemoglobin and $^{15}$NO-Hb formation**

(A) Following oral $^{15}NO_3^-$, no significant increase in methemoglobin (MetHb) occurs. (B) Following oral $^{15}NO_2^-$, a significant rise in MetHb occurs through 1 hr. (C) A representative EPR spectra (red) shows the characteristic hyperfine splitting of $^{15}$NO-Hb from one subject following oral $^{15}NO_2^-$ (raw tracings shown in blue). The fit is composed of 42% pentacoordinate $^{15}$N alphanitrosyl Hb, 21% hexacoordinate alphanitrosyl Hb, and 38% betanitrosyl Hb. Inclusion of pentacoordinate $^{14}$N alphanitrosyl Hb does not substantially improve the fit (Fig. S3B). (D) $^{15}$NO-Hb formation is detected with oral $^{15}NO_2^-$ treatment only at 0.5 to 1 hours, with no $^{15}$NO-Hb detected with oral $^{15}NO_3^-$ treatment. NS = not significant, N.D. = not detected. Repeated measures ANOVA with time as the within-subject effect was used to evaluate response to drug treatment for the endpoint measures in A–B.

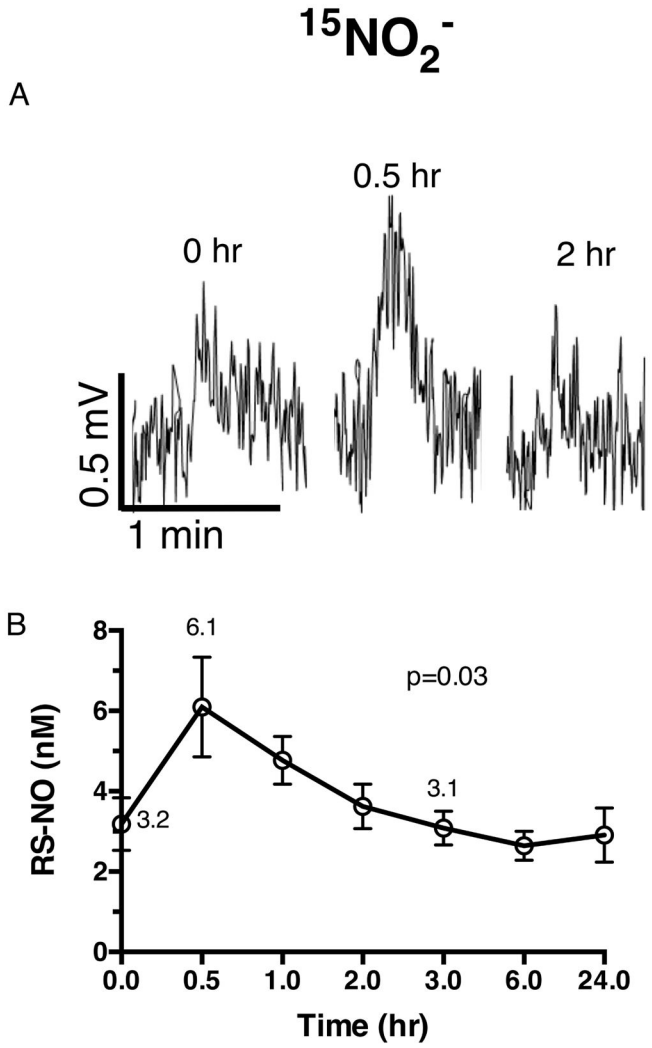


**Figure 4. $NO_2^-$ signaling and RS-NO**

(A) Representative plasma RS-NO traces at baseline and the time when peak RS-NO concentrations were detected with oral $^{15}NO_2^-$ followed by a time point after $^{15}NO_2^-$ treatment. (B) Following oral $^{15}NO_2^-$, plasma RS-NO concentrations increase significantly, but after 3 hr, approach baseline. Repeated measures ANOVA with time as the within-subject effect was used to evaluate response to drug treatment for the endpoint measure in B.

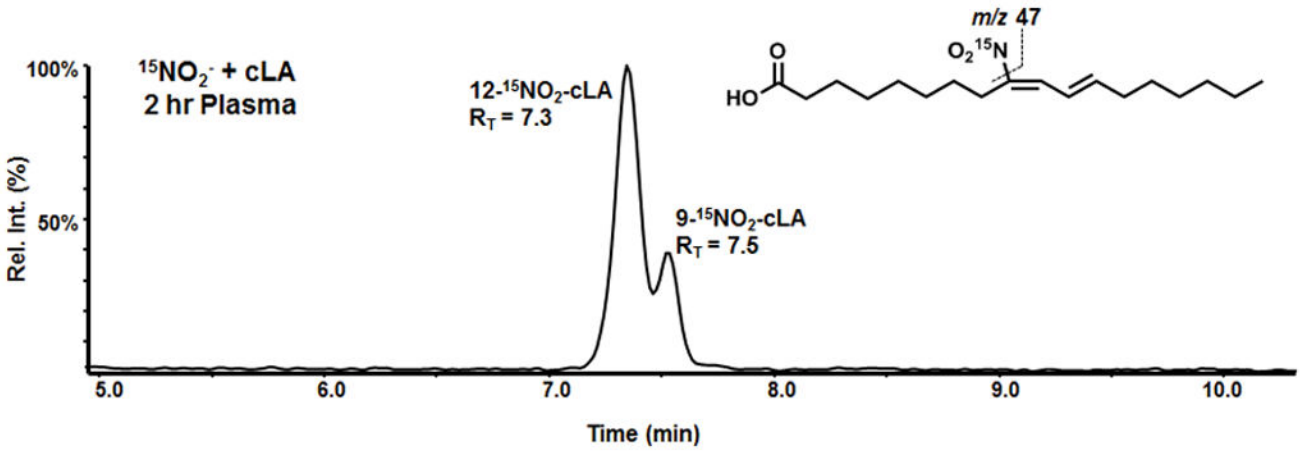


**Figure 5. $NO_2^-$ signaling and $NO_2$-cLA formation**

A representative LC-ESI-MS/MS chromatogram of plasma lipid extract shows $^{15}NO_2$-cLA present in plasma of volunteers treated with oral $^{15}NO_2^-$ plus cLA (Adapted with permission from Delmastro-Greenwood et al. Nitrite and Nitrate-Dependent Generation of Fatty Acid Nitroalkenes. *Free Radic Biol Med*. 2015;89:333–341.).

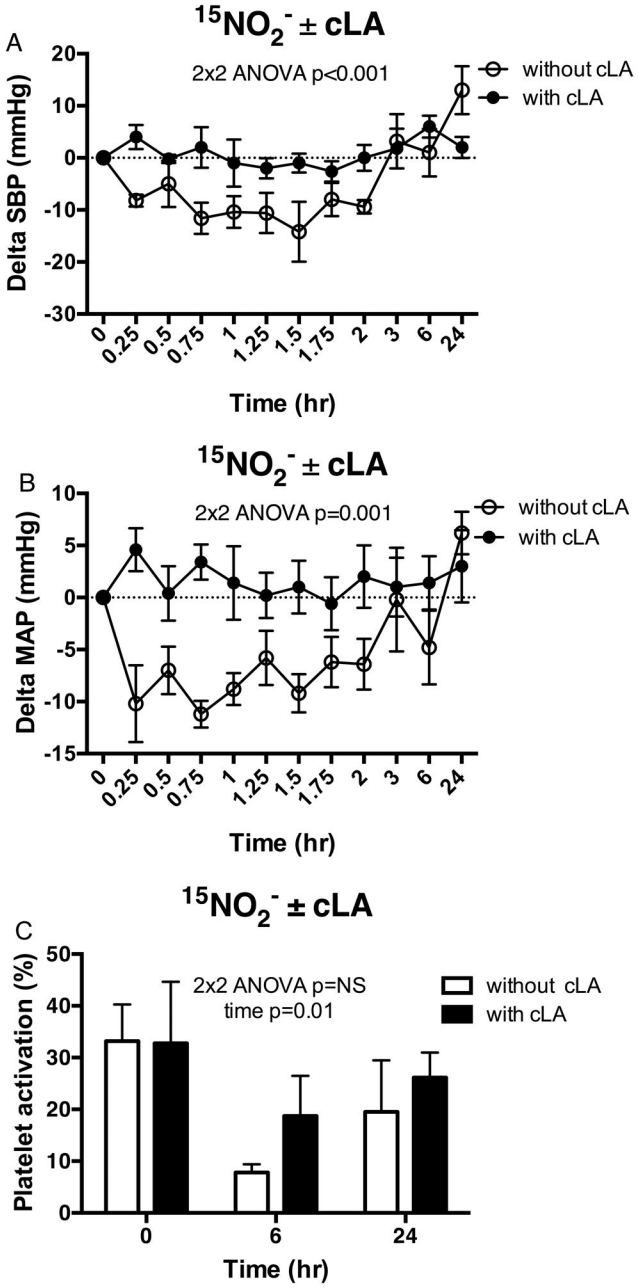


**Figure 6. Physiologic effects and $NO_2^-$ signaling without and with cLA**

Significant decreases in SBP (A) and MAP (B) but not DBP that persisted through 1.5 hr after subjects were treated with oral $^{15}NO_2^-$ alone (open circles) were abolished by cLA supplementation (closed circles). (C) No differences in the % of platelet activation were detected when comparing oral $^{15}NO_2^-$ treatment alone (open bars) versus oral $^{15}NO_2^-$ with cLA (dark bars) over 24 hr. 2×2 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.