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Postprandial lipids accelerate and redirect nitric oxide consumption in plasma

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Abstract

Nitric oxide (NO) and O₂ are both three- to four-fold more soluble in biological lipids than in aqueous solutions. Their higher concentration within plasma lipids accelerates NO autoxidation to an extent that may be of importance to overall NO bioactivity. This study was undertaken to test the hypothesis that increased plasma lipids after a high-fat meal appreciably accelerate NO metabolism and alter the byproducts formed. We found that plasma collected from subjects after consumption of a single high-fat meal had a higher capacity for NO consumption and consumed NO more rapidly compared to fasting plasma. This increased NO consumption showed a direct correlation with plasma triglyceride concentrations ($p=0.006$). The accelerated NO consumption in postprandial plasma was reversed by removal of the lipids from the plasma, was mimicked by the addition of hydrophobic micelles to aqueous buffer, and could not be explained by the presence of either free hemoglobin or ceruloplasmin. The products of NO consumption were shifted in postprandial plasma, with 55% more nitrite ($n=12$, $p=0.002$) but 50% less SNO ($n=12$, $p=0.03$) production compared to matched fasted plasma. Modeling calculations indicated that NO autoxidation was accelerated by about 48-fold in the presence of plasma lipids. We conclude that postprandial triglyceride-rich lipoproteins exert a significant influence on NO metabolism in plasma.

Keywords

Nitric oxide; nitrite; S-nitrosothiol; high-fat meal; plasma; lipid

1. INTRODUCTION

Nitric oxide (NO) is a potent vasodilator produced primarily in the vascular endothelium, from where it is thought to diffuse rapidly and randomly in all directions [1]. NO diffusing abluminally into the surrounding vascular smooth muscle results in vasodilation.

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Alternatively, if the NO diffuses into the blood vessel lumen, it can interact with substances in either the plasma or erythrocyte [1]. Therefore, the amount of endothelium-derived NO available for vasodilation is a consequence of not only the rate of NO production, but also the rate at which it is consumed in the blood. Any increase in the rate of NO metabolism in the blood decreases the amount that can reach the vessel wall to cause vasodilation.

Although much of the NO entering the blood is converted to nitrate via reaction with oxyhemoglobin in red blood cells [2], various barriers to NO diffusing into the erythrocyte exist [3; 4; 5]. This enables a significant portion of NO to be consumed in the plasma even when red blood cells are present, as has been previously observed [6; 7]. The reactions consuming NO in plasma are not well characterized however. Although NO can react with O₂ in an autoxidation reaction that is second order with respect to NO [8; 9], in oxygenated aqueous buffer this reaction progresses too slowly to account for measured rates of plasma NO disappearance [6; 10; 11]. The copper-containing protein ceruloplasmin (Cp) has been proposed to mediate conversion of NO to nitrite and S-nitrosothiols (SNO) in plasma [6; 12], but the relatively rapid rate of NO disappearance from plasma is sustained even in the absence of Cp [13], suggesting other pathways are involved.

NO and O₂ are both three- to four-fold more soluble in biological lipids than in aqueous solutions. As a result, both reactants preferentially partition into hydrophobic compartments such as lipid micelles [14; 15; 16; 17; 18; 19; 20; 21; 22]. The resulting concentration increase of NO and O₂ within lipids is termed a “lens effect” [10; 15] and accelerates the rate of NO disappearance approximately 30-fold [19]. Thus, the presence of a hydrophobic lipid phase in plasma could “steal” NO from the reactions that would otherwise occur in the aqueous phase and significantly increase the overall NO autoxidation rate.

A single high-fat meal induces acute, transient hyperlipidemia and a concurrent loss of NO-mediated vasorelaxation known as *postprandial endothelial dysfunction* [23] (reviewed in [24]). This phenomenon has been proposed to result from a decrease in NO bioavailability (reviewed in [25] and [26]). Postprandial hyperlipidemia is characterized by triglyceride-rich lipoproteins (TRL) such as chylomicrons, very low-density lipoproteins and their remnants. Both endothelial dysfunction and elevated plasma triglycerides are thought to play important roles in atherosclerosis [26; 27; 28; 29; 30; 31; 32; 33; 34; 35], but the influence the lens effect may have on NO bioavailability, however, does not appear to have been fully considered. At a constant rate of endothelial NO production, increased NO consumption within TRL could decrease the amount of NO available for direct vasodilation and proper endothelial function. Furthermore, this diversion of NO to the lipid phase may result in a product profile which differs from that produced by NO consumption in aqueous phases.

The major products of NO consumption in the blood that are stable enough to circulate systemically are nitrate [36], nitrite [36], and SNO [7; 37]. Although nitrate is of physiological relevance via its conversion to nitrite by commensal bacteria in the mouth, it is inert in mammalian cells [38] and thus the bioactivity of NO is lost upon conversion to nitrate. In contrast, both nitrite [39; 40; 41] and especially SNO [42; 43; 44; 45; 46; 47] are potent vasodilators. Yet, despite much interest in their bioactivity (see review by Lundberg

and Weitzberg, 2010 [38]), relatively little is known about how these NO metabolites are produced in plasma.

In this study, we tested the hypothesis that increased plasma lipids after a single high-fat meal accelerate plasma NO consumption. We also hypothesize that NO reactions in postprandial plasma result in a different product profile than in fasted plasma. We test these hypotheses by measuring rates and products of NO consumption in plasma collected from normal healthy male subjects before and after ingestion of a high-fat meal.

2. MATERIALS AND METHODS

All procedures were conducted in accordance with human protocols pre-approved by the Loma Linda University Institutional Review Board and animal protocols pre-approved by the Loma Linda University Institutional Animal Care and Use Committee. Human volunteers were normal healthy men between 21 and 78 years of age (mean: 28 ± 3 , median: 24) with no known history of hypertension, hyperlipidemia or lipid metabolism disorder, and who gave informed consent prior to study. Sheep were used when large amounts of plasma were needed. Ewes obtained from Nebeker Ranch (Lancaster, CA) were between one and two years of age. Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. PROLI 1-(hydroxy-NNO-azoxy)-L-proline disodium salt (PROLI-NO) and triglyceride standard were obtained from Cayman Chemicals (Ann Arbor, MI).

2.1 Blood collection

uman blood was collected by forearm venipuncture. Sheep blood was collected from the external jugular vein. Blood samples were collected into heparinized syringes and plasma was prepared by centrifugation of the blood at 2,500 rpm for 15 minutes. Unless otherwise stated, experiments were performed at room temperature without shielding the samples from exposure to room air.

2.2 Addition of NO to samples

For *in vitro* experiments NO was derived from the donor compound PROLI-NO. It decomposes with a half-life ($T_{1/2}$) of ~1.8 sec at pH 7.4 and 37°C in a mono-exponential kinetic to produce two molecules of NO per molecule of donor. PROLI-NO is stable at high pH, and was made fresh daily and stored on ice at pH 12 (0.01 M NaOH) until injection into experimental samples. Experiments were performed by instillation of PROLI-NO to achieve final calculated concentrations of 0.5, 0.75, or 50 μM , in order to achieve NO concentrations of 1, 1.5 or 100 μM . This is referred to as “addition of 1, 1.5 or 100 μM NO” throughout the text.

The presence and disappearance of free NO was measured in a sealed reaction vessel (Harvard Apparatus, Holliston, MA) at 37°C which was equipped with a built-in propeller to ensure near instantaneous mixing of reactants. The vessel was filled to the brim to minimize any potential headspace gas and closed with a gas-tight seal. The vessel held a highly selective amperometric NO probe (ISO-NOP, World Precision Instruments, Sarasota, FL) with a response time of about two seconds and a detection limit of about 1 nM. NO

concentrations were recorded at a sampling rate of 1 Hz, and the probe was calibrated before each experiment over a range of 0.2 to 5 μM NO.

2.3 Elimination of NO in plasma

NO added to plasma disappears in a biphasic manner as shown in Figure 1A and as we and others have previously described [13, 37, 48, 49]. The first 1 to 2 μM of free NO added to plasma disappears faster than the response time of our NO probe. We herein refer to this process as the 'fast mode' of NO disappearance. Following saturation of the fast mode, further NO additions result in detectable NO with a measurable $T_{1/2}$, referred to here as the 'slow mode' of NO disappearance. However, the relative physiological importance of the fast and slow modes *in vivo* is unknown, as we do not know whether the fast mode is saturated *in situ*. Hence we investigated the NO disappearance and products formed from low amounts of NO (1 to 1.5 μM NO) to explore the reactions in the fast mode, and relatively high concentrations of NO (100 μM NO) to investigate the NO reactions in the slow mode.

As noted, the rate of NO disappearance from plasma during the fast mode was too rapid to quantify directly with our techniques. Instead, we used two different methods to quantify the amount of NO required to saturate the fast mode. For both methods, plasma was pretreated with carbon monoxide (CO) to block hemoglobin from reacting with NO. Sulfuric acid (18 M) was reacted with formic acid (24 M) to form CO gas, which was then scrubbed of particulates by passage through a 0.2 μm filter. Plasma was agitated with this CO (1:1 v/v) for three 10-minute intervals. CO dissolved in plasma was removed immediately before an experiment by agitating the plasma with room air for two 10-second intervals. Additionally, the effect of plasma thiols on NO metabolism was evaluated by inhibiting SNO formation. 10 mM NEM (N-ethylmaleimide) was used to irreversibly alkylate free thiols and prevent their reaction with NO.

In the first method, described in more detail earlier [13], PROLI-NO was added to stirred plasma as bolus injections to generate 1 μM NO each until the injections produced reproducible peaks as detected by the amperometric probe (Fig 1A). The amount of NO consumed by the fast mode reaction was determined as the amount of NO injected until consistent peaks were observed (Fig 1B). In the second method, we measured the amount of NO remaining in plasma after addition of various concentrations of NO (1 to 4 μM) to separate samples. After one minute, plasma samples were injected into a purge vessel containing PBS buffer (pH 7.4), sparged with argon and flowing into a chemiluminescence NO detector (Fig 1C). The difference between the calculated concentration of NO added to the plasma and the concentration of NO recovered one minute later was taken as the amount of NO consumed by the fast mode reaction (Fig 1D). See legend to Figure 1 for further description.

Note that the bimodal rate of NO disappearance is a property of the *plasma* because introduction of PROLI-NO into PBS results in an immediate, full and reproducible peak (Fig 1B, D and [13]).

2.4 Measurement of NO metabolites

NO metabolites were measured using triiodide chemiluminescence (NOA 280, Sievers, Boulder, CO), which detects NO gas and other NO_x including nitrite, SNO, iron-nitrosyls, and N-nitrosyls, but not nitrate [50, 51]. To distinguish between these detectable species, NO_x concentrations were determined before and after treatment of the samples with acidified sulfanilamide (0.5% for 3 min) which selectively removes nitrite. Concurrently, volatile free NO escapes the samples as they are agitated vigorously while open to the air for three minutes. Similarly, SNO concentrations were measured by the difference of NO_x concentrations in sulfanilamide-treated samples before and after treatment with 5 mM HgCl₂ (3 min), which selectively removes SNO. The NO_x signal remaining after sulfanilamide and HgCl₂ treatment was considered to reflect combined iron-nitrosyl and N-nitrosyl concentrations. However, these mercury-stable NO_x were below the limit of detection (15 nM) in our experiments. To measure only free and reversibly bound NO, samples were injected into PBS in a separate purge vessel that had never been exposed to triiodide, a precaution taken to eliminate any potential triiodide reagent contamination that would cause false-positive measurement of NO from reduction of other NO_x species. Fast mode NO metabolites were measured 1 minute after addition of 1.5 μM NO, while slow mode metabolites were measured over a time course of 20 seconds to 30 minutes after addition of 100 μM NO.

2.5 High-fat meal protocol

Subjects refrained from consuming food, alcohol, caffeine, over-the-counter medications, vitamin supplements and tobacco products for 12 hours before a study period. At 9 a.m. a fasting blood sample was collected. Subjects then ate a high-fat meal, consisting of 1 cup of whole milk, 2 hash brown patties fried in butter, 2 eggs fried in butter and 3 pieces of buttered, white bread toast. This meal has about 1100 calories with 680 calories from fat, 76 grams of fat (of which 43 grams are saturated fat) and 5633mg of cholesterol. Three hours after the meal, the postprandial blood sample was taken. This design was patterned after previous studies summarized by Mihos *et al* [52].

2.6 Triglyceride-rich lipoprotein particle isolation

In Thin-wall Ultra-Clear 17 mL tubes (Beckman-Coulter, Brea, CA), 8 mL of postprandial plasma was layered underneath 7 mL of 5 mM sodium phosphate buffer with 100 μM of the metal chelator DTPA at pH 7.4. Samples were centrifuged for 60 min at 25,000 rpm using an Optima XE ultracentrifuge with an SW 28.1 Ti Rotor (Beckman-Coulter, Brea, CA). The TRL micelles stratified atop the buffer were aspirated using sterile, low retention pipet tips. The density of the phosphate buffer was 1.002 ± 0.0005 g/mL, heavier than the average density of chylomicrons (<0.95 g/mL) and a majority of the very low density lipoproteins (0.95–1.006 g/mL), the largest low-density lipids micelles in the circulation [32].

2.7 Plasma delipidation and deoxygenation

Plasma was delipidated according to the method of Cham and Knowles [53], a procedure that removes triglycerides, cholesterol, phospholipids, and unesterified fatty acids from plasma without protein denaturation or altering ionic strength and pH. Briefly, plasma was

agitated for 30 min with a mixture of 1-butanol and di-isopropyl ether in a 50:40:60 (v/v/v) ratio. Phases were then separated by 2 min of centrifugation at 2000 rpm and the aqueous phase aspirated. The delipidation procedure was performed twice on each sample to assure maximal removal of the plasma lipids.

Deoxygenation experiments were performed with samples agitated four times for ten minutes with nitrogen (1:1 v/v), before being transferred to a hypoxic glove box capable of maintaining oxygen levels at <0.2%.

2.8 Ceruloplasmin oxidase activity

Cp oxidase activity was measured as described by Schosinsky *et al* [54]. Briefly, Cp oxidizes the substrate o-dianisidine dihydrochloride to a colored substance that is detected spectrophotometrically, as previously detailed [13].

2.9 Triglyceride measurement

Total plasma triglycerides were measured using the TR0100 Triglyceride Determination Kit according to manufacturer specifications (Sigma-Aldrich, St. Louis, MO).

2.10 Mathematical analysis of the effect of lipids on nitrite and s-nitrosothiol production

A simple mathematical analysis of plasma NO autoxidation was carried out based on the results of experiments described in section 3.3 and shown in Figure 4A–C, in which 1.5 μM NO was allowed to react in plasma for one minute. We assumed that nitrite forms at the same rate in the lipid phase, whether it resides in fasted or postprandial plasma. It was also assumed that nitrite production in the aqueous phase is the same in fasted and postprandial plasma. Then, based on these differential rates in lipid and aqueous phases and the measured volumes of the two phases, we calculated the increase in rate of NO autoxidation observed following the high-fat meal (described in detail in Appendix A).

2.11 Statistical analysis

Results are presented as mean $\pm$ SE. Normality was tested and non-parametric tests used when appropriate. Significance was determined using paired Student's t-test, Wilcoxon matched pairs test, 1-way ANOVA, or 2-way ANOVA for differences in NO_x in fasting and postprandial plasma over time. The $T_{1/2}$ of NO in plasma was determined from the rate of NO disappearance during the slow mode. The NO disappearance from the peak, down to 20% of peak was used to calculate the apparent $T_{1/2}$ by fitting a mono-exponential decay equation to the NO concentration versus time. Half-lives were determined for each plasma sample separately and averaged by group. Pearson correlation coefficients were determined for the relationships between triglyceride content and the NO concentration needed for the initial NO probe response (Fig 3B), and between triglyceride content of isolated TRL and nitrite formation from NO (Fig 4D). Statistical analyses were carried out using Prism 6.0 (Graphpad Software, La Jolla, CA) with significance accepted at $p = 0.05$.

3.1 Effect of a high-fat meal on plasma triglycerides and ceruloplasmin oxidase activity

Total plasma triglycerides were increased three hours postprandially in each of seven subjects, on average from 44 ± 8 mg/dL to 78 ± 12 mg/dL (Figure 2A, $p=0.001$). The plasma

delipidation protocol reduced triglyceride concentrations in fasted plasma to 7 ± 1 mg/dL ($p=0.008$) and in postprandial plasma to 6 ± 1 mg/dL ($p=0.002$). Cp oxidase activity was not affected by postprandial status or delipidation of the plasma (Fig 2B).

3.2 The fast mode of plasma NO disappearance

The concentration of NO needed to saturate the fast mode reaction and thus elicit a response by the amperometric probe was 1.5 ± 0.3 μ M in sheep plasma (Fig 1B). Less NO was required to saturate the fast mode reaction as measured by chemiluminescence (0.73 ± 0.1 μ M and 1.1 ± 0.1 μ M in human and sheep plasma respectively; Fig 1C, D). This suggests a portion of the NO added to plasma remained labile to sparging with argon but was not available to react with the NO probe, i.e. was reversibly sequestered. To account for this reversibly bound fraction of NO, the NO-consuming capacity of the fast mode as determined by the amperometric probe was used for metabolite experiments and to compare fasted and postprandial samples. Results in sheep plasma were qualitatively similar to those in human plasma with the distinction of a greater capacity of the fast mode to consume NO. They also show that free hemoglobin contributed less than 15% to NO consumption in plasma, and that thiols made no detectable contribution (Fig 1B).

In sheep plasma, removal of the lipid phase reduced the concentration of NO required to saturate the fast mode reaction from 1.5 ± 0.3 μ M to 0.8 ± 0.1 μ M (Fig 1B; $p=0.001$). In human plasma, consuming a high-fat meal increased the capacity of this fast mode reaction from 0.9 ± 0.2 μ M to 1.6 ± 0.4 μ M (Fig 3A; $p=0.03$). Additionally, the increase in the amount of NO needed to saturate the fast mode response in postprandial plasma correlated directly with the triglyceride concentration of the samples (Fig 3B; $r=0.62$, $p=0.006$). These findings demonstrate that lipids contribute to the fast mode consumption of NO in plasma.

3.3 Effect of high-fat meal on fast mode plasma NO reactions

We next investigated the products of NO reactions in the fast mode reaction by measuring free NO, SNO, and nitrite in fasted and postprandial plasma one minute after addition of 1.5 μ M NO. Less free NO remained in postprandial plasma (0.11 ± 0.05 μ M) than in matched fasting plasma (0.34 ± 0.09 μ M) (Fig 4C; $p=0.009$). Also, less SNO was formed in postprandial plasma (0.06 ± 0.02 μ M) than in fasted plasma (0.12 ± 0.02 μ M) (Fig 4B; $p=0.03$). At the same time, more nitrite was produced in postprandial plasma (0.17 ± 0.03 μ M) than in fasted plasma (0.11 ± 0.03 μ M) (Fig 4A; $p=0.002$). Consistent with NO autooxidation in lipid micelles, nitrite was produced when NO was introduced into samples of buffer with TRL isolated from postprandial plasma using ultracentrifugation (Fig 4D). In addition, the amount of nitrite formed one minute after addition of 2 μ M NO correlated positively with the triglyceride concentration in these samples ($r=0.79$, $p=0.02$), indicating that the rate of nitrite production from NO was increased in the presence of lipids.

3.4 Effect of plasma delipidation and deoxygenation on fast mode NO metabolism

Because NO, O₂, and lipid micelles are the three required components of the lens effect, we next investigated the role of the lens effect in determining the products of NO reaction in plasma by performing experiments after delipidation or deoxygenation of the plasma. In

general, plasma delipidation and deoxygenation resulted in metabolite changes opposite to the differences observed following the consumption of a high-fat meal.

One minute after addition of 1.5 μM NO, more free NO remained in deoxygenated ($1.08 \pm 0.1 \mu\text{M}$) and delipidated plasma ($0.87 \pm 0.07 \mu\text{M}$) than in untreated plasma ($0.47 \pm 0.1 \mu\text{M}$) collected from non-fasting subjects (Fig 5A, B; $p=0.003$ and $p=0.0002$ respectively). At the same time, more SNO was produced in deoxygenated ($0.44 \pm 0.05 \mu\text{M}$) and delipidated ($0.22 \pm 0.04 \mu\text{M}$) than in untreated plasma ($0.16 \pm 0.05 \mu\text{M}$) (Fig 5C, D; $p<0.0001$ and $p=0.02$ respectively). Contrastingly, less nitrite was produced in deoxygenated plasma ($0.12 \pm 0.06 \mu\text{M}$) than in untreated samples ($0.27 \pm 0.08 \mu\text{M}$) (Fig 5E; $p=0.04$). Because the chemicals used in the delipidation method introduced high nitrite contamination to our samples, we were unable to compare nitrite formation between untreated and delipidated plasma.

Despite the significant changes in mean concentrations noted above, considerable variation existed between subjects for both absolute concentrations of free NO, SNO and nitrite within a group (i.e. fasted and postprandial in Fig 4; and untreated, deoxygenated, and delipidated in Fig 5) and for the product yield ratios from the instilled NO. For instance, a relatively large nitrite yield did not necessarily correspond to an inversely proportionate small SNO yield in the same subject. We also noted significant differences in the combined amounts of free NO, SNO, and nitrite remaining after 1 minute. For Figure 4A–C we recovered $0.6 \pm 0.1 \mu\text{M}$ of the instilled NO from fasted plasma samples and $0.3 \pm 0.1 \mu\text{M}$ from postprandial samples ($p=0.05$). Compared to untreated plasma, delipidation resulted in an increase in the combined concentrations of NO and SNO ($0.6 \pm 0.1 \mu\text{M}$ versus $1.1 \pm 0.1 \mu\text{M}$ respectively; $p<0.0001$). And equally, compared to controls, deoxygenation resulted in an increase in the total NO, SNO and nitrite yield ($0.9 \pm 0.2 \mu\text{M}$ versus $1.6 \pm 0.1 \mu\text{M}$, respectively; $p=0.008$).

3.5 The slow mode of plasma NO disappearance

To assess the effect of postprandial plasma lipids on NO in the slow mode, we next studied the NO disappearance after saturation of the fast mode of NO handling. To saturate the fast mode reaction, we repeatedly injected 1 μM NO at 15-second intervals into samples until reproducible free NO peaks were observed with the amperometric probe, as in Fig 1A arrows 3–5. Figure 6 shows the NO concentration versus time curve of 1 μM NO in buffer samples with and without a synthetic hydrophobic phase of 100 mg/mL Triton X-100. The area under the curve (AUC), which is inversely proportional to the rate of NO disappearance, was used to quantify the overall rate of NO disappearance. Rates of NO disappearance were increased by $26 \pm 0.01\%$ when hydrophobic Triton X-100 micelles were added to the buffer (Figs 6A, B; $p=0.0002$). Additionally, with the micelles present the $T_{1/2}$ of NO in phosphate buffer was shortened from 142 ± 13 seconds to 99 ± 2 seconds ($p=0.02$). Addition of TRL, isolated from postprandial plasma, increased the rate of NO disappearance by $23 \pm 0.08\%$ (Figs 6C, D; $p=0.02$) and shortened the $T_{1/2}$ from 164 ± 17 to 117 ± 10 seconds ($p=0.009$). Additionally, the rate of NO disappearance in postprandial plasma samples increased by $20 \pm 0.08\%$ over that observed in fasted samples (Figs 6E, F; $p=0.05$). The $T_{1/2}$ of NO in postprandial plasma was shorter than in fasted plasma (90 ± 11 versus 117 ± 12 seconds;

p=0.02). These various results all demonstrate an increased rate of NO disappearance when a hydrophobic lipid phase (Triton X-100, TRL) is present in an aqueous solution.

3.6 Effect of a high-fat meal on slow mode plasma NO metabolism

To characterize the products of the slow mode NO reaction we added 100 μM NO to fasted and to postprandial plasma, reasoning that at this concentration <2% of the added NO would be consumed by the saturable fast mode reaction, leaving >98% of the NO to be consumed by the slow mode. We measured free NO, SNO, and nitrite concentrations at 20, 40 and 60 seconds and 5, 15 and 30 minutes after addition of PROLI-NO. Free NO fell to undetectable levels during the 30-minute period, and no differences between fasted and postprandial plasma were noted. SNO concentrations peaked at $9.5 \pm 0.4 \mu\text{M}$ within 20 seconds after addition of NO and then gradually declined to $6.9 \pm 0.3 \mu\text{M}$ during the 30 minutes. Nitrite concentrations continued to increase as long as free NO remained and were higher in postprandial samples (Fig 7A; 2-way ANOVA; $p < 0.01$). At the one minute time point, when a large majority of the reacted NO had been consumed by the slow mode reaction, $75 \pm 4 \mu\text{M}$ nitrite was formed in postprandial plasma versus $64 \pm 5 \mu\text{M}$ in fasted plasma (Fig 7B; $p = 0.02$).

3.7 Mathematical determination of the effect of lipids on NO metabolism

The total nitrite measured in the high-fat meal experiment (Fig. 4A) is the sum of nitrite formed in the lipid and aqueous phases of the plasma. In order to estimate the extent to which NO consumption was increased in the lipid phase versus the aqueous phase, we made a numerical determination of the amount of nitrite formed with respect to the estimated lipid volume in fasted and postprandial plasma. We assumed that the rate of nitrite formation per volume is the same in the lipid phase whether it is present in fasted (3.4% v/v lipids) or postprandial plasma (6% v/v lipids). Likewise, we assumed that the rate of nitrite formation per volume is the same in the aqueous phase in either plasma. These assumptions allowed us to calculate the nitrite formation in the lipid and aqueous phases. We found that our assumptions held true when applied to the experimental results and calculated that the nitrite formation rate was 48 times faster in lipids than in the aqueous plasma (see detailed calculation in Appendix A). Similar calculations were performed for SNO concentrations. However here our assumptions that the rates of SNO formation in the hydrophobic and aqueous phase respectively are the same whether the phase resides in fasted or postprandial plasma did not hold true. Hence no further conclusions could be made about the effect of lipids on the rate of plasma SNO formation.

4. DISCUSSION

The results of this study show significant effects of a single high-fat meal on NO consumption in plasma. NO disappears more rapidly from plasma collected 3 hours after ingestion of a high-fat meal, when triglyceride levels are elevated. This acceleration is reversed by the selective removal of these lipids, and is simulated by the addition of hydrophobic micelles, in the form of triglyceride-rich lipoproteins or Triton X-100 micelles, to phosphate buffer. The results of the study also show the product yields of NO reactions are altered in postprandial plasma, with more nitrite and less SNO produced compared to

fasted and delipidated plasma. These results are consistent with accelerated NO autoxidation in the presence of plasma lipids due to the concentration of NO and O₂ within TRL micelles, a lens effect.

4.1 Effect of a hydrophobic phase on nitrite and SNO production in plasma

NO consumption in oxygenated liquids occurs via an autoxidation reaction that is second order with respect to NO as follows:

NO disappearance by this reaction is also autocatalytic as NO reacts rapidly with the NO₂ produced in *Reaction 1* as follows:

It is noted that the rate laws of *Reactions 1* and *2* are largely unaffected by changes in pH or whether the NO and O₂ are dissolved in an aqueous or hydrophobic matrix [8, 15]. It should also be noted that nitrite and SNO production from NO are negligible in the absence of O₂ [9, 11, 56, 57], indicating that the oxidation of NO per *Reaction 1* is a prerequisite. The NO₂· and N₂O₃ produced in *Reaction 1* and *2* respectively, can serve as substrates for subsequent reactions that produce either nitrite or SNO, and it is likely that both sets of pathways occur in plasma [56] (also reviewed by Broniowska *et al* [58]).

Although chemical pathways have been described for the production of SNO from the NO₂· and N₂O₃ derived from *Reactions 1* and *2*, these reactions, as measured in aqueous buffer, are too slow to explain the yield of SNO from near-physiological concentrations of NO added to plasma [6, 11, 57, 58]. For example, the addition of NO to an oxygenated aqueous buffer with an excess of thiol substrate (such as glutathione), results in very little SNO production and virtually all of the NO being recovered as nitrite [6, 48, 59]. This suggests the existence of a specialized mode of SNO production from NO in plasma. One possibility is that SNO production is favored due to the localization of NO autoxidation within the hydrophobic regions of proteins [20], such as albumin [48, 60]. By this model, the products of *Reactions 1* and *2* would be preferentially localized near protein thiols, increasing the chance of SNO production versus nitrite. Although this model is supported by experiments using a high ratio of albumin to NO (150μM:6μM in Rafikova *et al* [48]), which emulates the physiological situation in plasma, it is not supported by experiments with lower (and less physiological) ratios (17μM:50μM in Keszler *et al* [56]). This suggests that the prevalence of this pathway of SNO production, compared to the competing nitrite-producing pathways, may vary with the concentration of NO added, which could explain why our yield of SNO did not vary between fasted and postprandial samples following addition of 100 μM NO.

The production of SNO from NO in plasma may also be catalyzed by Cp, a copper-containing oxidase [13, 61, 62]. Similar to the case of SNO production in hydrophobic regions of proteins, the role of Cp in SNO production was not observed experimentally at supraphysiological (100 μM) NO concentrations [6, 13], again suggesting that the rate of

SNO-producing pathways, relative to nitrite-producing pathways, may be decreased at higher NO concentrations.

Whether SNO production in plasma occurs via hydrophobic catalysis within proteins or by the action of Cp, our results are consistent with the idea that NO autooxidation within lipid micelles effectively competes with these pathways by diverting the production of the SNO-producing intermediates of NO autooxidation away from pathways that utilize them for SNO production, and resulting in nitrite production instead. Notably, previous work by Rafikova *et al* [59] and Ortiz *et al* [63] suggests that the effect of hydrophobic micelles on the production of SNO from NO in plasma may be more complex than simply diverting NO away from SNO-producing pathways. Their work demonstrates that the presence of synthetic perfluorocarbons (PFC) in an aqueous buffer containing glutathione actually potentiates the production of SNO from NO. Interestingly, this potentiating effect is dependent upon on the volume of hydrophobic phase, relative to the aqueous phase, with optimal activity at 0.4 to 1.0% v/v [20, 59]. Below 0.4% the SNO formation rate decreases very rapidly to zero in the absence of lipids. And as PFC concentrations increase above 1% v/v, the production of SNO from NO again decreases markedly, falling by 90% when PFCs are at 4% v/v. The results of the current experiments are consistent with this finding, since we observed peak SNO formation in delipidated samples that retained 0.5% v/v lipids (the level at which peak SNO-formation occurs according to Gordin *et al* [20] and Rafikova *et al* [59]). Likewise, in fasted (3.4% v/v lipids) less SNO formation was found, and postprandial samples (6% v/v lipids) had the least amount of SNO formation.

4.2 Effect of hydrophobic phases on the rate of NO consumption in plasma

The rates of NO disappearance and production of nitrite and SNO in aerated plasma are markedly faster than would be expected based on rates measured in aqueous buffer [6; 7; 11; 13], suggesting the existence of a catalyzed reaction in plasma. One possible explanation for this rapid disappearance is that NO may be oxidized by reaction with free hemoglobin in the plasma. However, our experiments indicate that the role of free hemoglobin in normal plasma is minor, since the amount of NO consumed by the fast mode reaction was decreased only by ~15% following repeated equilibration of the plasma with CO (Figure 1B and [13]). In addition, rapid NO consumption is found in plasma with undetectable hemoglobin or in which hemoglobin has been immunodepleted to low levels [49]. Cp, the major copper-containing oxidase, has been proposed to catalyze the production of both SNO [13; 61; 62] and nitrite [6] from NO in plasma (see above), but its overall contribution to the rate of NO consumption appears to be small, as demonstrated by similarly fast rates of NO disappearance in fetal and adult plasma compared to aqueous buffer, even though fetal sheep plasma is devoid of Cp activity [13]. Interestingly, Wang *et al* isolated an NO-consuming plasma fraction of only a dozen proteins that included apolipoproteins A, B, and E [37], a protein combination only found on TRL.

The lipid lens effect offers an alternative explanation for the accelerated rate of NO consumption in plasma. Due to its relative hydrophobicity, NO may partition initially into the plasma lipid phase. This effect may contribute to the fast mode of NO disappearance in plasma as shown in Fig 1B. Delipidation of the plasma caused a greater decrease in the

capacity of the fast mode of NO disappearance than blockade of plasma hemoglobin or thiols. However, it is worth noting that the abruptly saturable nature of the fast mode may indicate that NO entering the lipid phase binds tightly or reacts rapidly with a depletable component of the lipids. In addition, a significant portion of the fast mode reaction persisted after a combination of thiol and heme blockade and delipidation, suggesting other mechanisms may also play a role. In earlier work the addition of lipid micelles has been shown to accelerate the rate of NO autoxidation about 30-fold compared to the aqueous phase without micelles [10]. In the current experiments we also observed an increased rate of NO consumption in phosphate buffer following addition of either Triton X-100 micelles or the TRL isolated from postprandial plasma. We calculate that NO autoxidation is accelerated by ~48-fold in the presence of postprandial lipid micelles, a value in reasonable agreement with previous reports [10].

4.3 Hyperlipidemia and endothelial dysfunction

Many studies have shown that a single high-fat meal induces postprandial endothelial dysfunction (reviewed in [64, 65] and [24]) and that this dysfunction correlates with the presence of TRL micelles in plasma (reviewed in [24]). Likewise, various plasma hydrophobic phases attenuate endothelial NO-mediated vasorelaxation *in vivo* [66, 67, 68, 69, 70, 71, 72, 73], and a similar inhibition is observed *in vitro* where triglyceride-rich lipoprotein micelles inhibit NO-dependent vasodilation of arteries suspended in tissue baths [27, 28, 29, 74, 75]. Studies such as these have also determined that the oxidation of TRL increases their vasoconstriction potential [31] and, interestingly, that NO and O₂ are about twice as diffusible in oxidized than native TRL [18]. These studies point to the sequestration of NO in lipid micelles and its increased rate of oxidation as a contributing mechanism to postprandial endothelial dysfunction.

In vitro we found that the lipid phase in plasma accelerates the NO autoxidation and nitrite formation about 48-fold relative to that in an equal volume of aqueous phase, as determined in calculations included in Appendix A. Recent public health data now shows that about 25% of US adults overall have fasting triglyceride levels >150 mg/dL, the top-end of the normal range ([76] and Appendix B). However, even with fasting levels below this threshold, a typical American consuming a Western diet, spends about two-thirds of the day with triglyceride levels >150 mg/dL postprandially [77, 78]. At 150 mg/dL or 11.5% v/v lipids, more than 86% of nitrite formed would originate from the lipid phase of plasma.

Furthermore, as part of their normal metabolism, circulating TRL micelles localize at the glycocalyx, in between the flowing erythrocytes and the luminal wall of the endothelial cell [79, 80] where they can remain attached for minutes [81]. At this location TRL would have first-exposure to newly synthesized NO, potentially sequestering and metabolizing it before exposure to hemoglobin, a hypothesis of high interest to us. Similarly, hydrophobic atherosclerotic plaques of the luminal vessel wall could also potentially intercept endothelium derived NO and further disrupt vascular homeostasis.

Additionally, whether NO consumption in plasma produces nitrite as opposed to SNO may also be of importance for vascular homeostasis. Side-by-side comparisons available from the literature show that in normoxia the vasodilating potencies of SNO are markedly greater

than that of nitrite [42; 43; 44; 45; 46; 47]. Thus, the extent to which hydrophobic phases in the vascular lumen might alter the vasoactivity of NO and contribute to endothelial dysfunction *in vivo* should be explored in future studies.

4.4 Study Limitations

It should be reiterated that the current experiments were conducted in plasma only and hence further work is needed to establish the relevance of our findings to NO metabolism and bioactivity in whole blood and the intact vasculature.

We also noted a large and unexplained inter-subject variability in absolute concentrations of free NO, SNO and nitrite within treatment groups in our NO metabolism experiments (Figs 4 and 5). This finding suggests there is significant variation between individuals in plasma NO metabolism that is independent of plasma lipid or oxygen concentrations. And while we detected significant effects of plasma lipids in these experiments, the large variability between subjects may indicate that other factors not studied in this work play a significant role in plasma NO metabolism.

4.5 Conclusions

In summary, triglyceride-rich lipoproteins of dietary origin are capable of accelerating the disappearance of NO in isolated plasma. Additionally, autoxidation of NO to nitrite is found to occur at a rate 48 times faster within these TRL compared to the aqueous phase, markedly increasing the ratio of nitrite to SNO produced from NO reaction in plasma. These results raise the possibility that the lipid lens effect on NO metabolism may contribute to postprandial endothelial dysfunction, a hypothesis worthy of future study.

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ABBREVIATIONS

PROLI-NO	PROLI 1-(hydroxy-NNO-azoxy)-L-proline disodium salt
CO	carbon monoxide
NEM	N-ethylmaleimide
Cp	ceruloplasmin
DTPA	diethylene triamine pentaacetic acid
TRL	triglyceride-rich lipoproteins

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6. APPENDIX A: Calculation of nitrite production in lipid versus aqueous phases of plasma

Table A.1

Knowns and unknowns of the effect of a high-fat meal on plasma nitrite formation

	Total nitrite measured (amount in nanomoles)	Lipid volume (as fraction of total)	Aqueous volume (as fraction of total)	Nitrite of lipid origin (concentration in nM)	Nitrite of aqueous origin (concentration in nM)
Fasted plasma	0.226 (N_F)	0.034 (V_{Fl})	0.966 (V_{Fa})	? ($[N_{Fl}]$)	? ($[N_{Fa}]$)
Postprandial plasma	0.332 (N_P)	0.06 (V_{Pl})	0.94 (V_{Pa})	? ($[N_{Pl}]$)	? ($[N_{Pa}]$)

A final concentration of 1.5 μM free NO was incubated in 2 mL of plasma for one minute. The nitrite formed is a combination of that from the lipid and aqueous phases. In Table 1 N is nitrite, F indicates fasted plasma, P postprandial plasma, I denotes the lipid phase of plasma, and a the aqueous phase. Square brackets signify a concentration which, increasing from *zero* in a fixed volume during a one minute interval, becomes equivalent to the nitrite production rate.

$$N_F = [N_{Fl}] \bullet V_{Fl} + [N_{Fa}] \bullet V_{Fa} \quad \text{Eq. (A.1)}$$

$$N_P = [N_{Pl}] \bullet V_{Pl} + [N_{Pa}] \bullet V_{Pa} \quad \text{Eq. (A.2)}$$

We assume nitrite is made at the same rate in the lipid phase whether it is present in fasted or postprandial plasma, and thus:

$$[N_{Fl}] = [N_{Pl}] = [N_I] \quad \text{Eq. (A.3)}$$

Similarly, we assume that nitrite is made at the same rate in the aqueous phase whether it is present in fasted or postprandial plasma.

$$[N_{Fa}] = [N_{Pa}] = [N_a] \quad \text{Eq. (A.4)}$$

We can now rewrite equations (A.1) and (A.2) as

$$N_F = [N_l] \bullet V_{Fl} + [N_a] \bullet V_{Fa} \quad \text{Eq. (A.5)}$$

$$N_P = [N_l] \bullet V_{Pl} + [N_a] \bullet V_{Pa} \quad \text{Eq. (A.6)}$$

Because the total amount of nitrite formed has been measured and the respective volumes of lipid and aqueous phases are known we have two equations and two unknowns, namely the rates of nitrite formation in the two phases ($[N_l]$ and $[N_a]$).

Rearranging Eq. (A.5) for $[N_a]$:

$$[N_a] = \frac{(N_F - [N_l] \bullet V_{Fl})}{V_{Fa}} \quad \text{Eq. (A.7)}$$

And substituting into Eq. (A.6)

$$N_P = [N_l] \bullet V_{Pl} + \frac{(N_F - [N_l] \bullet V_{Fl})}{V_{Fa}} \bullet V_{Pa} \quad \text{Eq. (A.8)}$$

Solve Eq. (A.8) for $[N_l]$:

$$[N_l] = \frac{V_{Fa} \bullet N_P - V_{Pa} \bullet N_F}{V_{Pl} \bullet V_{Fa} - V_{Fl} \bullet V_{Pa}} \quad \text{Eq. (A.9)}$$

$$[N_l] = \frac{0.966 \bullet 0.332 - 0.94 \bullet 0.226}{0.06 \bullet 0.966 - 0.034 \bullet 0.94}$$

$$[N_l] = 4.16 \text{ nM}$$

This is the rate of nitrite production (per minute) in the lipid phase of postprandial plasma $[N_{Pl}]$. Substituting the result back into Eq. (A.6) we find the rate in the postprandial aqueous phase $[N_{Pa}]$:

$$[N_a] = \frac{(N_P - [N_l] \bullet V_{Pl})}{V_{Pa}} \quad \text{Eq. (A.10)}$$

$$[N_a] = \frac{(0.332 - 4.16430769 \bullet 0.06)}{0.94} \quad \text{Eq. (A.11)}$$

$$[N_a] = 0.0874 \text{ nM}$$

Comparing rates in lipid and aqueous phases of postprandial plasma.

$$\frac{[N_{Pl}]}{[N_{Pa}]} = \frac{4.16 \text{ nM}}{0.0874 \text{ nM}} = 47.6$$

Thus in postprandial plasma collected after the high-fat meal experiment, the lipid phase made about 48 times more nitrite per volume per minute than the aqueous phase.

A similar calculation may now be repeated for fasting plasma. And we note that if our assumptions that the nitrite formation rates are the same in the lipid and aqueous phases irrespective of whether they reside in the fasted or postprandial plasma are valid, then $[N_{Fl}]$ and $[N_{Fa}]$ will equal the $[N_{Pl}]$ and $[N_{Pa}]$ calculated above.

Solving Eq. (A.6) for $[N_l]$:

$$[N_l] = \frac{(N_P - [N_a] \bullet V_{Pa})}{V_{Pl}} \quad \text{Eq. (A.12)}$$

And substituting Eq. (A.12) into Eq. (A.5)

$$N_F = [N_a] \bullet V_{Fa} + \frac{(N_P - [N_a] \bullet V_{Pa})}{V_{Pl}} \bullet V_{Fl} \quad \text{Eq. (A.13)}$$

Rearranging Eq. (A.13) for $[N_a]$.

$$[N_a] = \frac{V_{Pl} \bullet N_F - V_{Fl} \bullet N_P}{V_{Pl} \bullet V_{Fa} - V_{Fl} \bullet V_{Pa}}$$

$$[N_a] = \frac{0.06 \bullet 0.226 - 0.034 \bullet 0.332}{0.966 \bullet 0.06 - 0.94 \bullet 0.034} \quad \text{Eq. (A.14)}$$

$$[N_a] = 0.0874 \text{ nM}$$

This is the rate of nitrite production for the lipid phase in fasted plasma $[N_{Fl}]$. Substituting the result back into Eq. (A.5) we find the rate in the fasted aqueous phase $[N_{Fa}]$:

$$[N_l] = \frac{(N_F - [N_a] \bullet V_{Fa})}{V_{Fl}} \quad \text{Eq. (A.15)}$$

$$[N_l] = \frac{(0.226 - 0.08738462 \bullet 0.966)}{0.034} \quad \text{Eq. (A.16)}$$

$$[N_l] = 4.16 \text{ nM}$$

This is the value for the rate of nitrite production in the lipid phase of plasma obtained from fasting subjects $[N_{Fl}]$. Comparing rates in lipid and aqueous phases:

$$\frac{[N_{Fl}]}{[N_{Fa}]} = \frac{4.16 \text{ nM}}{0.0874 \text{ nM}} = 47.6$$

The lipid phase of fasted plasma is again calculated to make about 48 times more nitrite per minute than it's aqueous phase. This indicates that the original assumption that the nitrite formation rate in lipid and aqueous phases is the same regardless of whether present in fasted or postprandial plasma is supported and the calculations are warranted.

7. APPENDIX B: Triglyceride ranges in plasma of fasting adults

	Normal	Borderline high	High	Very high
Triglycerides (mg/dL)	< 150	150 – 199	200 – 499	> 500
Lipid phase (%)	< 11.5 %	11.5 – 15.2 %	15.3 – 38.2 %	> 38.3 %

Taken from: Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA*. 2001;285:2486–2497

HIGHLIGHTS

- A major portion of plasma NO metabolism occurs in lipid micelles.
- Triglyceride-rich lipoproteins following a high-fat meal increase the rate of NO consumption in isolated plasma.
- Postprandial hyperlipidemia favors conversion of NO to nitrite over S-nitrosothiols in isolated plasma.

Fig. 1.

(A) Representative trace showing NO measured after repeated injections of 1 μM NO into a sample of human plasma. Arrow 1 shows missing peak and Arrow 2 shows a reduced peak where NO is consumed more rapidly than detectable by the sensing probe, termed the fast mode of NO disappearance. Arrows 3–5 show reproducible peaks after saturation of the fast mode consumption, termed the slow mode. (B) In sheep plasma, the fast mode is saturated after addition of $1.5 \pm 0.3 \mu\text{M}$ NO ($n=7$). The capacity of the fast mode for NO was not affected by blockade of thiols with NEM ($n=6$), but was decreased following blockade of plasma free hemes with carbon monoxide (CO, $n=6$) and plasma delipidation ($n=7$). (C) Plots of NO recovered (measured via chemiluminescence method) after adding NO to PBS and plasma pre-treated with NEM and CO. The depressed Y-intercept again suggests NO is rapidly cleared from plasma despite blockade of plasma hemoglobin and thiols. (D) Depressed Y-intercepts for human ($n=5$) and sheep ($n=6$) samples compared to PBS are -0.73 ± 0.1 and $-1.1 \pm 0.1 \mu\text{M}$ respectively. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Fig. 2.

(A) Triglyceride levels in plasma of subjects are shown to increase 77% after eating a high fat meal (n=6). Delipidation reduces triglycerides by 84% in fasted and 92% in postprandial plasma. (B) Cp oxidase activity does not change postprandially or with delipidation. (** p<0.01).

Fig. 3.

(A) NO metabolized in the fast phase increases ~80% in plasma from subjects after eating a high-fat meal. (B) Fast phase consumption of NO varies with triglyceride concentration in the plasma. Individual responses from fasted (●) to postprandial (○) are shown for 8 subjects. (* $p < 0.05$).

Fig. 4.

NO metabolites formed during the fast mode. More nitrite (A) but less SNO (B) is formed, and less free NO (C) remained in plasma from postprandial subjects than when they were fasting ($n=12$). (D) Nitrite formed after addition of NO into phosphate buffer with triglyceride-rich lipoproteins taken from human postprandial plasma after ultracentrifugation ($n=8$). A positive correlation between the amount of triglycerides in buffer and nitrite yield is shown ($r=0.79$; $p=0.02$). (* $p<0.05$, ** $p<0.01$).

Fig. 5.

NO metabolites formed during the fast mode reaction in untreated, deoxygenated ($\text{O}_2 < 0.2\%$) and delipidated human plasma. A, C, and E show that decreasing O_2 levels result in more SNO, less nitrite formation, and more remaining free NO one minute after addition of $1.5 \mu\text{M}$ NO. Similarly, B and D show more free NO remains and more SNO is formed in delipidated than untreated plasma. (n=8). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fig. 6.

Time course of changes in free NO levels (nA) measured in buffers (A–D) and plasma (E, F) after NO is introduced and the fast phase of NO consumption has been saturated. A and B show less free NO following addition of 100 mg/ml Triton X-100 to the buffer (which creates a suspension of hydrophobic micelles), than in buffer alone (n=4). Similarly, C and D show less NO after addition of postprandial TRL (triglyceride-rich lipoproteins) to buffer (n=9). E and F show less NO after addition of NO to postprandial plasma than to plasma from fasted subjects (n=6). (* $p < 0.05$, *** $p < 0.001$).

Fig. 7.

(A) Time course of metabolites formed following addition of 100 μM NO to plasma samples collected from subjects before and after a high-fat meal consumption (n=7). Significantly more nitrite is formed in postprandial than in fasted plasma at each time point (2-way ANOVA). Differences for fasted and postprandial plasma SNO and free NO are not significant. B shows differences for the individual subjects at one minute where postprandial samples produced measurably more nitrite (* p<0.05).