Characterization of Linoleic Acid Nitration in Human Blood Plasma by Mass Spectrometry†

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ABSTRACT: Nitric oxide (NO) is a pervasive free radical species that concentrates in lipophilic compartments to serve as a potent inhibitor of lipid and low-density lipoprotein oxidation processes. In this study, we synthesized, characterized, and detected nitrated derivatives of linoleic acid (18:2) in human blood plasma using high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. While the reaction of nitronium tetrafluoroborate with 18:2 presented ions with a mass/charge (m/z) ratio of 324 in the negative ion mode, characteristic of nitrolinoleate (LNO2), the reaction of nitrite (NO2 ) with linoleic acid hydroperoxide yielded nitrohydroxylinoleate (LNO2OH, m/z 340). Further analysis by MS/MS gave a major fragment at m/z 46, characteristic of a nitro group (–NO2) present in the parent ion. This was confirmed by using [15 N]NO2, which gave products of m/z 325 and 341, that after fragmentation yielded a daughter ion at m/z 47. Moreover, a C—NO2 structure was also demonstrated in LNO2OH by nuclear magnetic resonance spectroscopy (15 N NMR, δ 375.9), as well as by infrared analysis in both LNO2OH (νmax = 3427, 1553, and 1374 cm−1) and LNO2 (νmax = 1552 and 1373 cm−1). Stable products with m/z of 324 and 340, which possessed the same chromatographic characteristics and fragmentation pattern as synthesized standards, were found in human plasma of normolipidemic and hyperlipidemic donors. The presence of these novel nitrogen-containing oxidized lipid adducts in human plasma could represent “footprints” of the antioxidant action of NO on lipid oxidation and/or a pro-oxidant and nitrating action of NO-derived species.

The simultaneous production of nitric oxide (NO)1 and oxygen free radicals, observed in different pathological states, can result in the formation of NO-derived reactive intermediates such as the nitrogen dioxide radical (NO2) and the peroxyxynitrite anion (ONOO−). Even though NO-derived metabolites may exert oxidative modifications in membranes and on low-density lipoprotein (LDL) (1—6), NO itself inhibits lipid oxidation-dependent processes. In fact, NO has multiple physicochemical properties that make it an effective lipid antioxidant. Nitric oxide (a) more avidly reacts with unsaturated lipid reactive species such as alkyl (R*), epoxy-allylic [R(O)•], alkoxyl (RO•), or peroxy (ROO•) radicals to yield nitrogen-containing radical—radical termination products (5, 7, 8), (b) has a partition coefficient of 6.5 for n-octanol/H2O, permitting concentration in lipopholic milieu such as the hydrophobic core of LDL (9, 10), (c) does not affect the physical properties of membranes or LDL because of its small molecular radius, and (d) by virtue of its high reactivity with lipid radicals will protect other lipophilic antioxidants from oxidation (11).

Several in vitro studies have demonstrated the presence of nitrogen-containing products of polyunsaturated fatty acids, including alkynitrates (RONO), alkynitrates (ROONO and RONO3), alkylepoxyxynitrite R(O)NO2, alkylhydroxynitrite (RNO2OH), and nitrolipids (RONO3) that could represent “footprints” of the in vivo pro-oxidant and/or antioxidant actions of NO (12—17). Moreover, antiplatelet actions of a nitrated lipid (nitrolinoleate) have recently been observed, an effect that would be considered vascular-protective (12). Little is known about the chemistry of these nitrogen-containing oxidized lipids. Some products appear to be highly unstable and may decompose to reinitiate radical processes. In particular, the product of the ROO•NO condensation reaction in aqueous solution may be hydrolyzed to form a lipid hydroperoxide and nitrite (NO2−) (12, 14). Alternatively, ROONO may be cleaved by homolysis to RO• and NO2− with rearrangement of RO• to R(O)•, followed by recombina-
tion of R(O·) with *NO 2 to form R(O)NO 2 , a more stable product (14). Importantly, reaction products of *NO-derived species with arachidonic acid include nitrohydroxy eicosanoids (RNO2 OH), which release *NO and may be important endogenous mediators of vascular relaxation by soluble guanylate cyclase activation (16).

Herein, we report for the first time the presence of stable linoleic acid-derived nitrated products in human plasma of normolipidemic and hyperlipidemic subjects. The in vivo detection of these novel products can be an important way to better elucidate the role of *NO and reactive nitrogen species in regulating vascular oxidative reactions.

MATERIALS AND METHODS

Materials. Linoleic acid (18:2) was obtained from Nu-Check-Prep (Elysian, MN). 2,2'-Azobisis(2,4-dimethylvaleronitrile) (AMVN) was from Wako Chemicals USA (Richmond, VA). Chromatographic grade 2-propanol and methanol were obtained from Merck (Gibbstown, NJ). All other reagents were from the Sigma Chemical Co. (St. Louis, MO).

Synthesis of Linoleic Acid Hydroperoxide. Hydroperoxylinolenate (18:2-OOH) was synthesized as previously described (18) with modifications. Briefly, 18:2 (0.16 mmol) was incubated in 1 mL of a 2:1 (v/v) chloroform/methanol mixture for 120 min at 37 °C in the presence of 2 mM AMVN. Subsequently, 18:2-OOH was purified by high-pressure liquid chromatography (HPLC) using a 5 μm particle size, semipreparative LC18DB column (Supelco, Bellefonte, PA) eluted with a 1:1 (v/v) methanol/tert-butyl alcohol mixture and monitoring at 234 nm. For the nuclear magnetic resonance spectroscopy (NMR) and infrared (IR) analysis, 18:2-OOH was synthesized by photooxidation using methylene blue as the singlet oxygen source and further purified by a silica gel chromatographic column, according to the method of ref 19.

Nitration of Linoleic Acid. Sodium nitrite (either 14N or 15N, 1 mM) was added to 0.10 mmol of 18:2-OOH or 0.16 mmol of 18:2 in 200 μL of a 2:1 (v/v) chloroform/methanol mixture, following acidification to pH 3.0 with 1 N HCl and incubation at 25 °C for 15 min. One milliliter of 0.1 M phosphate buffer (pH 7.4) was added, and extraction was carried out with diethyl ether. The organic layer was separated, dried, and analyzed by liquid chromatography–electrospray ionization tandem mass spectrometry analysis (LC–ESI/MS/MS). Linoleic acid nitration was also carried out with nitronium tetrafluoroborate (NO2 BF4). The reaction was performed as described previously (20) with modifications. In brief, a solution of either 18:2 (0.16 mmol) or 18:2-OOH (0.10 mmol) in chloroform (2 mL) was purged with nitrogen, and solid NO2 BF4 was added (0.12 mmol). The mixture was kept under a nitrogen atmosphere at room temperature overnight, and then 1 mL of 0.1 M phosphate buffer (pH 7.4) was added. The organic layer was separated, dried, and analyzed by LC–ESI/MS/MS.

Nuclear Magnetic Resonance Spectroscopy (NMR) and Infrared (IR) Analysis. Na15NO2 (1.07 mmol) was added to 0.64 mmol of 18:2-OOH in 3 mL of methanol at pH 3.0. Following incubation at 25 °C for 15 min, lipids were extracted using diethyl ether and dried under a nitrogen flow. Lipids were dissolved in 800 μL of CdCl2, and spectra were collected at 300 K on a Bruker DRX500 spectrometer with the following acquisition parameters: 9888 scans, a 15 s recycle time, composite-pulse decoupling during the acquisition time (0.33 s), a 30° pulse width, and a 22 831 Hz spectral width. Data were expressed as chemical shifts in reference to nitromethane. Similar synthesis procedures were performed to obtain samples for infrared analysis. Infrared spectra were obtained with a Bomem MB 100 spectrometer by accumulating 128 scans between 400 and 4000 cm−1.

Lipid Extraction from Human Blood Plasma. Human blood from five normolipidemic (<200 mg of cholesterol/dL) and four hyperlipidemic subjects (200–400 mg of cholesterol/dL) was collected after overnight fasting in tubes containing ethyldiaminetetraacetic acid (EDTA). Plasma was obtained after blood centrifugation at 2500 rpm for 10 min at 4 °C, immediately extracted, and analyzed. Plasma (500 μL) was mixed with 500 μL of acidified methanol (pH 3.0) containing an internal standard (heptadecanoic acid, 17:0, 100 pmol) and stirred at 8 rpm for 20 min. Then, 5 mL of diethyl ether containing 0.02% butylated hydroxytoluene (BHT) previously treated with Chelex, to avoid further lipid oxidation during lipid extraction, was added. Samples were vortexed (2 min) and centrifuged at 2500 rpm for 5 min at 4 °C. The upper layer was collected, filtered (0.22 μm), and evaporated to dryness in a vacuum rotary evaporator. Lipids were dissolved in 100 μL of a 1:1 (v/v) 2-propanol/methanol mixture, and then 10 μL was immediately injected into the
Linoleic Acid Nitration. When 18:2 was incubated with NO₂BF₄, the main reaction products, ions with m/z 324 ([M – H]⁻), eluted at 10–12 min, being characteristic of nitrolinoleate (LNO₂, Figure 1A). After fragmentation, a

\[ \text{N} = \text{O of nitro (asym), RNO}_2 \]
\[ \text{N} = \text{O of nitro (sym), RNO}_2 \]
\[ \text{N} = \text{O of nitrito, RONO} \]
\[ \text{N} = \text{O of nitrate, RONO}_2 \]
\[ \text{OH of hydroxyl} \]
\[ \text{C} = \text{O of carbonyl} \]
\[ \text{C} = \text{H} \]

<table>
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<tr>
<th>functional group stretch</th>
<th>linoleate</th>
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<th>nitrohydroxylolate</th>
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<tr>
<td>N=O of nitro (asym), RNO₂</td>
<td>-</td>
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<td>2930</td>
<td>2927</td>
<td>1725–1695</td>
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Stability Studies. The stability of the 18:2 derived nitrated standards at 4 and −70 °C was evaluated by analyzing aliquots at the same day of preparation (day 0) and daily for 7 days. In addition, blood plasma was analyzed at day 0 and plasma aliquots were kept at −20 °C and further analyzed by LC–ESI/MS/MS in intervals of 24 h over the course of 7 days.

RESULTS AND DISCUSSION

Linoleic Acid Nitration. When 18:2 was incubated with NO₂BF₄, the main reaction products, ions with m/z 324 ([M – H]⁻), eluted at 10–12 min, being characteristic of nitrolinoleate (LNO₂, Figure 1A). After fragmentation, a
daughter main ion having an \( m/z \) of 46 (\( [^{14}\text{NO}_2^-] \)) was obtained. Ions of \( m/z \) 306, formed by loss of water (\( [(M - H_2O) - H^-] \)), and of \( m/z \) 277, formed by the loss of \( -\text{NO}_2 \) (\( [(M - ^{14}\text{NO}_2) - H^-] \)), were also observed (Figure 1B). Under these conditions, no 18:2 oxidation products were detected. On the other hand, when 18:2 was reacted with Na\(^{14}\text{NO}_2 \) at acidic pH, a small amount of the ion with \( m/z \) 324, having the same chromatographic characteristics and fragmentation pattern as the product synthesized in larger amounts with \( \text{NO}_2\text{BF}_4 \), was formed. The presence of an \(-\text{NO}_2 \) group on the ion with an \( m/z \) of 324 was confirmed.

**Table 2: Presence of LNO\(_2\) (m/z 324) and LNO\(_2\)OH (m/z 340) in Blood Plasma of Normolipidemic and Hyperlipidemic Subjects**

<table>
<thead>
<tr>
<th>Subject</th>
<th>LNO(_2) (m/z 324)</th>
<th>LNO(_2)OH (m/z 340)</th>
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<tr>
<td>5N (M, 26)</td>
<td>0.58</td>
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<tr>
<td>6N (F, 25)</td>
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<td>7N (F, 47)</td>
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<tr>
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<td>0.43</td>
<td>ND*</td>
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<tr>
<td>9N (F, 20)</td>
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</tr>
<tr>
<td>1H (F, 57)</td>
<td>0.96</td>
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</tr>
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<td>2H (F, 58)</td>
<td>0.57</td>
<td>0.18</td>
</tr>
<tr>
<td>3H (F, 56)</td>
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<td>0.59</td>
</tr>
<tr>
<td>4H (M, 61)</td>
<td>2.50</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* M, male; F, female (M/F, age); N, normolipidemic; H, hyperlipidemic. * Results are expressed as the ratio of total integrated peak areas for \( m/z \) species of interest and the internal standard (17:0, \( m/z \) 269). * Not detected.
by reacting Na^{15}NO_3 with 18:2. This reaction yielded a main ion at \( m/z \) 325, that upon fragmentation resulted in an ion characteristic of \([^{15}\text{NO}_2]^{-}\) at \( m/z \) 47 (Figure 1C). Whereas LC-MS verifies the presence of a compound with \( m/z \) 324 and a MS/MS daughter ion of \( m/z \) 46, this does not distinguish between a nitro (\(-\text{NO}_2\)) or a nitrite (\(-\text{ONO}\)) functional group. It is important to distinguish between the formation of LNO_2 and LONO because a great difference in stability and possibly biological activity of these products is expected. The IR spectrum of the compound with \( m/z \) 324 showed a characteristic absorption of a nitro group directly attached to the carbon chain (1552 and 1373 cm\(^{-1}\), Table 1). No bands occurred in the 1600–1680 cm\(^{-1}\) region where the N=O bond of a RONO or RONO_2 strongly absorbs (21). It is clearly demonstrated that the product formed from the reaction between 18:2 and NO_2BF_4 is LNO_2, which is consistent with other reports (14, 17). Moreover, products having main ions of \( m/z \) 324 were previously detected by reaction of 18:2 with peroxynitrite at pH 7.4 (14), clearly showing the possibility of formation of this product in vivo.

The reaction of 18:2-OOH with sodium \([^{14}\text{N}]\text{nitrite under acidic conditions formed products having mainly ions with } m/z \) 340 ([M – H]\(^{-}\)) that eluted at 6–8 min (Figure 2A). When fragmented on MS/MS, a major ion with \( m/z \) 46 (\([^{14}\text{NO}_2]^{-}\)) and minor ions with \( m/z \) 322, formed by the loss of water \([([M – \text{H}_2\text{O}] – \text{H}]^{-}\), and others of \( m/z \) 293, formed by the loss of \(-\text{NO}_2 \) \([([M – \text{H}^{14}\text{NO}_2] – \text{H}]^{-}\) ] (Figure 2B), were observed. Moreover, when 18:2-OOH reacted with sodium \([^{15}\text{N}]\text{nitrite at acidic pH, products with the same retention time having } m/z \) 341 were formed, showing similar daughter ions mainly of \( m/z \) 47 (Figure 2C). These results strongly suggest the formation of LOONO, LONO_2, L(O)NO_2, and/or LNO_2OH structures. The presence of a nitro group was demonstrated by \([^{15}\text{N}]\)NMR analysis of the \( m/z \) 340 compound which exhibited a chemical shift of 375.9 ppm, which confirms previously described data (17) and suggests the presence of a nitro group directly bound to a carbon atom (Figure 3B). The IR spectrum of this product, when compared with that of 18:2, exhibited novel bands at 1553 and 1374 cm\(^{-1}\) (Figure 3A), corresponding to the N=O binding of RNO_2 and a strongly absorbing band at 3427 cm\(^{-1}\), corresponding to a hydroxy group (21). Neither bands in the 1640–1620 cm\(^{-1}\) range, characteristic of RONO_2 species, nor bands at 877–830 cm\(^{-1}\), characteristic of an epoxy group (21), were observed. This strongly suggests that the main product formed in this reaction is LNO_2OH, as recently described (17). Finally, the reaction of NO_2BF_4 with 18:2-OOH yielded both \( m/z \) 324 and 340 products, having retention times of 10–12 and 6–8 min, respectively (data not shown). This is in agreement with an alkyl linoleate radical (L’) reaction with \(^{15}\text{NO}_2 \) which forms LNO_2, and an alkoxyl linoleate radical (L^0) reaction with \(^{15}\text{NO}_2 \) which yields LNO_2OH (17). The mean yield of both LNO_2 and LNO_2OH products was approximately 37.5\% of 18:2 or 18:2-OOH, respectively, as estimated in relation to heptadeca-noic acid (\[m/z \) 269 ([M – H]\(^{-}\)) used as an internal standard. Even though a significant contribution toward the elucidation of the structure of nitrated lipids was achieved, it was not possible to determine the position of the nitro group in LNO_2 or LNO_2OH because several positional isomers can be formed in the reaction systems used to synthesize these compounds (14, 17).

Identification of Nitrated Lipids in Plasma. The analysis of human blood plasma showed a major product having ions of \( m/z \) 324 and exhibiting the same fragmentation pattern and retention time as products synthesized by the reaction of 18:2 with NO_2BF_4 or NO_2^{-} at acidic pH (Figure 4). A second group of products was found in a smaller amount, eluting at 6–8 min and having an \( m/z \) of 340 with the same fragmentation pattern as the standards synthesized with 18:2-OOH and sodium nitrite at acidic pH (Figure 5). Thus, LNO_2 and LNO_2OH were detected and characterized in fresh
human plasma of normolipidemic and hyperlipidemic donors (Table 2). These products represent percent ion intensities of 4.1 and 0.7% for the ions at m/z 324 and 340, respectively, in plasma samples, as evaluated by using heptadecanoic acid (10 pmol) as the internal standard. Small differences in the spectra fragmentation pattern between standards and plasma products could be explained by the presence of different positional isomers and/or functional group orientations in vivo. For instance, it is possible that the ion with m/z 324, detected in plasma samples, is LNO; and/or LONO. Similar nitrated products were found when plasma extraction was carried out at either pH 3 or 7, indicating that linoleate nitration products are not artifacts formed due to acidic extraction/hydrolysis conditions (Figure 6).

A significant increase in the amount of 18:2-nitrated products was observed in the hyperlipidemic group compared with the amounts in their normolipidemic counterparts (Figure 7). First, this might be favored because of the increase in the level of reactive nitrogen species formation during lipid peroxidation in vivo (Figure 7). First, this might be favored because of the increase in the level of reactive nitrogen species formation during lipid peroxidation in vivo (Figure 7). For instance, it is possible that the ion with m/z 324, detected in plasma samples, is LNO; and/or LONO. Similar nitrated products were found when plasma extraction was carried out at either pH 3 or 7, indicating that linoleate nitration products are not artifacts formed due to acidic extraction/hydrolysis conditions (Figure 6).

In summary, as far as we know, this is the first report of the presence of nitrated lipids in human blood plasma. These compounds may act as potential markers of the chain-breaking antioxidant role of *NO, during lipid peroxidation (5–8, 11), reinforcing the in vivo antioxidant activity of *NO. Due to its relative hydrophobicity, *NO readily partitions into hydrophobic lipid membranes and lipoproteins (10), greatly increasing its local concentration. This property will significantly increase the efficiency of *NO as a terminator of lipid peroxidation in vivo (34). Therefore, these nitrated lipids in blood plasma can be primarily considered as biomarkers of the inhibitory role of *NO on lipid peroxidation, and/or a footprint of the presence of oxidative and nitrating agents in the vascular system.

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REFERENCES


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