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

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Received January 7, 2002; Revised Manuscript Received June 11, 2002

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-pressure 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 (LNO_2), the reaction of nitrite (NO_2^-) with linoleic acid hydroperoxide yielded nitrohydroxylinoleate (LNO_2OH, *m*/*z* 340). Further analysis by MS/MS gave a major fragment at *m*/*z* 46, characteristic of a nitro group ($-NO_2$) present in the parent ion. This was confirmed by using $[^{15}N]O_2$, which gave products of *m*/*z* 325 and 341, that after fragmentation yielded a daughter ion at *m*/*z* 47. Moreover, a $C-NO_2$ structure was also demonstrated in LNO_2OH by nuclear magnetic resonance spectroscopy (^{15}N NMR, δ 375.9), as well as by infrared analysis in both LNO_2OH ($\nu_{max} = 3427$, 1553, and 1374 cm^{-1}) and LNO_2 ($\nu_{max} = 1552$ 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 ($•NO_2$) and the peroxynitrite 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•), epoxyallylic [R(O)•], alkoxyl (RO•), or peroxyl (ROO•) radicals to yield nitrogen-containing radical−radical termination products (*5*, *7*, *8*), (b) has a partition coefficient of 6.5 for *n*-octanol/H_2O, permitting concentration in lipophilic 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 alkylnitrites (RONO), alkylnitrates (ROONO and $RONO_2$), alkylepoxynitrite $R(O)NO_2$, alkylnitrohydroxy (RNO_2OH), and nitrolipids (RNO_2) 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 (NO_2^-) (*12*, *14*). Alternatively, ROONO may be cleaved by homolysis to RO• and $•NO_2$, with rearrangement of RO• to R(O)•, followed by recombina-

† This work was supported by FAPESP (Grant 97/05090-3 to D.S.P.A. and scholarship to E.S.L.), PRONEX (D.S.P.A.), and Fogarty-NIH (H.R.).

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[1] Abbreviations: •NO, nitric oxide; $•NO_2$, nitrogen dioxide; 17:0, heptadecanoic acid; 18:2, linoleic acid; 18:2-OOH, linoleic acid hydroperoxide; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); HPLC, high-pressure liquid chromatography; IR, infrared; R•, alkyl radical; R(O)•, epoxyallylic radical; $L(O)NO_2$, nitroepoxylinoleate; LC−ESI/MS/MS, liquid chromatography−electrospray ionization tandem mass spectrometry; LDL, low-density lipoprotein; LNO_2, nitrolinoleate; RO•, alkoxyl radical; LONO, nitritelinoleate; $LONO_2$, nitratelinoleate; ROO•, peroxyl radical; LNO_2OH, nitrohydroxylinoleate; LOOH, lipid hydroperoxide; MRM, multiple-reaction monitoring; MS/MS, tandem mass spectrometry; NO_2^-, nitrite; NO_2BF_4, nitronium tetrafluoroborate; NMR, nuclear magnetic resonance spectroscopy.

10.1021/bi025504j CCC: $22.00 © 2002 American Chemical Society
Published on Web 07/31/2002

tion of R(O)$^{\bullet}$ with $^{\bullet}NO_2$ to form R(O)NO_2, a more stable product (*14*). Importantly, reaction products of $^{\bullet}$NO-derived species with arachidonic acid include nitrohydroxy eicosanoids (RNO_2OH), which release $^{\bullet}$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 $^{\bullet}$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, MI). 2,2′-Azobis(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. Hydroperoxolinoleate (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 ^{14}N or ^{15}N, 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 (NO_2BF_4). 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 NO_2BF_4 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. $Na^{15}NO_2$ (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. Samples were dissolved in 800 μL of $CdCl_3$, 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}.

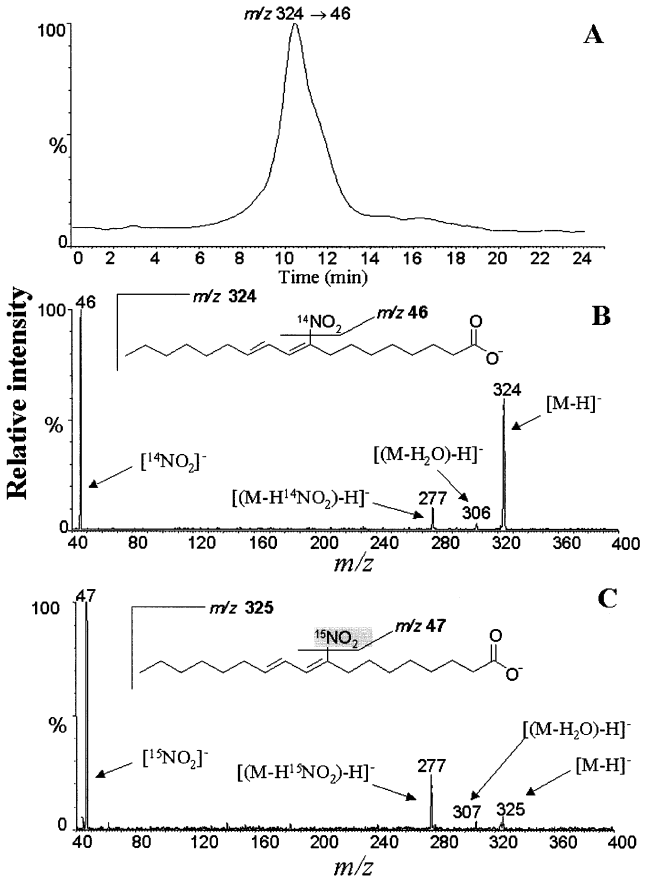

FIGURE 1: Detection of the LNO_2 (*m*/*z* 324) standard by LC−ESI/MS/MS analysis. (A) MRM [*m*/*z* 324 → 46 (selecting ion of *m*/*z* 324 in Q_1 and *m*/*z* 46 in Q_3)] chromatogram. (B) MS/MS spectra fragmentation patterns of the product generated by reaction of 18:2 with NO_2BF_4. (C) MS/MS spectra fragmentation patterns of product with *m*/*z* 325 generated by reaction of 18:2 with sodium [^{15}N]nitrite.

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

Table 1: Infrared Wavenumbers (cm^{-1}) of Linoleic Acid Nitrated Products

functional group stretch	linoleate	nitrolinoleate	nitrohydroxylinoleate	literature data (*21*)
N=O of nitro (asym), RNO_2	–	**1552**	**1553**	1560–1548
N–O of nitro (sym), RNO_2	–	**1373**	**1374**	1397–1310
N=O of nitrito, RONO	–	–	–	1680–1613
N=O of nitrate, $RONO_2$	–	–	–	1640–1620
OH of hydroxyl	–	–	**3427**	3570
C=O of oxide	–	–	–	877–830
OH of carboxylic acid	3009	3010	3009	3335–2500
C=O carbonyl	1709	1709	1712	1725–1695
C–H	2919	2930	2927	2940

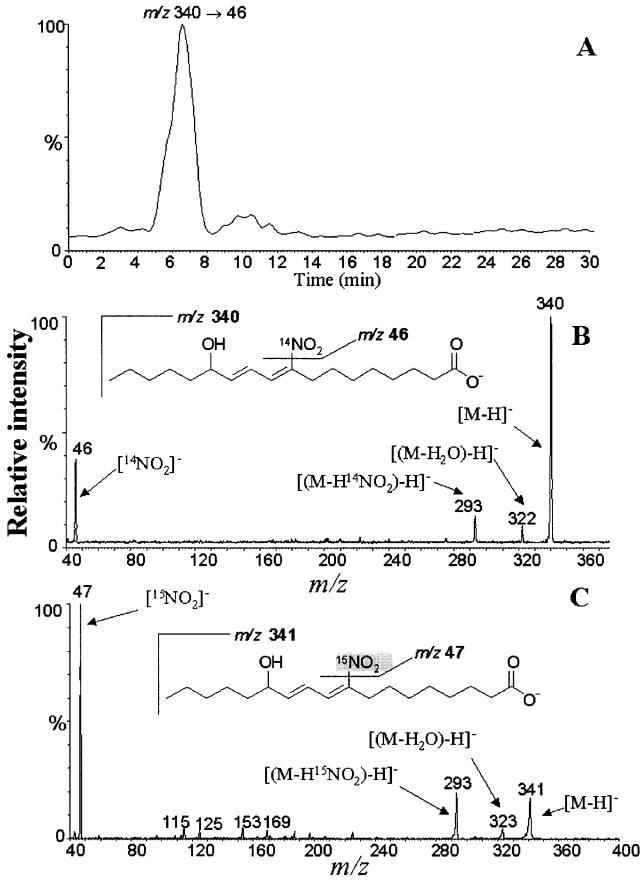

FIGURE 2: Detection of LNO_2OH (*m*/*z* 340) standards by LC–ESI/MS/MS analysis. (A) MRM [*m*/*z* 340 → 46 (selecting ion of *m*/*z* 340 in Q_1 and *m*/*z* 46 in Q_3)] chromatogram. MS/MS spectra fragmentation patterns of products generated by reaction of 18:2-OOH with sodium [^{14}N]nitrite (B) or sodium [^{15}N]nitrite (C).

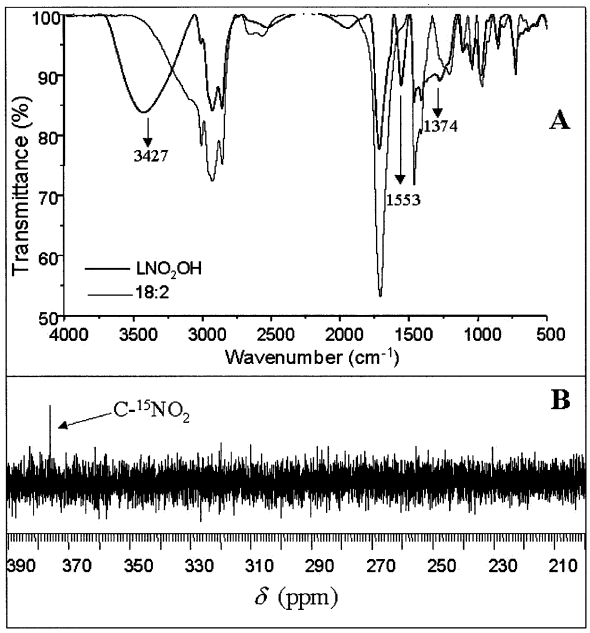

FIGURE 3: Infrared (A) and ^{15}N NMR (B) spectra of the reaction products of $Na^{15}NO_2$ with 18:2-OOH at pH 3.0. Sample preparation and spectra acquisition parameters are described in Experimental Procedures.

LC–MS system. Alternatively, lipid extraction was carried out in the presence of methanol/potassium phosphate buffer at pH 7.0 to avoid acidic conditions.

Mass Spectrometry Analysis (LC–ESI/MS/MS). Sample analyses were performed on an Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, U.K.) following reverse-phase HPLC on a 20 mm × 4.0 mm (inside diameter), MercuryMS, 3 μm, column (Phenomenex, Torrance, CA) using a gradient from 0 to 50% 2-propanol over the course of 15 min in buffer A [80:20 (v/v) methanol/water mixture] at a flow rate of 0.2 mL/min. The column eluent was totally inserted into the ion spray interface. Negative ion mass spectra were recorded with an orifice potential of −20 V. The source temperature was kept at 120 °C. Daughter ion and multiple-reaction monitoring (MRM) mass spectra were obtained with a collision energy of −20 V and a gas (Ar) pressure of 6.0×10^{-4} mbar. The full scan was made over an interval between *m*/*z* 40 and 400. The detection of nitrated products was performed in MRM mode, selecting the ions of *m*/*z* 340 and 46 in the first (Q_1) and third quadrupoles (Q_3) and the ions of *m*/*z* 324 and 46 in Q_1 and Q_3, for LNO_2OH and LNO_2, respectively. Quantitative yields of products were calculated by normalizing the total integrated peak area for *m*/*z* species with the internal standard 17:0 (*m*/*z* 269). Statistical analysis was performed by an unpaired "*t*" test with the level of significance defined as $p < 0.05$.

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 NO_2BF_4, the main reaction products, ions with *m*/*z* 324 ([M − H]$^-$), eluted at 10–12 min, being characteristic of nitrolinoleate (LNO_2, Figure 1A). After fragmentation, a

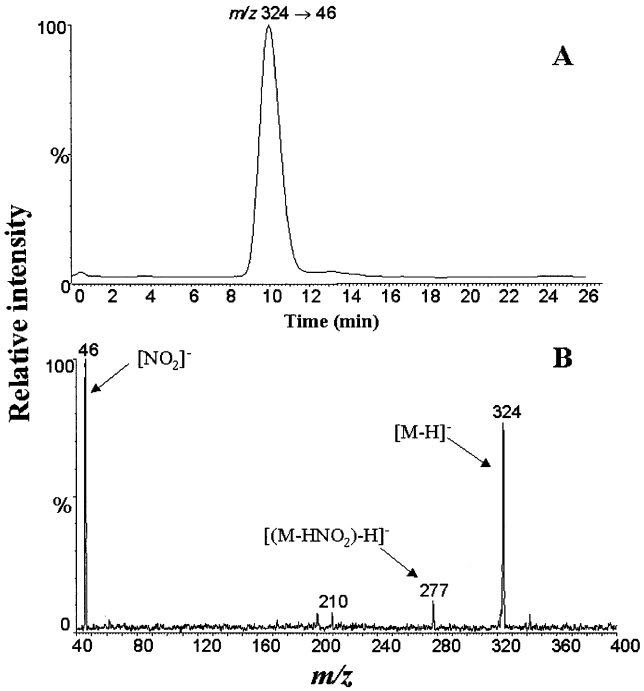

FIGURE 4: Detection of LNO_2 (*m*/*z* 324) in human blood plasma by LC−ESI/MS/MS analysis. (A) Representative MRM [*m*/*z* 324 → 46 (selecting ion of *m*/*z* 324 in Q_1 and *m*/*z* 46 in Q_3)] chromatogram and (B) MS/MS spectra fragmentation patterns of products of *m*/*z* 324 isolated from blood plasma of a normolipidemic subject.

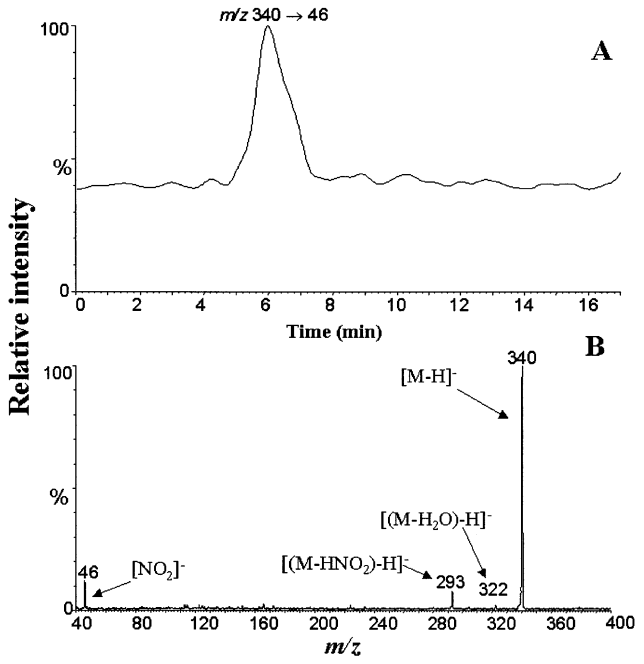

FIGURE 5: Detection of LNO_2OH (*m*/*z* 340) in human blood plasma by LC−ESI/MS/MS analysis. (A) Representative MRM [*m*/*z* 340 → 46 (selecting ion of *m*/*z* 340 in Q_1 and *m*/*z* 46 in Q_3)] chromatogram and (B) MS/MS spectra fragmentation patterns of products of *m*/*z* 340 isolated from blood plasma of a normolipidemic subject.

daughter main ion having an *m*/*z* of 46 ($[^{14}NO_2]^-$) 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 $-NO_2$ {[(M − $H^{14}NO_2$) − 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

Table 2: Presence of LNO_2 (*m*/*z* 324) and LNO_2OH (*m*/*z* 340) in Blood Plasma of Normolipidemic and Hyperlipidemic Subjects

subject[a]	LNO_2 (*m*/*z* 324)[b]	LNO_2OH (*m*/*z* 340)
5N (M, 26)	0.58	0.05
6N (F, 25)	0.17	0.04
7N (F, 47)	0.05	ND[c]
8N (F, 27)	0.43	ND[c]
9N (F, 20)	0.34	0.03
1H (F, 57)	0.96	0.22
2H (F, 58)	0.57	0.18
3H (F, 56)	1.23	0.59
4H (M, 61)	2.50	0.19

[a] M, male; F, female (M/F, age); N, normolipidemic; H, hyperlipidemic. [b] 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). [c] Not detected.

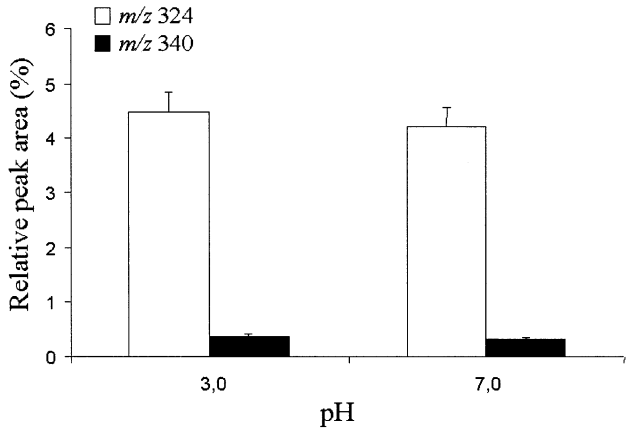

FIGURE 6: Detection of nitrated products in human blood plasma by LC−ESI/MS/MS analysis. Effect of pH on extraction of the nitrated products from blood plasma ($n = 3$). Results (means ± standard deviation) are expressed as the percentage of total integrated peak areas for *m*/*z* species of interest and the internal standard (17:0, *m*/*z* 269).

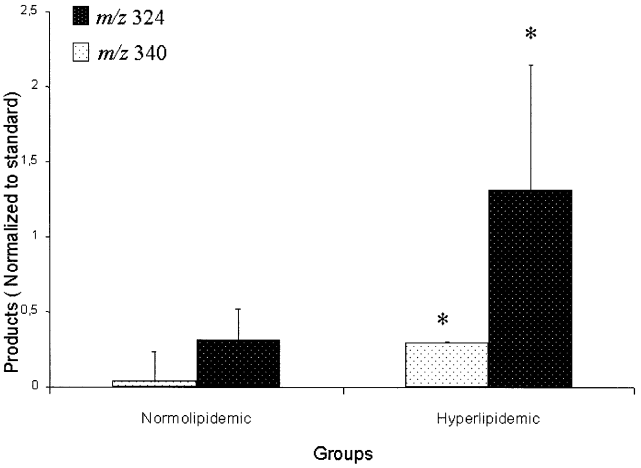

FIGURE 7: Presence of LNO_2 (*m*/*z* 324) and LNO_2OH (*m*/*z* 340) in blood plasma of normolipidemic and hyperlipidemic subjects. The results (means ± standard deviation) are expressed as the ratio of total integrated peak areas for *m*/*z* species of interest and the internal standard (17:0, *m*/*z* 269). The asterisks denote values statistically different from that of the normolipidemic group (*t* test, $p < 0.05$).

$Na^{14}NO_2$ 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 NO_2BF_4, was formed. The presence of an $-NO_2$ group on the ion with an *m*/*z* of 324 was confirmed

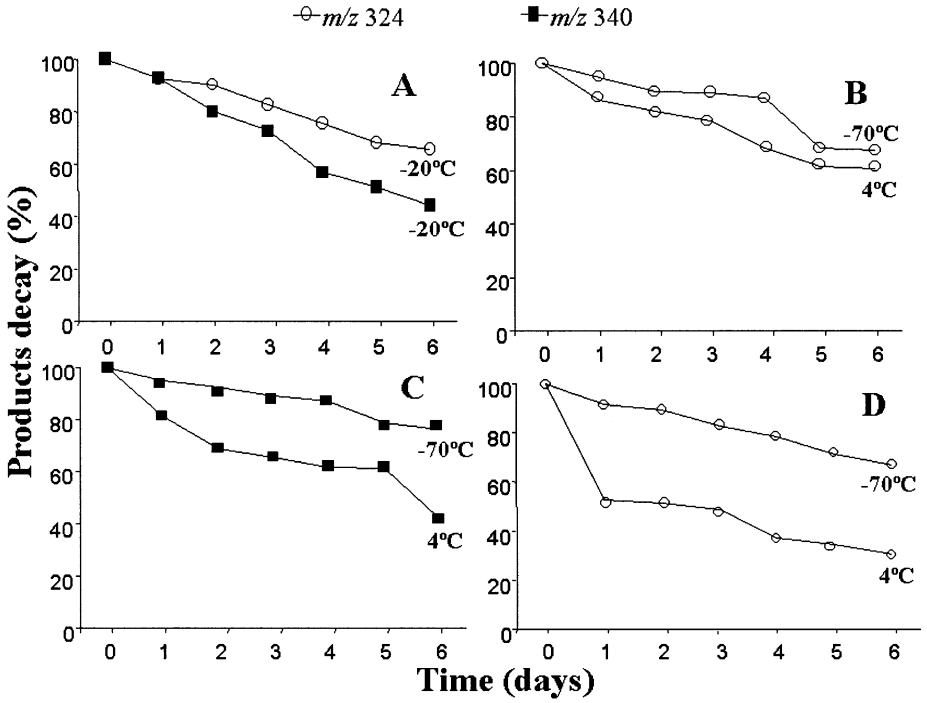

FIGURE 8: Stability of (A) LNO_2 (*m*/*z* 324) and LNO_2OH (*m*/*z* 340) in blood plasma at −20 °C, (B) the LNO_2 (*m*/*z* 324) standard, (C) the LNO_2OH (*m*/*z* 340) standard, and (D) the LNO_2 (*m*/*z* 324) standard added to blood plasma stored at 4 and −70 °C.

by reacting $Na^{15}NO_2$ with 18:2. This reaction yielded a main ion at *m*/*z* 325, that upon fragmentation resulted in an ion characteristic of $[^{15}NO_2]^-$ 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 (−NO_2) or a nitrite (−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}N]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 ($[^{14}NO_2]^-$) and minor ions with *m*/*z* 322, formed by the loss of water $\{[(M - H_2O) - H]^-\}$, and others of *m*/*z* 293, formed by the loss of $-NO_2$ $\{[(M - H^{14}NO_2) - H]^-\}$ (Figure 2B), were observed. Moreover, when 18:2-OOH reacted with sodium [^{15}N]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}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 ($L^\bullet$) reaction with $^\bullet NO_2$ which forms LNO_2, and an alkoxyl linoleate radical ($LO^\bullet$) reaction with $^\bullet NO_2$ 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 heptadecanoic 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_2 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 plasma and/or tissue oxidative damage. Enhancement of •NO production during hypercholesterolemia, associated with a low •NO bioactivity, has been reported (*22*−*28*). The oxidative stress, associated with hypercholesterolemia (*29*−*32*), is implicated in the generation of reactive oxygen species that could also contribute to the formation of nitrated species in the vascular wall. Thus, the nitrated lipids could act as a storage form of •NO representing a compensatory mechanism for the impaired endothelial-dependent vasorelaxation characteristic in the early steps of atherosclerosis.

Stability Studies. The 18:2-nitrated products were detected in plasma on the day of collection (day 0) and daily, in plasma stored for up to 1 week at −20 °C (Figure 8A). However, the *m*/*z* 340 species were less stable than the *m*/*z* 324 product. Similar results were observed with standards (Figure 8B,C) or plasma with added standards (Figure 8D), when stored at 4 or −70 °C. Both *m*/*z* 340 and 324 products were less stable at 4 °C than at −70 °C. However, the synthesized standards were more stable when kept in 2-propanol that when added to plasma, probably due to hydrolysis in the aqueous phase as previously suggested (*33*).

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.

ACKNOWLEDGMENT

We thank Bruce A. Freeman, Ana Denicola, and Rafael Radi for helpful discussions. Additional thanks to Clecio F. Klitzke and Mirian Uemi for technical assistance in mass spectrometry and NMR analyses, respectively.

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BI025504J