Cholesteryl nitrolinoleate, a nitrated lipid present in human blood plasma and lipoproteins

Emersom S. Lima,* **Paolo Di Mascio,**[†] **and Dulcineia S. P. Abdalla**[1,*]

Departamento de Análises Clínicas e Toxicológicas,* Faculdade de Ciências Farmacêuticas, and Departamento de Bioquímica,[†] Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil
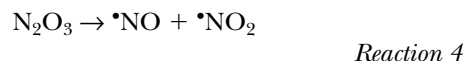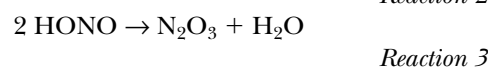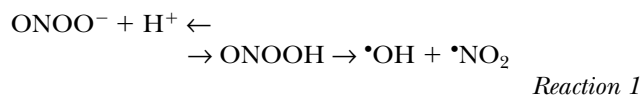
Abstract Nitric oxide (•NO) and •NO-derived reactive species (e.g., peroxynitrite anion, nitrogen dioxide radical) react with lipids containing unsaturated fatty acids to generate nitrated species. In the present work, we synthesized, characterized, and detected a nitrated derivative of cholesteryl linoleate (Ch18:2) in human blood plasma and lipoproteins using a high-pressure liquid chromatography coupled to electrospray ionization tandem mass spectrometry method. It was synthesized by a reaction of Ch18:2 with nitronium tetrafluoroborate, yielding a species with m/z 711, which is characteristic of the cholesteryl nitrolinoleate (Ch18:2NO$_2$) ammonium adduct. The presence of the nitro group was confirmed by using [^{15}N]nitrite, which gave a product with m/z 712, with the same chromatographic and spectrometric characteristics of those of m/z 711. Furthermore, a C-NO$_2$ structure was also demonstrated in Ch18:2NO$_2$ by infrared analysis (V_{max} 1549, 1374 cm^{-1}). A stable product with m/z of 711, showing the same chromatographic characteristics and fragmentation pattern as those of synthesized standard, was found in human blood plasma and lipoproteins of normolipidemic subjects.— The presence of this novel nitrogen-containing lipid product in human plasma and lipoproteins could represent a potential indicator of the oxidative/nitrative roles that •NO or its metabolites play during in vivo lipid oxidation, generating a compensatory mechanism of protection in vascular disease.— Lima, E. S., P. Di Mascio, and D. S. P. Abdalla. **Cholesteryl nitrolinoleate, a nitrated lipid present in human blood plasma and lipoproteins.** *J. Lipid Res.* 2003. **44:** 1660–1666.

Supplementary key words nitration • cholesteryl linoleate • lipoproteins • mass spectrometry

Nitric oxide (•NO) is an endogenously produced free radical that can modulate several relevant biological actions. •NO is the primary source of several substances with oxidant and nitrating activities [e.g., nitrogen dioxide radical (•NO$_2$), peroxynitrite anion] (**Reaction 1**) that can yield nitrated and/or nitrosylated endogenous substances (1, 2). Nitrated lipid formation was initially described in studies that aimed at modeling acute and chronic exposures of pulmonary lipids to NO$_2$ in severely polluted urban atmospheres, providing evidence that at low ppm levels, NO$_2$ reacts with unsaturated fatty acids, leading to nitrolipids formation (3–5).

Nitrated lipid formation has also been shown in a variety of in vitro model systems, including unsaturated free fatty acids, phosphatidylcholine liposomes, LDL oxidized by copper, endothelial cells, and macrophages (6–9). This formation can be primarily a consequence of •NO or its metabolites reacting with lipid-derived radicals (e.g., L•, LO•, LOO•) via diffusion-limited rates (10^9 to 10^{11} mol/l^{-1}/s^{-1}) (10, 11) leading to the formation of nitrated products with structural characteristics of nitrolinoleate, nitritelinoleate (LONO), nitratelinoleate (LONO$_2$), L(O)NO$_2$, and nitro-hydroxylinoleate (12–18). Nitrolipids can also be formed by a direct reaction of •NO$_2$ with nonoxidized lipids at physiological pH (16), or by a reaction of nitrous acid (HONO) (**Reaction 2**), a product yielded by nitrite acidification, with oxidized lipids (16, 18). At physiological pH, e.g., 7.4, NO$_2^-$ is reasonably stable. However, in an acidic media, as in inflammatory states, gastric compartment, and neutrophil phagocytic vesicles, NO$_2^-$ equilibrates with HONO (pK_a = 3.25), which, in turn, is readily converted to a range of potent nitrosating/nitrating species, such as •NO$_2$

$$ONOO^- + H^+ \leftarrow$$
$$\rightarrow ONOOH \rightarrow {}^\bullet OH + {}^\bullet NO_2$$
Reaction 1

$$NO_2^- + H \rightarrow HONO$$
Reaction 2

$$2\ HONO \rightarrow N_2O_3 + H_2O$$
Reaction 3

$$N_2O_3 \rightarrow {}^\bullet NO + {}^\bullet NO_2$$
Reaction 4

Abbreviations: Ch18:2, cholesteryl linoleate (cholesteryl 9,12-octadecadienoate); Ch18:2NO$_2$, cholesteryl nitrolinoleate; [^{3}H]-Ch18:2, [1α,2α (n)-^{3}H]cholesteryl linoleate; IR, infrared; LC-ESI/MS/MS, liquid chromatography/electrospray ionization tandem mass spectrometry; LONO, nitritelinoleate; LONO$_2$, nitratelinoleate; •NO$_2$, nitrogen dioxide radical.

[1] To whom correspondence should be addressed.
e-mail: dspa@usp.br

and N_2O_3, (**Reactions 3**, **4**) capable of inducing modifications of polyunsaturated fatty acids (18).

Recent studies have demonstrated the presence of these products in biological samples. A vicinal nitrohydroxy arachidonate derivative was detected in bovine coronary arteries, which spontaneously releases •NO, causing relaxation in rat coronary aorta rings (19). Nitrolinoleate and its nitrohydroxy derivative, nitrohydroxylinoleate, were detected in human blood plasma (20). Moreover, some studies have demonstrated biological activities of these nitrated products, such as endothelium independent vasorelaxation (19, 21), inhibition of platelet aggregation (22), and antinflamatory actions, such as inhibition of superoxide generation, neutrophil degranulation, and integrin expression (23), suggesting that these nitrated lipids could have vascular-protective effects.

In the present work, for the first time, is reported the presence of cholesteryl nitrolinoleate (Ch18:2NO₂), a stable cholesteryl linoleate (Ch18:2)-derived nitrated product, in human blood plasma and lipoproteins, using high-pressure liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS). The ex vivo detection of these products represents an important step in the understanding of the protective and/or deleterious effects of in vivo modified lipoproteins on blood vessels.

MATERIALS AND METHODS

Chemicals

Sodium [¹⁵N]nitrite and nitronium tetrafluoroborate were purchased from Aldrich Chemical Co. (Milwaukee, WI); [1α,2α(n)-³H] Ch18:2 was obtained from Amersham (Buckinghamshire, UK). *tert*-Butanol and chromatographic-grade methanol were obtained from Merck (Gibbstown, NJ). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Nitration of Ch18:2

A solution of Ch18:2 (0.07 mmol) in chloroform (2 ml) was purged with nitrogen, and solid NO₂BF₄ was added (0.12 mmol). The mixture was kept under nitrogen atmosphere at room temperature for 1 h, and then 1 ml of 0.1 M phosphate buffer, pH 7.4 was added. The organic layer was separated, dried, and redissolved in 1 ml of 2-propanol. To isolate the nitrated lipids, the extract was passed through a 2.5 cm × 5.5 cm silica gel (200–400 mesh) column equilibrated with hexane. Nitrated lipids were separated from Ch18:2 with a hexane-diethyl ether step gradient (5% increments from 0 to 20% of diethyl ether). The content of nitrated lipid in the eluted fraction was monitored by thin-layer chromatography (TLC) using a mixture of methanol-chloroform (1:1; v/v) as solvent. Lipid fraction (1 μl) was applied to RP-18 F₂₅₄s 5 × 10 cm TLC plates (Merck KgaA, Darmstadt, Germany), where the separated lipid components were detected with iodine. Fractions containing mainly nitrated lipids were separated, dried under a vacuum, redissolved in 2-propanol, maintained at −20°C, and analyzed by LC-ESI/MS/MS. Nitration was also done by adding sodium [¹⁵N]nitrite (0.1 mmol) to 0.10 mmol of Ch18:2 in 200 μl chloroform-methanol (2:1; v/v) following acidification to pH 3.0 with 1 N HCl and incubation at 25°C for 15 min under aerobic conditions. One milliliter of 0.1 M, pH 7.4, phosphate buffer was added, and extraction was done with 5 ml of diethyl ether. The organic layer was separated, dried, and maintained at −20°C until LC-ESI/MS/MS analysis.

Structural and quantitative analyses of nitrated lipids by LC-ESI/MS/MS and infrared spectroscopy

Mass spectrometry analysis was performed on a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK) following reversed-phase HPLC on a 20 × 4.0 mm id, 4 μ, Mercury MS column (Phenomenex, Torrance, CA) using an isocratic system with methanol-*tert*-butanol (3:1; v/v) containing 10 mM of ammonium acetate as mobile phase at a flow rate of 0.4 ml/min. The column eluent was totally inserted into the ion spray interface. Positive ion mass spectra were recorded with an orifice potential of 20 V. The source temperature was kept at 100°C. Daughter ion and multiple reaction monitoring (MRM) mass spectra were obtained with a collision energy of 10 eV or 20 eV, respectively, and gas (Ar) pressure at 6.0×10^{-4} mbar. The full scan was made at an interval between *m/z* 40 and 800. The nitrated product was detected in MRM mode as ammonium adduct $[M+NH_4]^+$, selecting the ions of *m/z* 711 in the first and 369 in the third quadrupole (*m/z* 711→369). The MRM transition for Ch18:2 and ³H-labeled cholesteryl linoleate (³H-Ch18:2) were also measured as *m/z* 666→369 and *m/z* 668→371, respectively. This detection mode was used to increase the specificity of the analysis. Quantitative yields of nitrated product (Ch18:2NO₂) were calculated by elemental analysis of nitrogen content using a chemiluminescent nitrogen detector (Sievers® NOA 280, Boulder, CO) using sodium nitrite as standard. The data obtained for Ch18:2NO₂ were used for its quantification in the LC-ESI/MS/MS system. A calibration curve was also performed with Ch18:2. The lipid residue obtained after treatment of Ch18:2 with NO₂BF₄ was used for infrared (IR) analysis. IR spectra were obtained with a Bomem MB 100 spectrometer by accumulating 32 scans between 400 and 4,000 cm⁻¹.

LDL isolation and lipid extraction

Human blood from four normolipidemic subjects (cholesterol <200 mg/dl) was collected after overnight fasting in tubes containing ethyldiaminetetraacetic acid (EDTA). Plasma was obtained after blood centrifugation at 2,500 rpm for 10 min at 4°C, and antiprotease inhibitors and antioxidants were immediately added to avoid lipoprotein degradation. Lipoproteins were separated from plasma by sequential ultracentrifugation using a Sorvall® Ultra Pro 80 (Sorvall Products L.P., Newtown, CT) and dialyzed against Tris buffer, pH 7.4, (150 mM NaCl, 1.0 mM EDTA, 3 mM NaN₃, and 10 mM Tris) for 12 h. Lipid extraction from VLDL, LDL, and/or HDL was done by mixing 1 ml of each lipoprotein fraction with 500 μl of methanol, with vortex stirring for 30 s. Then 5 ml of hexane-diethyl ether (80:20; v/v) containing 0.02% butylated hydroxytoluene, previously treated with Chelex® to avoid further lipid oxidation during lipid extraction, was added. Samples were vortexed (2 min) and centrifuged at 2,500 rpm for 5 min at 4°C. The upper layer was collected, filtered (0.22 μ), and evaporated to dryness in a vacuum rotary evaporator. Lipids were dissolved in 100 μl of *tert*-butanol-methanol (1:1; v/v), and then 10 μl was immediately injected into a LC-ESI/MS/MS system using the protocol described for the standards. The same extraction system was used in the blood plasma analyses. To verify a possible artifactual nitration of Ch18:2 during the handling of the samples, the extraction procedure was also performed by the addition of an internal standard ³H-labeled Ch18:2 in plasma (100 pmol in chloroform), which can also be followed by LC-ESI/MS/MS.

RESULTS

LC-ESI/MS/MS and IR spectroscopic analyses of Ch18:2 nitration

When Ch18:2 reacted with NO₂BF₄, the main reaction products, eluting at 5.9 min, were ions with *m/z* 711
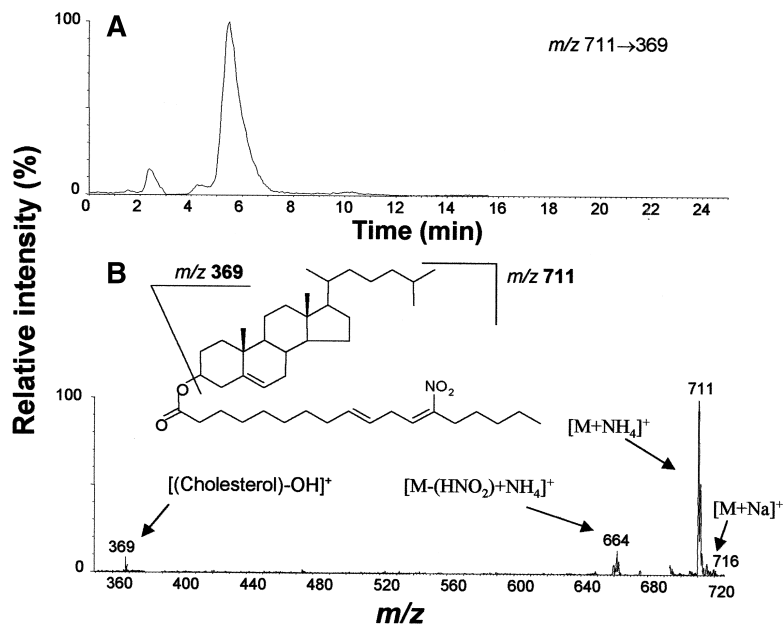
Fig. 1. Detection of cholesteryl nitrolinoleate (Ch18:2NO$_2$) ammonium adduct standard (m/z 711) by liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) analysis. A: Multiple reaction monitoring (MRM) chromatogram selecting ions with m/z 711 in Q$_1$ and 369 in Q$_3$. B: Spectra fragmentation patterns of peak eluted at 5.9 min.

[M+NH$_4$]$^+$ (**Fig. 1A**), characteristic of Ch18:2NO$_2$ ammonium adduct. After fragmentation, a daughter main ion having m/z 369 was obtained, characteristic of the cholesteryl group in the parent molecule [(cholesterol)-OH]$^+$. Ions of m/z 716, indicating a sodium adduct [M+Na]$^+$ and of m/z 664, formed by the loss of $-$NO$_2$ [M$-$(HNO$_2$)+H]$^+$, were also observed (Fig. 1B). The presence of a $-$NO$_2$ group on the ion with m/z 711 was confirmed by synthesizing Ch18:2NO$_2$ by reacting Na^{15}NO$_2$ with Ch18:2. This reaction yielded a molecular ion of m/z 712 that fragments given a main ion of m/z 369, which showed the same isotopic abundance pattern of those of m/z 711→369 ion (**Fig. 2**). The Ch18:2 used in the synthesis yielded ∼70% of Ch18:2NO$_2$.

The IR spectrum of the compound with m/z 711 showed a characteristic absorption of a nitro group directly attached to the carbon chain (1,549 cm^{-1} and 1,374 cm^{-1}, **Fig. 3**). No bands occurred in the 1,600–1,680 cm^{-1} region where the N $=$ O binding of a LONO or LONO$_2$ strongly absorbs.

Determination of nitrated products in plasma and lipoproteins

When human blood plasma and LDL samples were analyzed by the same LC-ESI/MS/MS method used for synthesized standards, a major product having an ion of m/z 711→369 was found. Moreover, the m/z 711 ion showed a fragmentation pattern and retention time identical to those of products synthesized by the reaction of Ch18:2 with NO$_2$BF$_4$ or NO$_2^-$ in acid pH [**Fig. 4A** (trace a), **B**]. The product with m/z 711 that fragments in m/z 369 was detected in LDL (**Fig. 5**), as well as in VLDL and HDL samples (data not shown). The addition of [^{3}H]-Ch18:2 to samples before the extraction procedure did not yield the
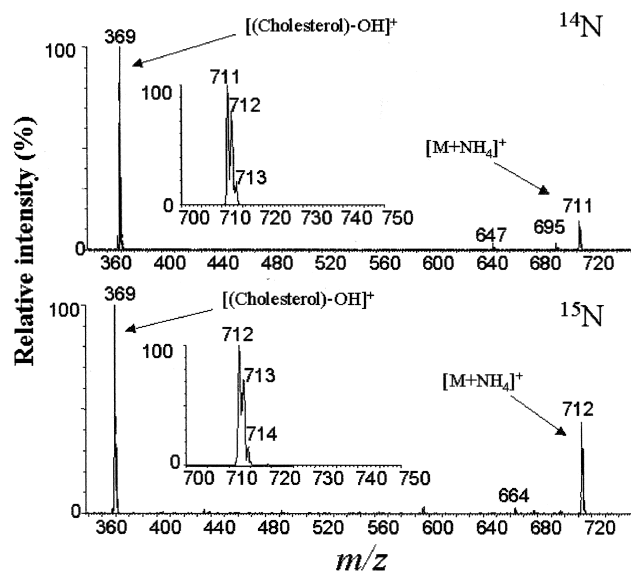

Fig. 2. Detection of Ch18:2NO$_2$ ammonium adduct standard by LC-ESI/MS/MS analysis. MS/MS* spectra fragmentation patterns of products generated by reaction of cholesteryl linoleate (Ch18:2) with NO$_2$BF$_4$ (top) or sodium [^{15}N]nitrite (lower), showing an enlargement of the [M+NH$_4$]$^+$ region (inside). * Selecting daughter ions mode for ions with m/z 711 or 712.
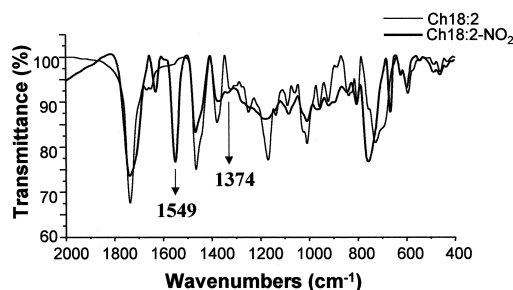

Fig. 3. Infrared spectra of products derived from reaction of Ch18:2 with NO$_2$BF$_4$. Sample preparation and spectra acquisition parameters are described in Materials and Methods.
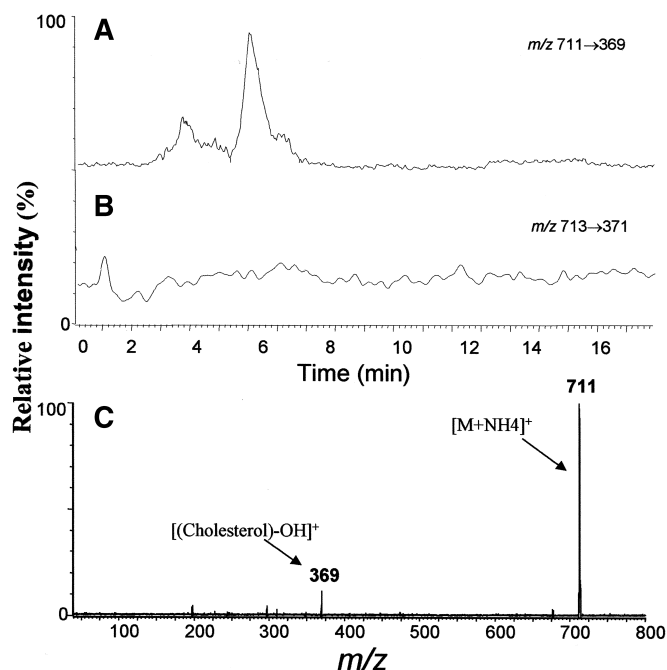
Fig. 4. Human blood plasma analysis by LC-ESI/MS/MS. MRM chromatogram (A) (711→369)* and (B) (713→371)*. C: MS/MS spectra fragmentation patterns of peak eluted at 5.9 min. * Selecting ion of m/z 369 or 371 in Q_3 and m/z 711 or 713 in Q_1 for (A) and (B), respectively.

[3H]-Ch18:2 nitrated product. This is indicated by the absence of a molecular ion of m/z 713 that fragments in a daughter ion of m/z 371 [Fig. 4A (trace b)].

The Ch18:2 was also detected in plasma and lipoprotein samples in the same run of the nitrated product, eluting at ∼14.0 min. It appears as an ion with m/z 666, relative to ammonium adduct $[M+NH_4]^+$, that fragments given a main daughter ion of m/z 369, characteristic of the cholesteryl
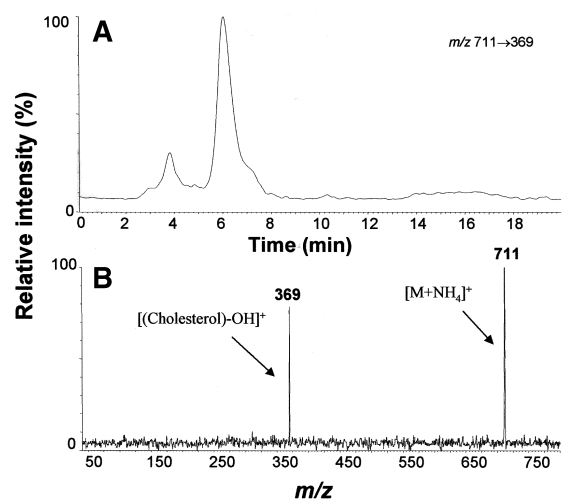

Fig. 5. LDL samples analysis by LC-ESI/MS/MS. A: MRM chromatogram (711→369)*. B: MS/MS spectra fragmentation patterns of peak eluted at 5.9 min. * Selecting ion of m/z 711 in Q_1 and m/z 369 in Q_3.

group (**Fig. 6A**, **B**). The [3H]-Ch18:2 added to plasma samples, which has the same retention time as Ch18:2 (Fig. 6C), appears as a molecular ion of m/z 668 $[M+NH_4]^+$, which fragments given a main ion of m/z 371 (Fig. 6D), characteristic of [3H]-labeled cholesterol [([3H]-cholesterol)-OH]$^+$. The nitrated product represents ∼0.26% of the total of plasmatic Ch18:2, maintaining practically the same proportion in different lipoproteins (**Fig. 7**). The concentrations observed in plasma samples (mean ± SD, n = 4) were 76.9 ± 10.7 (nmol/l) and 30 ± 8.7 (μmol/l) for Ch18:2NO$_2$ and Ch18:2, respectively.

DISCUSSION

The reaction of Ch18:2 with NO_2BF_4 yielded a main product that exhibited a molecular ion with m/z 711, when analyzed by LC-ESI/MS/MS, characteristic of a nitrated Ch18:2 ammonium adduct. The MS/MS fragmen-
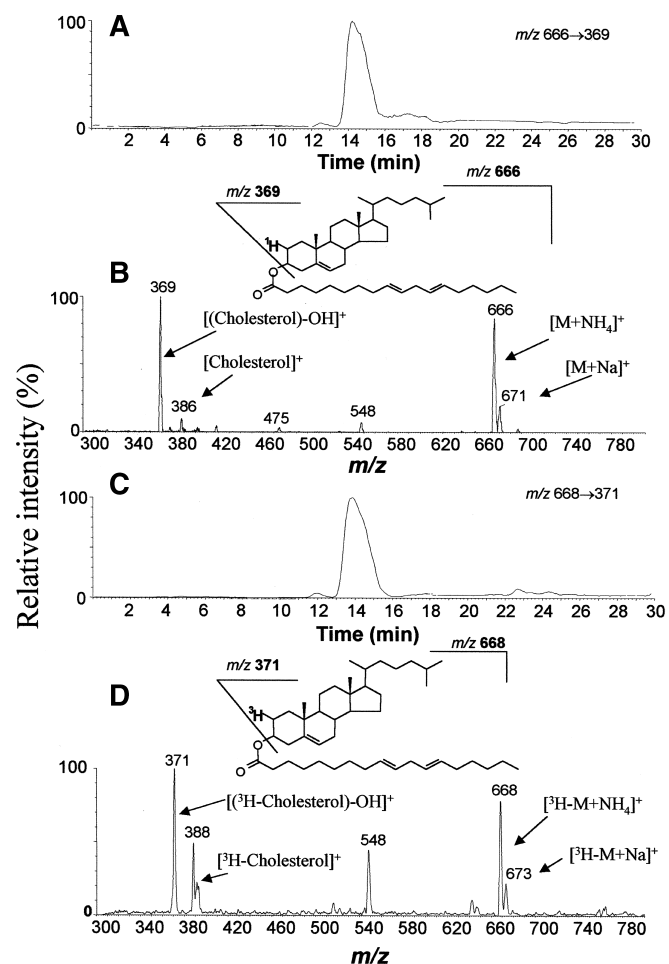

Fig. 6. Ch18:2 and [3H]-labeled cholesteryl linoleate analysis in human blood plasma samples by LC-ESI/MS/MS. A: MRM chromatogram (666→369)*. B: MS/MS spectra fragmentation patterns of peak detected in A (daughter ions of m/z 666). C: MRM chromatogram (668→371)*. D: MS/MS spectra fragmentation patterns of peak detected in C (daughter ions of m/z 668). * Selecting ion of m/z 666 or 668 in Q_1 and m/z 369 or 371 in Q_3 for A and C, respectively.
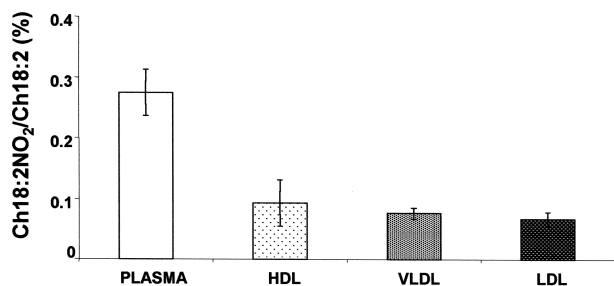
Fig. 7. Presence of Ch18:2NO$_2$ (m/z 711) in blood plasma and lipoproteins of normolipidemic subjects. The results (mean $\pm$ SD; n = 4) are expressed as the percent amount of nitrated and nonnitrated Ch18:2. The content of lipoprotein was corrected taking into consideration dilution during sample preparation.
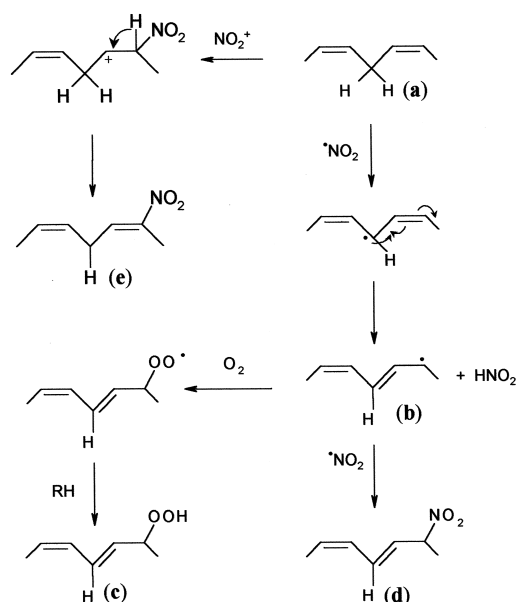

Fig. 8. Schematic representation of nitrated lipid formation. First, the nitrogen dioxide radical, produced during nitric oxide metabolism, or any other radical, abstracts an H atom from the allylic position of an alkene (a), forming a resonance-stabilized carbon radical (b). In the presence of oxygen, this radical combines with an oxygen molecule, forming a peroxyl radical that ultimately forms allylic hydroperoxides (c). This resonance-stabilized radical (b) can also react with another nitrogen dioxide molecule, forming a conjugated nitrolipid molecule (d). Second, the nitration can occur in the presence of the nitronium ion (NO$_2^+$) by electrophilic addition to the unsaturated carbon chain, yielding an unconjugated nitrolipid molecule (e).

tation spectra give a major peak with m/z 369, characteristic of the cholesteryl group [(cholesterol)-OH]$^+$, as previously reported (24), indicating that nitration was occurring in the carbon chain instead of the cholesterol ring. The same product was obtained when Ch18:2 was treated with aqueous acidic sodium nitrite under aerobic conditions. The synthesis with NO$_2$BF$_4$ was chosen for IR and LC-ESI/MS/MS experiments due to a higher yielding of the nitrated Ch18:2 product when compared with the synthesis performed with nitrite. The nitration mechanisms for these synthesis procedures must be different (12, 13). When NO$_2$BF$_4$ is used, the nitration occurs by nitronium ion (NO$_2^+$) addition in the double bond on the carbon chain (**Fig. 8**). In the reaction with sodium nitrite under acidic and aerobic conditions, the nitration occurs when $^\bullet$NO$_2$ formed by nitrite acidification in the presence of oxygen abstracts an H atom from the allylic position of the fatty acid, forming a resonance-stabilized radical. This radical further reacts with another $^\bullet$NO$_2$ molecule, forming a nitrolipid derivative (Fig. 8). In vivo, lipid nitration probably occurs by a radical-mediated mechanism in which $^\bullet$NO$_2$ is generated by peroxidase-dependent oxidation of nitrite (25). The $^\bullet$NO$_2$ can also be formed by other biological systems, such as in inflammatory states, gastric compartment, and neutrophil phagocytic vesicles (18).

The characterization of nitration by mass spectrometry was performed by reacting Ch18:2 with [^{15}N]nitrite, in acidic media, obtaining a main ion with m/z 712, which showed the same fragmentation and isotopic abundance patterns of those of m/z 711 ions. The isotopic abundance pattern confirms that the spectra obtained are relative to compounds containing $\sim$50 carbons, such as Ch18:2 (26). The increase of one mass unit using [^{15}N]nitrite confirms that the ion contains one nitrogen atom per molecule and is compatible with the nitrated Ch18:2 structure. This evidence was reinforced by IR analyses of the nitrated product, as compared with Ch18:2, that showed novel bands at 1,549 and 1,373 cm^{-1}, corresponding to the N = O binding of RNO$_2$ (27). These results clearly showed that the product formed in this synthesis is Ch18:2NO$_2$. It was not possible to determine the position of the nitro group in the carbon chain of the fatty acid. It is possible that a mixture of stereo and positional isomers (i.e., at C$_9$, C$_{10}$, C$_{12}$,

or C$_{13}$ and/or *cis-trans* isomers) is formed that is not possible to discriminate by mass spectrometry analysis.

The analysis of blood plasma and lipoproteins showed an ion with m/z 666, characteristic of the Ch18:2 ammonium adduct, as previously described (28). Ch18:2 was chosen because it is the predominant lipid component in the LDL core (28) and linoleic acid (18:2) is the most abundant polyunsaturated fatty acid in human blood plasma (29). As the presence of double-bonded carbons is an initial condition for $^\bullet$NO$_2$ attack (18), polyunsaturated fatty acids would be suitable substrates for $^\bullet$NO$_2$-mediated nitration. Fragmentation of the ion with m/z 666 showed a main daughter ion with m/z 369, characteristic of the cholesteryl group, as previously described (28).

Products given molecular ions with m/z 711, showing the same chromatographic characteristics and fragmentation pattern as those of synthesized standards, were found in human blood plasma and lipoproteins of normolipidemic donors. A possible artifactual nitration that could occur during the extraction procedure was tested by adding [^{3}H]-labeled Ch18:2 to plasma samples. As the corresponding nitrated [^{3}H]-Ch18:2 was not detected, an artifactual nitration during sample workup was excluded. The possibility that azide contributes to nitration was also excluded, because azide was used only for isolation of lipoproteins and not for plasma samples. Small differences in MS/MS fragmentation spectra patterns between synthe-

sized standards and products of plasma or lipoproteins could be explained by the presence of different positional isomers and/or functional group orientation (e.g., $-NO_2$ or $-ONO$) in vivo. Furthermore, the possibility cannot be excluded that Ch18:2-ONO (cholesteryl nitritelinoleate) could be formed in vivo and decomposed by transnitrozation or other routes during sample extraction and workup.

Nitrated lipids present in plasma and lipoproteins can be considered as potential indicators of the chain-breaking antioxidant role of $^\bullet$NO during lipid peroxidation, as previously reported (7, 8, 11), indicating that reactions of $^\bullet$NO, or its derivatives, with lipids may be important in vivo. Due to its relative hydrophobicity, $^\bullet$NO readily diffuses into hydrophobic lipid membranes, greatly increasing its local concentration (30). This property significantly increases the chain-breaking efficiency of low concentrations of $^\bullet$NO, favoring lipid peroxidation inhibition. Nitrated lipids could also be formed in vivo in the lipid microenvironments where the rate of $^\bullet$NO autoxidation is high (e.g., with concurrent $^\bullet$NO and O_2 therapy during lung disease) or where high NO_2^- concentrations are combined with low pH (e.g., gastric compartment, phagolysosome, and ischemic events) (16, 18).

In the context of hypercholesterolemia, we recently described increased levels of nitrated linoleic acid derivatives in plasma of hyperlipidemic compared with normolipidemic subjects (20). These could be implicated in the formation of reactive nitrogen species during plasma and/or tissue oxidative damage in hyperlipidemia. In fact, enhancement of $^\bullet$NO production during hypercholesterolemia has been associated with low $^\bullet$NO bioactivity (31–36). It is also well known that the increase of oxidative stress in hypercholesterolemia (37–39) implicated in the generation of reactive oxygen species could contribute to the formation of nitrated species in the vascular wall. Therefore, the presence of nitrated lipids in plasma can be an indicator of the inhibitory role of $^\bullet$NO in lipid oxidation, and/or a footprint of the presence of oxidants/nitrating agents in the vascular system. Finally, whereas it is suggested that these nitrated lipids cause vasorelaxation by $^\bullet$NO release (19, 21) and have other beneficial effects in the atherothrombotic process, such as inhibition of platelet aggregation and anti-inflamatory actions (22, 23), we postulate that the presence of these nitrated products in vivo would indicate primarily a storage form of $^\bullet$NO acting as a compensatory mechanism for the impaired endothelial-dependent vasorelaxation characteristic of the early steps of vascular disease.

REFERENCES

1. Eiserich, J. P., R. P. Patel, and V. B. O'Donnell. 1998. Pathophysiology of nitric oxide and related species: free radical reactions and modification of biomolecules. *Molec. Aspects Med.* **19:** 221–357.

2. Wink, D. A., and J. B. Mitchell. 1998. Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic. Biol. Med.* **25:** 434–456.

3. Pryor, W. A., and J. W. Lightsey. 1981. Mechanisms of nitrogen dioxide reactions: initiation of lipid peroxidation and the production of nitrous acid. *Science.* **214:** 435–437.

4. Pryor, W. A., J. W. Lightsey, and D. F. Church. 1982. Reaction of nitrogen dioxide with alkene and polyunsaturated fatty acids: addition and hydrogen abstraction mechanism. *J. Am. Chem. Soc.* **104:** 6685–6692.

5. Giamalva, D. H., G. B. Kenion, D. F. Church, and W. A. Pryor. 1987. Rates and mechanisms of reaction of nitrogen dioxide with alkenes in solution. *J. Am. Chem. Soc.* **109:** 7059–7063.

6. Rubbo, H., and B. A. Freeman. 1996. Nitric oxide regulation of lipid peroxidation reactions: formation and analysis of nitrogen-containing oxidized lipid derivatives. *Methods Enzymol.* **269:** 385–394.

7. Rubbo, H., S. Parthasarathy, S. Barnes, M. K. B. Kalyanaraman, and B. A. Freeman. 1995. Nitric oxide inhibition of lipoxygenase-dependent liposome and low-density lipoprotein oxidation: termination of radical chain propagation reactions and formation of nitrogen-containing oxidized lipid derivatives. *Arch. Biochem. Biophys.* **324:** 15–25.

8. Hayashi, K., N. Noguchi, and E. Niki. 1995. Action of nitric oxide as an antioxidant against oxidation of soybean phosphatidyl choline liposomal membranes. *FEBS Lett.* **370:** 37–40.

9. Yates, M. T., L. E. Lambert, J. P. Whitten, L. McDonald, M. Mano, G. Ku, and S. J. T. Mao. 1992. A protective role for nitric oxide in the oxidative modification of low-density lipoproteins by mouse macrophages. *FEBS Lett.* **309:** 135–138.

10. Maricq, M. J., and J. J. Szente. 1996. Kinetics of the reaction between ethyl peroxy radicals and nitric oxide. *J. Phys. Chem.* **96:** 986–992.

11. Padmaja, S., and R. E. Huie. 1993. The reaction of nitric oxide with peroxyl radicals. *Biochem. Biophys. Res. Commun.* **15:** 539–544.

12. Galon, A. A., and W. A. Pryor. 1993. The identification of the allylic nitrite and nitro derivatives of the methyl linoleate and methyl linolenate by negative chemical ionization mass spectrometry. *Lipids.* **28:** 125–133.

13. Galon, A. A., and W. A. Pryor. 1994. The reaction of low levels of nitrogen dioxide with methyl linoleate in the presence and absence of oxygen. *Lipids.* **29:** 171–176.

14. Rubbo, H., R. Radi, M. Trujillo, R. Telleri, B. Kalyanaraman, S. Barnes, M. Kirk, and B. A. Freeman. 1994. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. *J. Biol. Chem.* **269:** 26066–26075.

15. O'Donnell, V. B., J. P. Eiserich, A. Bloodsworth, P. H. Chumley, M. Kirk, S. Barnes, V. M. Darley-Usmar, and B. A. Freeman. 1999. Nitration of unsaturated fatty acids by nitric oxide-derived reactive species. *Methods Enzymol.* **301:** 455–471.

16. O'Donnell, V. B., J. P. Eiserich, P. H. Chumley, M. J. Jablonsky, M. Kirk, S. Barnes, V. M. Darley-Usmar, and B. A. Freeman. 1999. Nitration of unsatured fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide and nitronium ion. *Chem. Res. Toxicol.* **12:** 83–92.

17. Napolitano, A., E. Camera, M. Picardo, and M. D'Ischia. 2000. Acid-promoted reactions of ethyl linoleate with nitrite ions: formation and structural characterization of isomeric nitroalkene, nitro-hydroxy and novel 3-nitro-1,5-hexadiene and 1,5-dinitro-1, 3-pentadiene products. *J. Org. Chem.* **65:** 4853–4860.

18. Napolitano, A., E. Camera, M. Picardo, and M. D'Ischia. 2002. Reaction of hydro(pero)xy derivatives of polyunsaturated fatty acids/esters with nitrite ions under acidic conditions. Unusual nitrosative breakdown of methyl 13-hydro(pero)xyoctadeca-9,1-dienoate to a novel 4-nitro-2-oximinoalk-3-enal product. *J. Org. Chem.* **67:** 1125–1132.

19. Balazy, M., T. Iesaki, J. L. Park, H. Jiang, P. M. Kaminski, and M. Wolin. 2001. Vicinal nitrohydroeicosatrienoic acids: vasodilator lipids formed by reaction of nitrogen dioxide with arachidonic acid. *J. Pharmacol. Exper. Ther.* **299:** 1–9.

20. Lima, E. S., P. Di Mascio, H. Rubbo, and D. S. P. Abdalla. 2002. Characterization of linoleic acid nitration in human blood plasma by mass spectrometry. *Biochemistry.* **41:** 10717–10722.

21. Lim, D. G., S. Sweeney, A. Bloodsworth, C. R. White, P. H. Chumley, N. R. Krishna, F. Schopfer, V. B. O'Donnell, J. P. Eiserich, and B. A. Freeman. 2002. Nitrolinoleate, a nitric oxide-derived mediator of cell function: synthesis, characterization, and vasomotor activity. *Proc. Natl. Acad. Sci. USA.* **99:** 15941–15946.

22. Coles, B., A. Bloodsworth, J. P. Eiserich, M. J. Coffey, R. M. McLoughlin, J. C. Giddings, M. J. Lewis, R. J. Haslam, B. A. Freeman, and V. B. O'Donnell. 2002. Nitrolinoleate inhibits platelet activation by attenuating calcium mobilization and inducing phosphorylation of vasodilator-stimulated phosphoprotein (VASP) through elevation of cAMP. *J. Biol. Chem.* **277:** 5832–5840.

23. Coles, B., A. Bloodsworth, S. R. Clark, M. J. Lewis, A. R. Cross, B. A. Freeman, and V. B. O'Donnell. 2002. Nitrolinoleate inhibits superoxide generation, degranulation, and integrin expression by human neutrophils. *Circ. Res.* **91:** 375–381.

24. Lin, Y. Y., and L. L. Smith. 1978. Recognition of functional groups by chemical ionization mass spectrometry. *Biomed. Mass Spectrom.* **11:** 604–611.

25. Carr, A. C., M. R. McCall, and B. Frei. 2000. Oxidation of LDL by mieloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. *Arterioscler. Thromb. Vasc. Biol.* **20:** 1716–1723.

26. Havrilla, C. M., D. L. Hachey, and N. A. Porter. 2000. Coordination (Ag+) ion spray-mass spectrometry of peroxidation products of cholesterol linoleate and cholesterol arachidonate: high-performance liquid chromatography-mass spectrometry analysis of peroxide products from polyunsaturated lipid autoxidation. *J. Am. Chem. Soc.* **122:** 8042–8055.

27. Silverstein, R. M., G. C. Bassler, and T. C. Morril. 1991. Spectrometric Identification of Organic Compounds, 5th edition. John Wiley and Sons, New York. 65–74.

28. Hafidi, M. E., F. Michel, J. Bascoul, and A. C. Paulet. 1999. Preparation of fatty acid cholesteryl ester hydroperoxides by photosensitized oxidation. *Chem. Phys. Lipids.* **100:** 127–138.

29. Jira, W., G. Spiteller, W. Carson, and A. Schram. 1998. Strong increase in hydroxy fatty acids derived from linoleic acid in human low density lipoproteins of atherosclerotic patients. *Chem. Phys. Lipids.* **91:** 1–11.

30. Denicola, A., C. Batthyány, E. Lissi, B. A. Freeman, H. Rubbo, and R. Radi. 2002. Diffusion of nitric oxide into low density lipoprotein. *J. Biol. Chem.* **277:** 932–936.

31. Podrez, E. A., D. Schmitt, H. F. Hoff, and S. L. Hazen. 1999. Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro. *J. Clin. Invest.* **103:** 1547–1560.

32. Busse, R., and I. Fleming. 1996. Endothelial dysfunction in atherosclerosis. *J. Vasc. Res.* **33:** 181–194.

33. Moriel, P., I. R. Pereira, M. C. Bertolami, and D. S. P. Abdalla. 2001. Is ceruloplasmin an important catalyst for S-nitrosothiol generation in hypercholesterolemia? *Free Radic. Biol. Med.* **30:** 318–326.

34. Patel, R. P., A. L. Levonen, J. H. Crawford, and V. M. Darley-Usmar. 2000. Mechanisms of the pro- and anti-oxidant actions of nitric oxide in atherosclerosis. *Cardiovasc. Res.* **47:** 465–474.

35. Minor, R. L., P. R. Myers, R. Guerra, J. N. Bates, and D. G. Harrison. 1990. Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. *J. Clin. Invest.* **86:** 2109–2116.

36. Heineck, J. W. 1997. Mechanisms of oxidative damage of low density lipoprotein in human atherosclerosis. *Curr. Opin. Lipidol.* **8:** 268–274.

37. Chisolm, G. M., and D. Steinberg. 2000. The oxidative modification hypothesis of atherogenesis: an overview. *Free Radic. Biol. Med.* **28:** 1815–1826.

38. Moriel, P., F. S. Okawabata, and D. S. P. Abdalla. 1999. Oxidized lipoproteins in blood plasma: possible marker of atherosclerosis progression. *IUBMB Life.* **48:** 413–417.

39. Halliwell, B. 2000. Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward? *Cardiovasc. Res.* **47:** 410–418.