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# Peroxynitrite mild nitration of albumin and LDL–albumin complex naturally present in plasma and tyrosine nitration rate–albumin impairs LDL nitration

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**Abstract**

In blood, peroxynitrite ($ONOO^-$) and $CO_2$ react to form $NO_2^{\cdot}$ and $CO_3^{\cdot -}$ through the short-lived adduct $ONOOCO_2^-$, leading to protein-bound tyrosine nitration. $ONOO^-$-modified LDL is atherogenic. Oxidatively modified LDL generally appears in the vessel wall surrounded by antioxidants. Human serum albumin (HSA) is one of them, partly associated to LDL as a LDL–albumin complex (LAC). The purpose was to study the effect of a mild nitration on LAC and whether albumin may interfere with LDL nitration. To do so, SIN-1 was used as $ONOO^-$ generator in the presence or absence of 25 mM $HCO_3^-$. The human serum albumin (HSA)-bound tyrosine nitration rate was found to be $4.4 \times 10^{-3}$ mol/mol in the presence of $HCO_3^-$. HSA decreased the LAC-tyrosine nitration rate from $2.5 \times 10^{-3}$ to $0.6 \times 10^{-3}$ mol/mol. It was concluded that HSA impaired the apoB-bound tyrosine nitration. These findings raise the question of the patho-physiological significance of these nitrations and their interactions which may potentially prevent both atheromatous plaque formation and endothelium dysfunction in particular and appear to be beneficial in terms of atherogenic risk.

**Keywords:** *Nitration, tyrosine, albumin, low-density lipoprotein, peroxynitrite, GC-MS*

**Abbreviations:** *DTPA, diethylenetriamine pentacetic acid; HSA, human serum albumin; LAC, LDL–albumin complex; MTBSTFA,* N-tert-*butyldimethylsilyl*-N-*methyltrifluoroacetamide; 3NT, 3-$NO_2$-Tyr; ox-LDL, oxidized LDL; SIN-1, 3-morpholinosydnonimine hydrochloride; TFAA, trifluoroacetyl anhydride*

## Introduction

Nitric oxide ($NO^{\cdot}$) is an ubiquitous inorganic radical, which is produced by many cell types (endothelial cells, macrophages and neutrophils) by both constitutive and inducible nitric oxide synthases [1] and performs a range of biological functions, including the regulation of neurotransmission and vascular tone, inhibition of platelet aggregation, relaxation of blood vessel. It is also closely linked to the pathogenesis of many inflammatory and degenerative disorders [2]. $NO^{\cdot}$ can react rapidly at closely diffusion-limited rate with superoxide ($O_2^{\cdot -}$), a reactive oxygen species (ROS), to produce cytotoxic oxidizing agents called reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^-$) [3]. This molecule is a potent biological oxidant that can oxidize and nitrate the aromatic ring of free or protein-bound tyrosine residues to produce the stable compound 3-nitro-tyrosine (3-$NO_2$-Tyr, 3NT) [4], which is considered in turn as an important biomarker of endogenous RNS production in various tissues [5]. However, tyrosine is not the only amino

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acid susceptible to be nitrated by $ONOO^-$ [6–8] and the respective importance of nitration reactions with methionine, cysteine, tryptophane and tyrosine is not clear.

A predominant part of $ONOO^-$ readily reacts with $CO_2$ in equilibrium with bicarbonate present in plasma [9]. The reaction with $CO_2$ is of particular importance because it is one of the fastest reactions known for $ONOO^-$. It can lead to the formation of a postulated highly reactive short-lived secondary oxidant, the nitrosoperoxycarbonate adduct ($ONOOCO_2^-$) and of highly reactive intermediates ($NO_2^{\cdot}$ and $CO_3^{\cdot-}$) derived therefrom [10]. In blood plasma, bicarbonate has been shown to increase the one-electron aromatic nitration of protein-bound tyrosine residues and to inhibit sulfydryl oxidation, thus redirecting peroxynitrite reactivity [11,12].

Based on the detection of 3NT, peroxynitrite has been suggested to be involved in the pathogenesis of a wide range of diseases, including cancer, chronic inflammation [13], rheumatoid arthritis [14], neurodegenerative diseases [15], respiratory conditions [16] and atherosclerosis [17].

HSA is the most abundant protein in plasma. It has multiple, physiological roles [18,19], including that to be an antioxidant in plasma and the extracellular spaces. Human albumin contains 18 tyrosine residues in a total of 585 amino acid residues [20]. It has recently been suggested that both albumin—as an associated protein—and lipids of LDL are of prime importance in determining the resistance of the particle to $Cu^{2+}$-oxidation [21]. Mild nitration of albumin by peroxynitrite has also been studied, unveiling that two tyrosine residues were reactive belonging to subdomains IIIA and IB [22].

LDL is a major lipoprotein in humans. It consists of a lipid core and a protein moiety with generally an unique protein, the apolipoprotein B (apoB), containing 152 tyrosine residues in a total of 4536 amino acid residues [23]. A large body of evidence supports the key role of oxidized low-density lipoprotein (ox-LDL), in the early (inflammatory) and more advanced stages of the atherosclerotic lesion [17,24–26]. One among its main properties is to promote the type B scavenger receptor-mediated accumulation of cholesterol in macrophages and foam cells leading to the formation of the atheromatous plaque.

Many studies have demonstrated the ability of peroxynitrite to cause oxidative modifications of the LDL components, including the lipid [27–32] and protein moieties [33]. Peroxynitrite converts LDL into nitrated LDL, a form recognized by the scavenger receptor of macrophages [34]. This clearly shows that the LDL modification by peroxynitrite plays a significant role in atherogenesis [17]. Moreover, it has recently been reported that nitrated LDL could be a better ligand for scavenger receptor [35] than ox-LDL. It is therefore of interest to explore any process liable to participate in inhibiting or impairing this nitration.

LDL is generally surrounded by extrinsic (hydrophilic) antioxidants, including albumin [36]. The purpose was here to study the nitration of 1/a LDL/albumin complex (LAC)—previously recognized as the form of LDL prevailing in plasma [21]—2/albumin alone or 3/LAC and albumin together in order to examine whether their nitration may interact in a manner leading to protect LAC from nitration in mild, physiologically relevant conditions of peroxynitrite formation.

## Materials and methods

### *Chemicals*

HSA (99%, essentially fatty acid free and globulin free), 3-nitro-L-tyrosine (3-$NO_2$-Tyr or 3NT), diethylenetriamine pentacetic acid (DTPA), phenol and trifluoroacetic anhydride (TFAA) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), 2,3,5,6-$d_4$-L-tyrosine ([$d_4$]-Tyr, 98%) was provided by Cambridge Isotope Laboratories, Inc. (Gif-sur-Yvette, France), sodium bicarbonate ($NaHCO_3$), methanol and dichloromethane were from Prolabo (Vaulx en Velin, France), acetonitrile ($CH_3CN$), sodium phosphate dibasic ($Na_2HPO_4 \cdot 12H_2O$) were from Merck (Nogent-sur-Marne, France), 3-morpholinosydnonimine hydrochloride (SIN-1) was from Interchim (Montluçon, France), *N-tert*-butyldimetylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-butyldimethylchlorosilane (MTBSTFA) and nitronium borofluorate ($NO_2BF_4$) were obtained from Fluka (Saint Quentin Fallavier, France), sodium chloride and hydrochloric acid were from Carlo Erba (Val de Reuil, France), sodium phosphate monobasic ($NaH_2PO_4$) was from Labosi (Oulchy-le-Chateau, France). Solutions were prepared with distilled water, which was further purified using a Milli-Q water purification system.

### *Synthesis and purification of 2,5,6-[$d_3$]-nitrotyrosine (3-$NO_2$-[$d_3$]-Tyr) for internal standard*

The method used for the deuterated nitrotyrosine derivative preparation was adapted from Houli et al. [37]. Briefly, isotopically labelled 3-$NO_2$-[$d_3$]-Tyr was synthesized from [$d_4$]-Tyr with the nitrating agent $NO_2BF_4$. After the nitration step, sample was purified on a Sep-Pak C18 Cartridge column (Waters, Saint-Quentin-en-Yvelines, France). An aliquot of the eluate was evaporated under nitrogen, resuspended in the phosphate buffer and the deuterated nitrotyrosine was quantified by means of a Waters 510 HPLC system (Waters) equipped with a μBundapak C18 column (3.9 × 300 mm, 10 μm particle size, Waters). Elution was performed with a 50 mM $NaH_2PO_4$

(pH 6.5) solution for 15 min at a flow rate of 1 ml/min. The product was quantified by comparing the absorbance at 274 nm (Waters 484 tunable absorbance detector) to that of known authentic 3NT.

*Preparation and biochemical parameters of low-density lipoprotein (LDL)*

Samples of human plasma were collected from three healthy, normo-lipidemic subjects donating their blood to the Etablissement Français du Sang and pooled to be used immediately for LDL preparation. Preparation and determination of biochemical parameters of LDL (*d* 1.019–1.063 g/ml) were carried out as previously reported [21]. The LDL preparation led to an albumin-associated form, the LDL/albumin (1:1, mol/mol) complex (LAC) which was immediately frozen in the presence of 10% sucrose and stored at −80°C until utilization. LAC was thawed just before using and extensively dialyzed three times at 4°C in the dark for 24 h against a PBS-DTPA buffer (pH 7.4). Its apoB content was determined by immunonephelometry method using a turbitimer apparatus (Behring, Rueil-Malmaison, France), whereas the protein content was determined using the Markwell's modification of the Lowry's method [38]. Assimilating the concentration of albumin-free LDL to that of apoB allowed to confirm that the LDL/albumin molar ratio was 1.

*Nitration of HSA and LDL*

The stock solution of SIN-1 (1 mM) in 10 mM sodium phosphate buffer (pH 7.4) containing 10 μM DTPA (the PBS/DTPA buffer) [30] was stored at −20°C and spectrophotometrically verified before use. The nitration of each following substrate: albumin (0.1–1 μM) without/with LAC (0.1 μM, expressed as apoB) and LAC without albumin was carried out in the PBS/DTPA buffer without or with 25 mM $NaHCO_3$ in the presence of 72.5 μM SIN-1 for 5 h at 37°C. The nitration was carried out in triplicate. Once nitrated, albumin and/or LAC were precipitated and hydrolyzed as described by Crowley et al. [39] with minor modifications. Briefly, proteins were precipitated by addition of 6 ml of ice-cold acetonitrile and collected by centrifugation (3500 rpm, 20 min, 4°C) on a Beckman J-21B apparatus. Supernatants were removed, protein pellets were washed with acetonitrile (250 μl) and centrifuged (this procedure was repeated once more).

*Protein hydrolysis and isolation of amino acids*

A volume of 200 μl of a 20 μmol/l 3-$NO_2$-[*d3*]-Tyr solution was added to protein pellets before evaporating to dryness under nitrogen gas. Samples were re-suspended in HCl (6 M, 500 μl) containing 1% phenol, kept 18 h at 110°C and then evaporated to dryness at 37°C [39]. To remove contaminants that interfere with derivatization and ionization of amino acids, hydrolysates were submitted to a solid-phase extraction [40]. To do so, they were re-suspended in HCl (0.1 M) and passed through a C18 Sep-Pak Cartridge column (Waters) previously washed with methanol (5 ml) and HCl (0.1 M, 10 ml). Column was washed with HCl (0.1 M, 5 ml) and was performed with 3 ml of methanol. Finally, the eluting solvent was evaporated under vacuum at 37°C prior to conversion of 3NT and amino acids in appropriate derivatives.

*Derivatization procedure for 3-$NO_2$-Tyr*

Amino acids were first incubated in 200 μl of cold TFAA and 200 μl of cold acetonitrile for 10 min at ambient temperature [41]. Excess solvent and reagent were removed under $N_2$ at ambient temperature and their *bis*-O-*tert*-butyl-dimethylsilyl ester or ether derivatives were generated by addition of 50 μl of dichloromethane and 50 μl of *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) with 1% *tert*-butyldimethylchlorosilane and incubation for 60 min at 38°C [42]. Analyses were immediately performed or samples were stored at −20°C until analysis.

*Gas chromatography-mass spectrometry (GC/MS) analysis*

GC/MS analyses were carried out using a system consisting of a Trace 2000 series gas chromatograph equipped with a splitless injector on a Rtx®-1 fused-silica capillary column (30 m × 0.32 mm internal diameter with 0.1 μm stationary phase film thickness; Restek, Evry, France), directly connected to a ThermoFinnigan single-stage quadrupole (SSQ) AUTOMASS mass spectrometer (ThermoFinnigan, Les Ulis, France). About 2 μl of *N*-trifluoroacetyl amide and *bis*-O-*tert*-butyl-dimethylsilyl ester or ether derivatives (TFAA/TBDMSE derivatives) were injected manually. Helium was used as the carrier gas at a constant flow rate of 0.9 ml/min. The GC column was at the starting temperature of 120°C for the first 2 min, the temperature was increased to 280°C at the 50°C/min rate and it was then maintained for 10 min. The injector, transfer line and ion source were at 300, 280 and 250°C. Analyses were performed in electron impact mode (ionization energy of 70 eV; filaments at 800 μA) in full-scan (mass range 150–600 amu at a scan rate of 500 ms per scan) and in selected ion monitoring acquisition mode at a scan rate of 250 ms per ion. The data acquisition and instrument control were carried out using Xcalibur software (ThermoFinnigan). A standard curve was prepared by mixing increasing amounts of 3NT (0–500 pmol) and a constant amount (2000 pmol) of 3-$NO_2$-[$d_3$]-Tyr

as an internal standard. Each mixture was evaporated to dryness; the concentrate was derivatized and analyzed by GC/MS as described above. The amount of 3NT was calculated from a regression line (typically, $r^2 = 0.999$) obtained by plotting the peak area ratio of the unlabeled and labeled nitrotyrosine selected fragments. A new standard curve was plotted for each series of determinations (native LAC, treated LAC without added HSA, treated LAC with added HSA, treated HSA without LAC). Reproducibility of the standard curves has been tested.

## Results and discussion

Under the chosen derivatization conditions, deuterated and non-deuterated 3NT were converted to their *N*-trifluoroacetylamide-*bis*-O-*tert*-butyl-dimethylsilyl ester or ether derivatives (Figure 1). The derivatization procedure gave a derivative with a molecular mass of 553 for 3-$NO_2$-[$d_3$]-Tyr and 550 for 3NT. Analysis of derivatives in full-scan mode showed that deuterated and non-deuterated 3NT had a predominant ion at *m*/*z* 496 and *m*/*z* 493, respectively, formed by the loss of 57 mass units (M-*tert*-butyl). The molecular ions were not observed.

Using 3-$NO_2$-[$d_3$]-Tyr as an internal standard potentially improves quality and conditions of analyses. After complete derivatization samples were analyzed in selective ion monitoring (SIM) mode using *m*/*z* 496 for 3-$NO_2$-[$d_3$]-Tyr and *m*/*z* 493 for 3NT. Figure 2 shows that there was a linear relationship between the peak area ratio of ((*m*/*z* 493)/(*m*/*z* 496)) and the amount of 3NT added. It also shows that the reproducibility of three standard curves carried out separately was good. The limit of quantification was 4 pmol for a signal-to-noise ratio of 36.

We found that 3NT was present in native LAC (not exposed to SIN-1) in trace amount, i.e. $0.015 \pm 0.001$ μmol/l for 0.1 μmol/l LAC, leading to the molar ratio 3NT/LAC-bound tyrosines of $1.0 \pm 0.07 \times 10^{-3}$ mol/mol.

In order to elicit peroxynitrite nitration in mild, physiologically relevant conditions, the SIN-1 concentration was chosen according to conditions previously tested, i.e. the lowest concentration that simultaneously produced the highest level of conjugated dienes (CD) and the maximal loss of eicosapentaenoic acid and docosahexaenoic acid in the 0.1 μM LAC preparation [30]. In this condition, Figure 3 shows the SIN-1-generated production of 3NT as a function of increasing albumin-concentrations in the presence or absence of a constant LAC concentration. It clearly appears that the presence of $HCO_3^-$ increased the conversion yield of tyrosine into 3NT by a factor of about 2 (compare the slope value 0.0044–0.0014 or 0.0023). Interestingly, the total HSA-bound tyrosine residues added enhanced linearly the converted tyrosine (except for the concentrations ranged within 0–2 μmol/l of added HSA-bound tyrosines for which the conversion yield was higher). The slopes of the curves were identical in the presence and absence of LAC, which was indicative of a constant nitration yield of HSA-bound tyrosines ($4.4 \times 10^{-3}$ mol/mol in the presence of $HCO_3^-$), whereas the LAC-bound tyrosine residues converted into 3NT was uniformly higher (+27 nmol/l by comparing curve A and curve B, Figure 3), regardless of added HSA concentration, corresponding to an apparent nitration rate of LAC-bound tyrosines of $1.6 \times 10^{-3}$ mol/mol and $0.6 \times 10^{-3}$ mol/mol after subtracting baseline nitration (see also Tables I and II). However, in the absence of HSA the concentration of LAC-bound tyrosine residues converted into 3NT was 0.06 μmol/l (Figure 3), leading to an apparent nitration rate of $3.5 \times 10^{-3}$ mol/mol and a nitration rate of $2.5 \times 10^{-3}$ mol/mol after subtracting baseline nitration (Table II).

Altogether, these results suggest that the conversion rate of HSA-bound tyrosine into 3NT was not modified by the presence of LAC, whereas the LAC-bound tyrosine converted into 3NT was decreased by about 75% by added HSA. In other words, it turned out that LAC did not affect HSA nitration, whereas HSA impaired LAC nitration, in spite of much lower amounts of tyrosine residues in HSA than in LAC. An explanation could be that the tyrosine residues bound to HSA were more reactive than those bound to LAC, as accounted for by the nitration ratios of $4.4 \times 10^{-3}$ mol/mol and $2.5 \times 10^{-3}$ mol/mol, respectively. However, this difference is more probably explained by a "hiding effect" of albumin towards some nitratable apoB-bound tyrosine residues.

We have established that peroxynitrite production increases linearly with SIN-1 concentration ranged within 0–1 mM (data not shown). Gow et al. [11] assumed that the rate of peroxynitrite production by 1 mM SIN-1 was approximately 10 μM/min. It is allowed to conclude that in our hand (5-h incubation, 72 μM SIN-1) the peroxynitrite production was 0.7 μM/min or 200 μM for the complete incubation time. Comparing to the nitration rate found by others—ranged within $10 \times 10^{-3}$–$27 \times 10^{-3}$ mol/mol [33,43–45]—and keeping in mind that available data were for bovine serum albumin (BSA) at higher concentrations but without $HCO_3^-$, our data were expectedly lower. The conversion rate of BSA-bound tyrosine residues reported by Oshima et al. [40] was $3.9 \times 10^{-3}$ mol/mol, close to that presently found, once exposed to 150 μM peroxynitrite for only 5 min. However, when nitration was performed by means of 150 μM SIN-1 for 1 h, the conversion rate was $0.12 \times 10^{-3}$ mol/mol. Alvarez et al. [12] carried out HSA nitration and considered the nitrated tyrosines

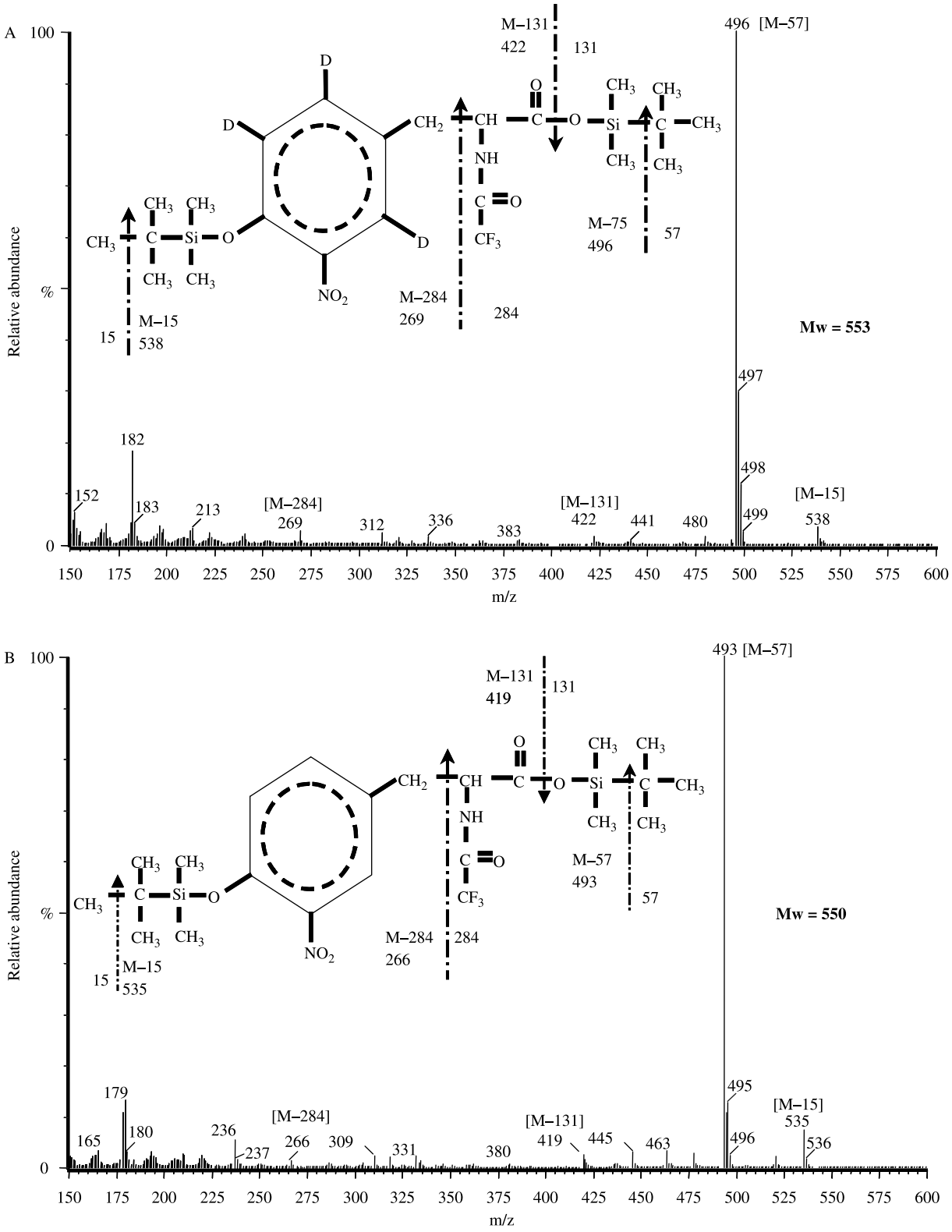


Figure 1. Chemical structure and full-scan mass spectrum of (A) 3-$NO_2$-[$d_3$]-Tyr and (B) 3-$NO_2$-Tyr. 3-$NO_2$-[$d_3$]-Tyr and 3-$NO_2$-Tyr were acylated with TFAA/$CH_3CN$ and then silylated with MTBSTFA/$CH_2Cl_2$ to form the *N*-trifluoroacetyl amide-*bis*-O-*tert*-butyl-dimethylsilyl ester or ether derivatives. The derivatives were analyzed by GC/MS in electron impact mode as described in Methods. The molecular mass of the final derivative was 553 mass units (for 3-$NO_2$-[$d_3$]-Tyr) and 550 mass units (for 3-$NO_2$-Tyr). The electron impact full-scan mass spectrum of the 3-$NO_2$-[$d_3$]-Tyr and 3-$NO_2$-Tyr gave a predominant ion at *m*/*z* 496 mass unit (for 3-$NO_2$-[$d_3$]-Tyr) and *m*/*z* 493 mass unit (3-$NO_2$-Tyr), formed by the loss of 57 mass units (M-*tert*-butyl).

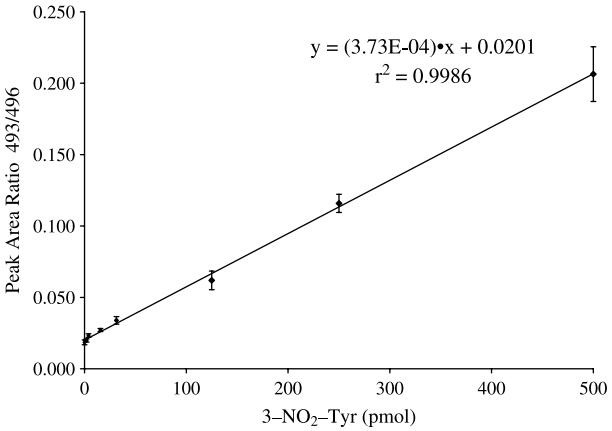


Figure 2. Standard curve for stable isotope dilution GC/MS measurement of 3-$NO_2$-Tyr. To a constant amount (2000 pmol) of 3-$NO_2$-[$d_3$]-Tyr as an internal standard was added increasing amounts (0–500 pmol) of 3-$NO_2$-Tyr. After derivatization, analyses were carried out by GC/MS in the electron impact mode with selected monitoring of ions arising from the target analyte (*m/z* 493) and the internal, deuterated standard (*m/z* 496). The peak-area ratio of the selected ions (non deuterated to deuterated nitrotyrosine) was plotted as a function of the amount of 3-$NO_2$-Tyr initially added. Here is shown the curve resulting from three typical standard curves obtained separately at different times.

in the presence and absence of $HCO_3^-$. They found $20 \times 10^{-3}$ and $4.7 \times 10^{-3}$ mol/mol, respectively. The conversion rate in the presence of $HCO_3^-$ was clearly higher (by a factor of 4.5) than in our hand, this being explained by higher concentrations in both HSA and SIN-1.

Data on the conversion rate of LDL-bound tyrosine residues are very few. Using 2 μM LDL incubated for 5 min without $HCO_3^-$, Leeuwenburgh et al. [33] found $0.8 \times 10^{-3}$ mol/mol and $20 \times 10^{-3}$ mol/mol in the presence of 30 μM and 300 μM peroxynitrite, respectively, vs. $1.3 \times 10^{-3}$ mol/mol in our hand (without $HCO_3^-$). In spite of rather different conditions, it can be concluded that there are no conflicting results between the conversion rate presently found and those found by others.

Since LAC was prepared from healthy subjects, the molar nitration ratio 3NT/LAC-bound tyrosine found in native LAC could be ascribed to the baseline oxidative stress. However, a molar ratio of $0.009 \pm 0.007 \times 10^{-3}$ mol/mol has been reported in LDL from plasma of healthy subjects [33]. Others found a molar

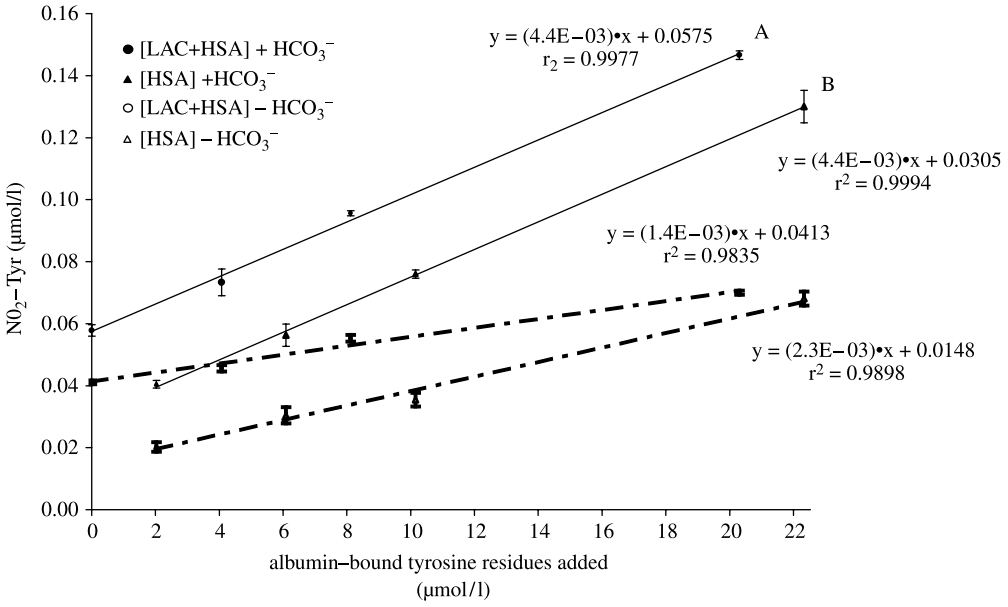


Figure 3. Formation of 3-$NO_2$-Tyr in LDL/HSA complex (LAC) and HSA alone exposed to SIN-1, a peroxynitrite generating system. HSA (0.1–1 μM) in the presence (0.1 μM) and absence of LAC were nitrated by SIN-1 (72.5 μM) in PBS/DTPA pH 7.4 (10 μM) without and with $HCO_3^-$ (25 mM) and incubated for 5 h at 37°C. After precipitation proteins were hydrolyzed and submitted to solid-phase extraction on a Sep-Pak C18 cartridge column. Protein-bound tyrosines and 3-$NO_2$-[$d_3$]-Tyr as an internal standard were then converted into the *N*-trifluoroacetyl amide-*bis*-O-*tert*-butyl-dimethylsilyl ester or ether derivatives. Analyses were performed by GC/MS as described in Methods. Values are the mean ± SEM of three independent determinations.

Table I. Calculation of protein-bound tyrosine conversion rate into nitrotyrosine.

| LAC (as apoB) | 0.1 μmol/l | HSA in LAC | 0.1 μmol/l |
|---|---|---|---|
| Tyrosine residues: | | Tyrosine residues | 1.8 μmol/l |
| •ApoB | 15.2 μmol/l | | |
| •HSA | 1.8 μmol/l | | |
| •Total | 17.0 μmol/l | | |
| LAC-bound $NO_2$-tyrosine | 27 nmol/l | HSA-bound $NO_2$-tyrosine | 8 nmol/l |
| Tyrosine nitration rate (mol/mol) | $1.6 \times 10^{-3}$ | Tyrosine conversion rate (mol/mol) | $4.4 \times 10^{-3}$ |

Each value results from a single calculation which was based on the data from Figure 3.

ratio of $0.02 \pm 0.004 \times 10^{-3}$ mol/mol [44]. This discrepancy (a factor 100 with our result) highlights that the baseline nitration of LDL remains to be explored. The mode of LDL preparation leading to different types of complexes between LDL and albumin [21] may play a role in the final nitration ratio assessment and we have unfortunately no information on the potential occurrence of such complexes in [33]. For the same concentration of apoB, albumin associated to LDL increases the number of tyrosine residues to be nitrated. However, it is doubtful whether this explains the difference of a magnitude of $10^{-2}$ for at least two reasons: (i) 1 mol of HSA adds 2 nitratable tyrosines on a total of 152 in 1 mol of apoB; (ii) the potential occurrence of a "hiding effect" of HSA, as already noticed. These large differences could be attributable merely to the natural inter-individual variations. On the other hand, LDL isolated from the fatty streaks-containing thoracic aorta led to a molar nitration ratio of $0.84 \pm 0.14 \times 10^{-3}$ mol/mol as assessed from a single individual [33].

We can conclude that the tyrosine nitration rate brought about by the SIN-1 treatment with or without $HCO_3^-$ was higher for HSA than for apoB. In the present mild conditions, SIN-1 increased by a factor 3.5 the tyrosine nitration rate of the native LDL/HSA complex in the presence of a physiologically relevant concentration of $HCO_3^-$. HSA was found to partly prevent LDL nitration.

These results lead us to advance that nitration of both HSA and LDL may occur in pathophysiological conditions. They raise the question of the pathophysiological significance of the LDL (apoB) nitration and its decrease by albumin. In the state of knowledge, the consequences of the partial inhibition of apoB nitration by albumin and those of albumin nitration seem to be clear. It is likely that decreased apoB nitration leads to decreased scavenger receptor activation, then foam cell formation and finally atherogenic plaque deposition [17,34,35]. On the other hand, albumin nitration—more than apoB nitration according to the present data—leads to removal of peroxynitrite from plasma, which may be considered beneficial in an atherogenic context, since this potentially protects against the deleterious effects of peroxynitrite alterations such as tetrahydrobiopterin oxidation (and its consequence, the uncoupling of eNOS), activation of cyclooxygenase enzymes and inhibition of prostacyclin synthase, all events participating in the endothelium dysfunction[46,47]. However, LDL (apoB) nitration may also be considered more specifically as a process assuring a targeted transport of nitrite to monocytes/macrophages. Given that monocytes/macrophages express myeloperoxidase (MPO) and that MPO-released HOCl has been found to be decreased by $NO_2$ [48], we can assume that the formation of HOCl-modified LDL—which contributes together with other modified forms of LDL to the atheromatous plaque formation—could be slowed down. However, that $NO_2$ be released locally from the protein-bound nitrated-tyrosines remains to be explored. A better understanding requires new researches on the fate of the 3NT nitrite-groups.

Table II. Conversion rates of protein-bound tyrosine into nitrotyrosine before/after SIN-1 treatment related to the protein moiety or the tyrosine residues bound to the protein moiety.

| | Native LAC (no treatment) | Treated LAC w/o added HSA | Treated LAC with added HSA | Treated HSA w/o LAC |
|---|---|---|---|---|
| Protein moiety* with $HCO_3^-$ | 0.15 | 0.6 | 0.27 | 0.08 |
| Protein-bound tyrosine*: | | | | |
| •With $HCO_3^-$ | $1.0 \pm 0.07 \times 10^{-3\dagger}$ | $3.5 \times 10^{-3}$ | $1.6 \times 10^{-3}$ | $4.4 \times 10^{-3}$ |
| | – | $2.5 \times 10^{-3\ddagger}$ | $0.6 \times 10^{-3\ddagger}$ | – |
| •W/o $HCO_3^-$ | – | $2.3 \times 10^{-3}$ | – | – |
| | – | $1.3 \times 10^{-3\ddagger}$ | – | – |

Each value results from a single calculation which was based on the data from Figure 3; *Mol/mol of protein moiety or of total tyrosine residues bound to protein moiety; †In triplicate; ‡Once the native LAC nitration was subtracted.

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