MASSTRPLAN Protocol

Reversed-phase LC-MS and MS/MS analysis of nitroxidative modifications in Cardiolipin

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Rationale for the Protocol

Nitroxidative modifications are of increasing interest owing to the understanding that nitrating and oxidizing conditions occur in inflammation and several associated diseases, such as cardiovascular disease and neurodegenerative diseases, through formation of the highly reactive compound peroxynitrite ($ONOO^-$) and radical nitroxy species. These have long been known to attack unsaturated fatty acids, leading to the formation of nitrated fatty acids, well known endogenous lipid mediators. More recently, modification of phospholipids by these reactive nitrogen species was also reported, leading to formation of nitrated and nitroxy products that have a variety of biological activities. However, previously the formation of such products on the mitochondrial lipid cardiolipin had not be studied.

This protocol details how to obtain nitroxidation products of cardiolipin using an in vitro model of nitroxidation with NO_2BF_4, followed by methods for structural characterization by reversed-phase C30 liquid chromatography – high-resolution mass spectrometry. The protocol is based on the methods and data that we have reported in [1].

Protocol

Equipment:

UltiMate 3000RS liquid chromatography system with a reversed-phase C30 column (Accucore C30 column (150 mm*2.1 mm, 2.6 µm, 150 Å, Thermo Fisher Scientific)) coupled to a high-resolution Q-Exactive mass spectrometer. Data acquisition can be carried out using the Xcalibur data system (V3.3, Thermo Fisher Scientific, Waltham, MA, USA).

Materials:

3'-Bis[1,2-Di-(9Z-12Z-octadecadienoyl)-sn-glycero-3-phospho]-snglycerol or tetralinoleoyl cardiolipin (TLCL) can be bought from Avanti® Polar Lipids, Inc. (Alabaster, USA) and used without further purification. Nitronium tetrafluoroborate (NO2BF4) can be purchased from Sigma-Aldrich (Madrid, Spain).

In vitro nitroxidation:

Cardiolipin (TLCL) nitration (1 mg) is carried out with an excess of nitronium tetrafluoroborate (approximately 5 mg) in chloroform (1 mL) at room temperature for 1 h, under orbital shaking at 750 rpm. Nitration should be quenched with water (2 mL), vortexing for 30 s and centrifuged for 5 min at 1000 rpm in a Mixtasel Centrifuge (Selecta, Barcelona, Spain). Discard the water phase containing products from the hydrolysis of the excess of NO2BF4 and salts in general, while the lower organic phase containing the cardiolipin is kept and dried under a nitrogen stream and stored at −20 °C to be further quantified using a phosphorus assay [2] and analysed by C30-HPLC-ESI-MS and MS/MS.

LC-MS conditions:

Cardiolipin nitroxidation products formed as describbed above should be dissolved in pure methanol and a total amount of 5 µg (10 µL) were injected using an autosampler. A flow rate of 300 µL/min and a column temperature of 40 °C is used. The solvents are A: acetonitrile/water/formic acid (95/5/0.1; v/v/v), 5 mM ammonium acetate; and B: isopropanol/acetonitrile/water/ formic acid (85/10/5/0.1; v/v/v/v), 5 mM ammonium acetate. The gradient has been optimized as follows: 25% B for 2 min, 25–

86% B in 18 min, 86–95% B in 1 min, 95% B for 14 min, and 10 minutes of re-equilibration of the column to initial conditions.

The mass spectrometer is operated in negative ion mode with a HESI source (setup with needle voltage 2.7 kV; capillary temperature 350°C; sheath gas flow 45 units, auxiliary gas flow 15 units, probe heater temperature 350ºC): MS survey mass spectra should be acquired with resolving power of 70,000 (full width half maximum) in the m/z range of the products expected, m/z between 1395–1750, using an automatic gain control (AGC) target of 10E6 and a maximum injection time of 100 ms. Use data-dependent acquisition, with an inclusion list containing the m/z values of possible nitroxidative modifications identified in MS (Table 1) and dynamic exclusion of 30 seconds, to select the five most intense ions for higher-energy collisional dissociation (HCD) fragmentation (stepped normalized collision energy of 20, 23, and 25% with nitrogen gas) generating MS/MS spectra at resolution of 17,500, with an AGC target of 10E5 and a maximum injection time of 200 ms.

Data-independent acquisition (DIA), using a list of the target m/z values and the respective retention times, was also performed for the lowest abundant ions to obtain high-quality MS/MS spectra. The method scans only these ranges using a 1 m/z isolation window, maximum injection times of 300 ms, and AGC targets of 2x10E5.

Expected results and example data

A total of 11 different modifications were assigned with unequivocal LC-MS and MS/MS information, including functional and positional isomers (Table 1).

Table 1. Nitrated and nitroso products of tetralinoleoyl-cardiolipin (TLCL) formed by treatment with NO2BF4, identified by C30 RP LC-HR-MS as [M-H]- ions and confirmed by accurate mass measurements and MS/MS data analysis. The relative abundance RA (%) of the various nitrated species was determined by integrating the peak area for each species.

***m/z* theoretical**	***m/z* observed**	**Error (ppm)**	**Shift (Da)**	**Modification**	**RT (min)**	**%**
1447.9644	1447.9686	2.9	0	-	24.2	-
1476.9546	1476.9595	3.3	29	NO	21.9	60.9
1505.9447	1505.9497	3.3	58	$(NO)_2$	17.5	4.1
1505.9447	1505.9501	3.6	58	$(NO)_2$	18.3	3.8
1534.9349	1534.9396	3.1	87	$(NO)_3$	12.9	1.3
1563.9251	1563.9279	1.8	116	$(NO)_4$	6.2	2.9
1492.9495	1492.9542	3.2	45	NO_2	23.0	11.9
1492.9495	1492.9539	3.0	45	NO_2	21.8	2.6
1492.9495	1492.9518	1.6	45	NO+O	19.6	2.4
1521.9396	1521.9447	3.4	74	$(NO)(NO_2)$	21.9	1.7
1521.9396	1521.9446	3.3	74	$(NO)(NO_2)$	20.2	5.4
1537.9346	1537.9376	2.0	90	$(NO_2)_2$	21.3	1.3
1537.9346	1537.9373	1.8	90	$(NO)(NO_2)$+ O	18.3	1.5

Examples of the chromatographic separation of different nitroxidized cardiolipin species are shown in Figure 1; it can be seen that addition of multiple nitro- and nitroxy- groups has substantial effects of the retention time.

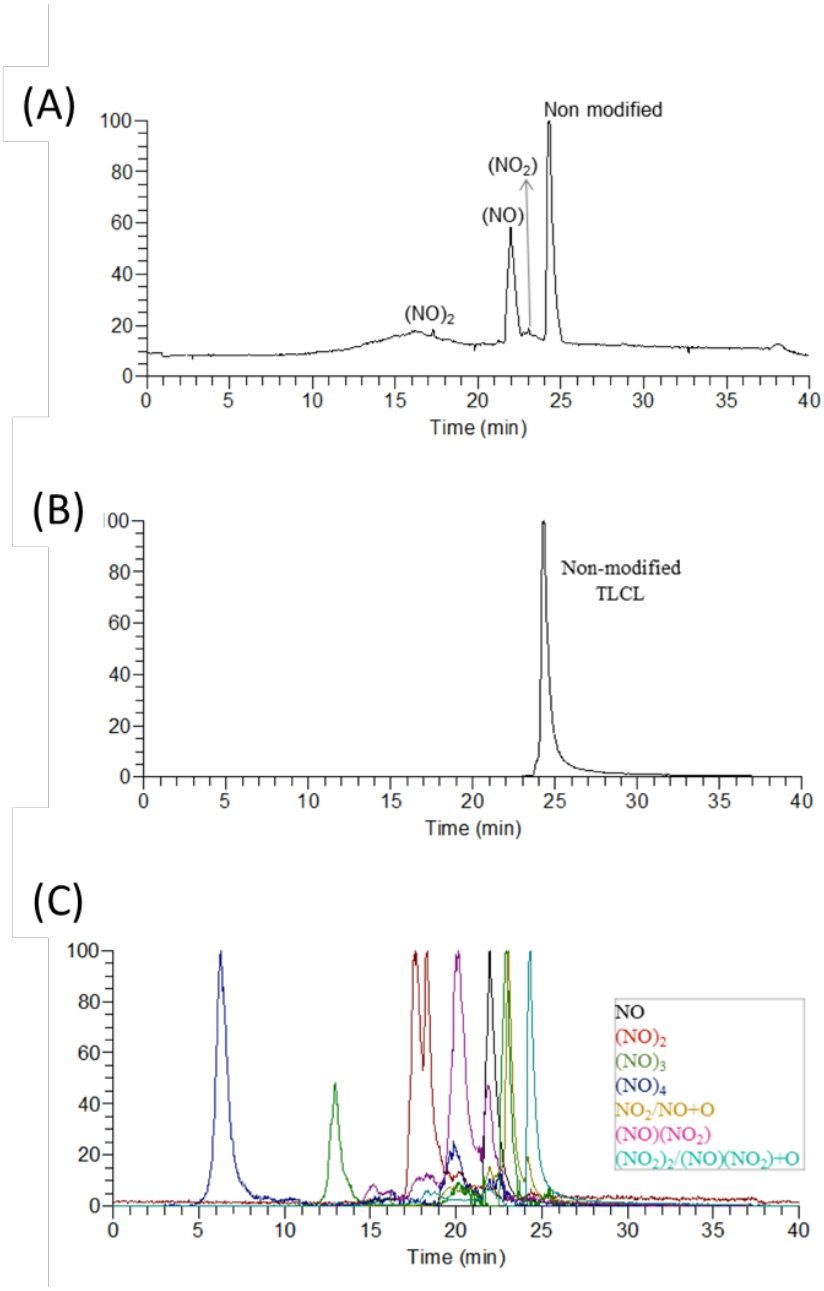

Figure 1. Total ion chromatogram obtained after nitroxidation of TLCL(A), reconstructed ion chromatogram (RIC) of non modified TLCL(B) and of the nitoso and nitrated derivatives (C) identified by C30 RP LC-HR-MS as $[M-H]^-$ ions and confirmed by accurate mass measurements and MS/MS data analysis shown in Table 1.

The fragmentation patterns of nitroxidized cardiolipin can be used to identify the modifications, as illustrated in Figure 2. The fragment ions have potential as reporters for modified cardiolipin containing nitro- or nitroxy- groups.

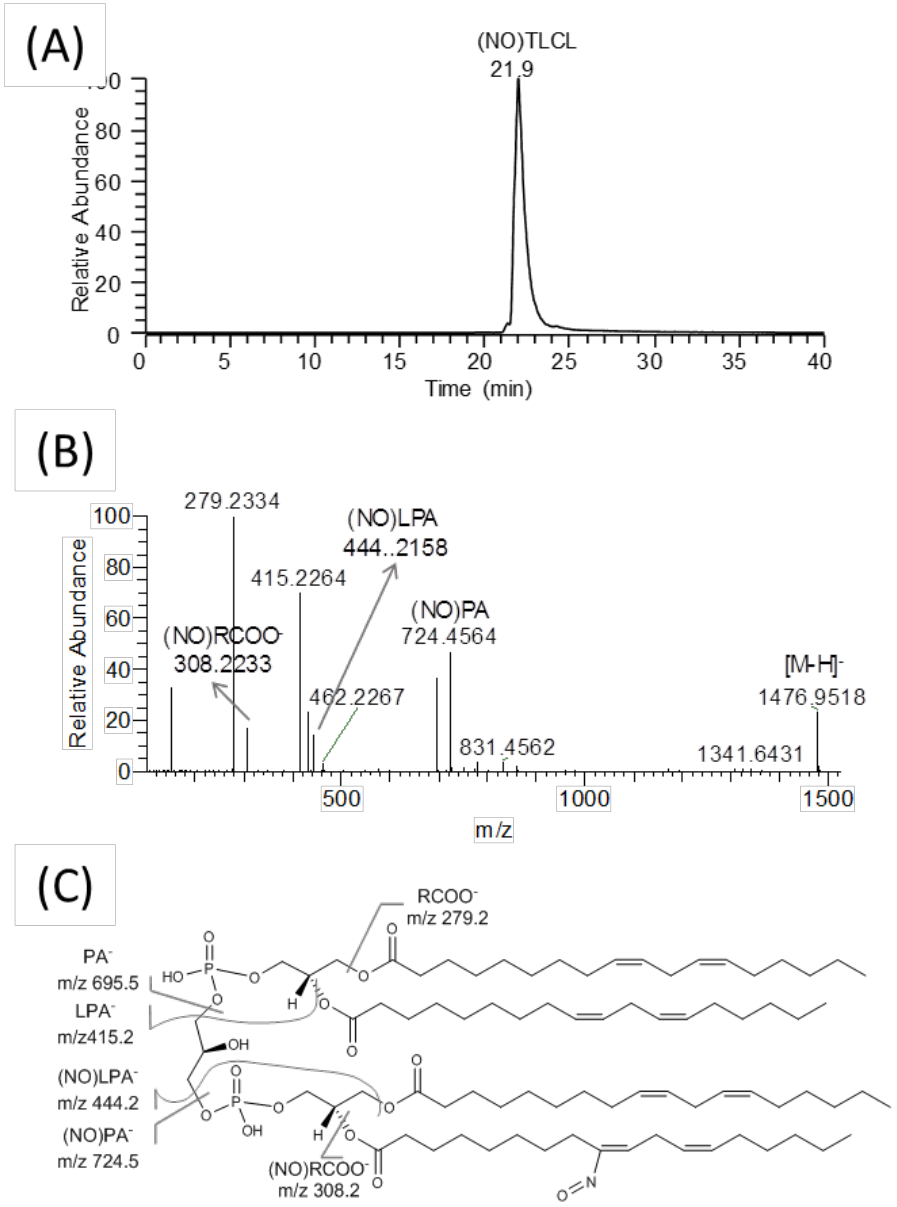

Figure 2: A) Reconstructed ion chromatogram for species with a mass shift of +29Da. B) Corresponding HCD MS/MS spectrum of the species acquired at RT 21.9 min. C) Structure of nitroxidized TLCL showing the main observed fragmentation pathways.

References

1. Javier-Fernando Montero-Bullon, Tânia Melo, M. Rosário M. Domingues, Pedro Domingues. Liquid chromatography/tandem mass spectrometry characterization of nitroso nitrated and nitroxidized cardiolipin products. Free Radical Biology and Medicine. https://doi.org/10.1016/j.freeradbiomed.2019.05.009

2. E.M. Bartlett, D.H. Lewis, Spectrophotometric determination of phosphate esters in the presence and absence of orthophosphate, Anal. Biochem. 36 (1970) 159–167, https://doi.org/10.1016/0003-2697(70)90343-X.