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Insight into the cellular effects of nitrated phospholipids: evidence for pleiotropic mechanisms of action

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Abbreviations : FAK, focal adhesion kinase; NO₂-FA, nitrated fatty acids; NO₂-POPC, nitrated 1-palmitoyl-2-oleyl-phosphatidyl choline; 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; PPAR LBD, peroxisome proliferator activated receptor ligand binding domain; PPRE, PPAR response element; DAF-2 DA, 4,5-Diaminofluorescein diacetate; EMSA, electrophoretic mobility shift assay ; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; SNP, sodium nitroprusside; lac-B, biotinylated iodoacetamide

Nitrated phospholipids have been recently identified in biological systems and showed to display anti-oxidant and anti-inflammatory potential in models of inflammation *in vitro*. Here, we have explored the effects of nitrated 1-palmitoyl-2-oleyl-phosphatidyl choline (NO₂-POPC) in cellular models. We have observed that NO₂-POPC, but not POPC, induces cellular changes consisting in cytoskeletal rearrangement and cell shrinking, and ultimately, loss of cell adhesion or impaired cell attachment. NO₂-POPC releases NO *in vitro* and induces accumulation of NO in cells. Nevertheless, the effects of NO₂-POPC are not superimposable with those of NO donors, which points to distinctive mechanisms of action. Notably, they show a stronger parallelism, although not complete overlap, with the effects of nitrated fatty acids. Interestingly, redistribution of vimentin by NO₂-POPC is attenuated in a C328S mutant, thus indicating that this residue may be a target for direct or indirect modification in NO₂-POPC-treated cells. Additionally, NO₂-POPC interacts with several typical lipoxidation targets *in vitro*, including vimentin and PPAR γ constructs, likely through cysteine residues. Therefore, nitrated phospholipids emerge as potential novel electrophilic lipid mediators with selective actions.

Keywords: lipoxidation; electrophilic lipid mediators; nitrated lipids; actin; vimentin

Under situations of oxidative stress, numerous lipid reactive species are generated that can react with various cellular components eliciting protective or damaging actions, depending on their levels, structure, and the cellular context, particularly the antioxidant defenses of the cell [1]. One of the main mechanisms for the action of electrophilic lipid species is their covalent binding to proteins, known as lipoxidation, which can alter their structure and functions [2, 3]. Indeed, covalent protein modification by oxidized lipids can elicit stress signaling pathways leading to activation of cellular defenses, or if these are surpassed, constitute pathogenic mechanisms that can contribute to cardiovascular, neurodegenerative or metabolic disease and cancer [4-6]. Several excellent reviews have been recently published on this topic [7, 8]. The functional consequences of protein modification will depend on the modification site and the structure of the modifying lipid [9]. Electrophilic lipids can have very diverse structure, from small molecules such as malondialdehyde or acrolein to complex structures like electrophilic prostaglandins or epoxy phospholipids [1, 10]. In certain proteins, it is clear that lipoxidation has different functional outcomes depending on the size and/or structure of the electrophilic lipid. For instance, the enzyme aldose reductase, involved in cancer resistance and diabetic complications, can be either activated or inhibited depending on the size of the electrophilic lipid binding cysteine residues close to its active site [11]. Similarly, lipoxidation of the cytoskeletal protein vimentin results in different assembly rearrangements depending on the structure of the electrophilic lipid [12]. As oxidants and electrophiles can elicit a chain reaction in cells, additional reactive species can be generated. Therefore, the potential functional outcomes of lipoxidation need to be characterized for every reactive species. Phospholipids bearing unsaturated fatty acids are important targets for these chain reactions and can be modified by oxygen or nitrogen reactive species leading to new electrophilic species [1]. Nitrated phospholipids (NO₂-PL) represent a recently studied class of electrophilic lipid that has been shown to be generated *in vitro* in biomimetic conditions [13, 14]. Moreover, the presence of NO₂-PL has been detected in samples from several origins, including H9c2 cardiomyoblasts and cardiac mitochondria from streptozotocin-treated diabetic rats [13, 14]. Nevertheless, knowledge about the biological actions of NO₂-PL is limited. Available data indicate that these molecules can act as radical scavengers *in vitro*. Indeed, NO₂-PL reduce the ABTS^{•+} and DPPH[•] radicals, suggesting antioxidant properties [15]. In cells, NO₂-POPC inhibits lipopolysaccharide-induced iNOS expression in RAW264.7 cultured macrophages at the protein level [16], for which they have been proposed to display anti-inflammatory actions in a way analogous to nitrated fatty acids [17].

Nitrated fatty acids (NO₂-FA) are considered signaling molecules with multiple biological functions [18]. They have been previously shown to inhibit the expression of vascular cell-adhesion molecule 1 (VCAM-1) in endothelial cells and reduce monocyte adhesion to endothelial cells induced by TNFα [19] in what it is considered an anti-inflammatory action. This effect appears to be independent from NO release and related to the electrophilic response elicited by these agents, which can affect targets such as NF-κB subunits [19], PPARγ [20], or the regulator of Nrf2, KEAP1 [21], as earlier shown for other electrophilic lipids [22-25].

Here we have explored the effects of NO₂-PL in several cell types in culture. We have observed morphological changes upon treatment with NO₂-PL, but not the corresponding PL. These changes are accompanied by cytoskeletal rearrangements and loss of cell adhesion to the substrate. Investigation of the processes taking place indicates that NO₂-PL can act through multiple mechanisms in cells, of which electrophilic actions appear to be an important component.

Materials and methods

Chemicals and reagents

Phospholipid 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, C16:0/C18:1), sodium nitroprusside (SNP), 4,6-diamidino-2-phenylindole (DAPI) and dimethyl sulfoxide (DMSO) were from Sigma. Lipid 9-Nitrooleate (9-NO₂-OA) and 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂) were from Cayman. Phalloidin-Alexa568 was from Thermo Fisher. 4,5-Diaminofluorescein diacetate (DAF-2 DA) was from Calbiochem. Antibodies used were anti-vimentin V9 clone (sc-6260) and anti-focal adhesion kinase (FAK)(sc-932), from Santa Cruz Biotechnology, anti-PPARγ from Millipore, and secondary antibodies from DAKO. Fetal Bovine Serum (FBS) was from Biowest. Nitrated POPC (NO₂-POPC) was prepared as previously described [16]. Recombinant hamster vimentin was from Cytoskeleton, and PPARγ ligand binding domain (LBD) purified as previously described [26], was the generous gift of Dr. Silvia Zorrilla (CIB, CSIC).

Cell culture and treatments

SW13/cl.2 cells, untransfected or stably transfected with RFP//vimentin wild type (wt), the RFP//vimentin C328S mutant, or GFP-vimentin wt were previously described [27]. U-251 MG glioblastoma astrocytoma cells (formerly known as U-373 MG) [28], were from ATCC and identity was confirmed by microsatellite amplification (short tandem repeat (STR)-PCR

profiling), (Secugen, S.L., Madrid, Spain). Cells were cultured in DMEM with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

Treatments were carried out in serum free medium, unless otherwise specified. For imaging, adhesion assays and nitrite quantification, cells were treated with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), nitrated POPC (NO₂-POPC) and 9-Nitrooleate (9-NO₂-OA), S-nitrosoglutathione (GSNO) at 10 µM or 30 µM, sodium nitroprusside (SNP) at 300 µM or vehicle DMSO (0.5%(v/v)) for 1 h 15 minutes.

Detection of apoptosis

Flow cytometry detection of apoptosis based on the binding of Alexa Fluor 488 Annexin V and propidium iodide staining was performed as previously described [29] using the “Dead cell apoptosis kit” from Invitrogen. Briefly, non-transfected SW13/cl.2 cells were treated with vehicle (DMSO), POPC or NO₂-POPC at 30 µM for 1 h 15 min. Cells were harvested, washed with PBS and counted using a hemocytometer chamber. 200,000 cells per condition were incubated with Alexa fluor 488 Annexin V 1:100 (v/v), and 10 µg/ml propidium iodide in Annexin buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) for 15 min at room temperature in the dark. Cells were analyzed on a Beckman-Coulter Epics XL cytometer. In this assay, early apoptotic cells are Annexin-V-positive and propidium-iodide negative, whereas late apoptotic cells are positive for both parameters. In addition, the cell cycle distribution was assessed as previously described [30] to monitor the appearance of cells with DNA content lower than G1, typical of apoptotic cells.

Fluorescence microscopy and image analysis

Cells treated with the different compounds were visualized live by confocal microscopy on Leica SP2 or SP5 microscopes. Images were acquired every 0.5 µm and single sections or overall projections are shown, as indicated. All scale bars are 20 µm. For immunofluorescence, cells were fixed with 4% (w/v) paraformaldehyde for 25 min at r.t., permeabilized with 0.1% (v/v) Triton-X100 in PBS and blocked with 1% (w/v) BSA in PBS. Antibodies were used at 1:200 dilution in blocking solution. For the detection of full-length vimentin the V9 antibody was employed. Filamentous actin (f-actin) was stained with Phalloidin-Alexa568 (Molecular Probes) following the manufacturer instructions. Nuclei were counterstained with DAPI (3 µg/ml). Fluorescence intensity profiles, measurements of cell area and DAF intensity, were obtained with ImageJ.

Cell adhesion assays

For assessment of initial attachment to substrate SW13/cl.2 stably transfected with GFP-vim wt were plated in the presence of the various agents. Cells were left at 37°C for 2 h until control cells started to adhere to the plate. For assessment of cell detachment, cells were plated and 24 h later the various agents were added to the cell culture medium at the indicated concentrations for 1 h 15 min. In both cases, non-adherent cells were removed by washing with PBS. Cells adhered to the plate were fixed with 5% glutaraldehyde for 20 min and stained with 0.5% (w/v) of crystal violet in 50% (v/v) methanol in water for 20 min. Crystal violet was solubilized with 10% acetic acid for 5 min at r.t. and absorbance was measured at 540 nm. Assays were performed in triplicate.

Nitrite quantification

SW13/cl.2 stably transfected with GFP-vim wt were treated with the different compounds in serum free DMEM without phenol red. Cultured medium was recovered after 1 h of treatment, centrifuged (13000 x g at 4°C to eliminate fragments of cells). Nitrate concentration in the medium was used as an estimate of NO generation, as previously described [31]. Griess reagent was prepared fresh with N-(1-naphtylethyl)-enediamine (0.1% in water) and Sulfanilamide (1% in phosphoric acid 5%) in equal volumes. Standards of NO₂Na were prepared in concentrations between 0 and 50 µM. One volume of Griess reagent was mixed with 3 volumes of samples or standards in a 96-well plate, incubated for 10 min at room temperature in dark and absorbance was measured at 540 nm.

Intracellular nitric oxide detection by DAF-2DA

SW13/cl.2 cells were incubated with 10 µM DAF-2-DA (dissolved in DMSO) for 20 min previous to the different treatments. Exposure to light was avoided as far as possible throughout experimentation. Images were acquired at 0, 15, 30 and 50 min of treatment in Leica SP2 or Nikon C-1 CLSM microscopes.

Analysis of the interaction between vimentin and PPAR γ with POPC and NO₂-POPC *in vitro*

This was performed by a gel competition assay as previously described [26]. Briefly, vimentin at 3 µM or PPAR γ -LBD at 1 µM, were incubated in 5 mM Pipes, pH 7.0, 0.1 mM DTT, in the presence of vehicle (DMSO, 10% (v/v)), POPC or NO₂-POPC at 100 µM or 15d-PGJ₂ at 10 µM, final concentrations, for 2 h at room temperature. Subsequently, 20 µM biotinylated iodoacetamide (Iac-B) was added and incubation was continued for 30 min. Incubation

mixtures were then denatured by incubation for 5 min at 95°C in Laemmli buffer and subjected to SDS-PAGE on 10% PA gels for vimentin and 12.5% PA gels for PPAR γ -LBD. Gels were transferred to Immobilon-P membranes (Millipore) using a semi-dry transfer system from Bio-Rad, following the instructions of the manufacturer. Detection of the biotin signal incorporated into the protein was achieved by incubation with HRP-Streptavidin and ECL from GE Healthcare. Blots were re-probed with the corresponding antibodies to correct for potential differences in protein levels. The intensities of the biotin and protein signals were quantitated by image scanning.

Preparation of nuclear extracts and Electrophoretic Mobility Shift Assays (EMSA)

SW13/cl.2 cells were treated with vehicle, POPC or NO₂-POPC at 30 μ M for 1 h 15 min. Nuclear extracts were obtained as we previously described in detail [32]. Briefly, cells were lysed in 10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiotreitol (DTT), 0.5% (v/v) NP-40, 0.1 mM sodium vanadate, 50 mM sodium fluoride, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml of leupeptin, antipain, trypsin inhibitor and pepstatin A. Nuclei were sedimented by centrifugation for 5 min at 15,000 g and extracted in 20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM sodium vanadate, 50 mM sodium fluoride, containing protease inhibitors as above. Levels of PPAR γ in nuclear extracts were assessed by SDS-PAGE and western blot, as described above. For EMSA, oligonucleotides with sequence: 5'-GGTAAAGGTCAAAGGTCAAT-3', and its complementary reverse, bearing a PPAR response element (PPRE), were annealed. Aliquots from nuclear extracts containing 10 μ g of total protein were incubated in binding buffer (4% glycerol (v/v), 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5) with 150 ng of annealed oligonucleotide, for 30 min at r.t. Protein-DNA complexes were separated by electrophoresis on non-denaturing 6% polyacrylamide gels and visualized by DNA staining with SYBR Gold (Invitrogen).

Statistical analysis

All experiments were performed at least three times. Results are presented as average values $\pm$ SEM, unless otherwise stated. Differences between average values were analyzed by Student's t-test and a value of $p < 0.05$ was considered significant.

Results

Nitrated POPC (NO₂-POPC) alters cell morphology. NO₂-POPC induced changes in the morphology of several cell types, including adrenal carcinoma (SW13/cl.2) and astrocytoma (U-251 MG) cells, the most consistent of which was cell shrinking (Fig. 1A), reflected by a significant decrease in cell area (Fig. 1A, right panels), affecting a broad proportion of cells (approximately 80%, see below). Importantly, these changes were not observed in cells incubated in the presence of an equivalent concentration of the parent POPC. First, we assessed whether these changes correlated with loss of cell viability through apoptosis (Fig. 1B). Treatment with NO₂-POPC induced a slight increase in the proportion of early apoptotic cells, although it was not significant with respect to cells treated with POPC (Fig. 1B, upper left quadrants in flow cytometry charts, quantitated in bar graph). The proportion of late apoptotic or necrotic cells (right quadrants in the flow cytometry charts) did not display significant differences under any condition (Fig. 1B). Moreover, other apoptotic features, such as chromatin condensation or nuclear fragmentation (Fig. 1C), or presence of cells with DNA content lower than G1 [30](Suppl. Fig. 1), were not observed. Thus, we explored additional factors potentially contributing to cell shrinking, including cytoskeletal rearrangement and/or loss of cell adhesion. Therefore, we monitored the reorganization of the vimentin cytoskeleton and of f-actin by immunofluorescence and phalloidin staining, respectively (Fig. 1C). For this, we used SW13/cl.2 cells stably expressing vimentin as the only cytoplasmic intermediate filament, which allow monitoring changes in the distribution of this protein without the interference of other systems [27]. In these cells, vimentin filaments display the typical distribution extending from the nuclear periphery, where filaments are more robust, towards the cell edge, where filaments are thinner. Actin displays a distribution along the cell cortex together with short cytoplasmic fibrils. Treatment with NO₂-POPC induced a clear condensation of both vimentin and f-actin at the cell edges coincident with cell shrinking (Fig. 1C, lower images), and enrichment at cell prolongations where some colocalization of the two proteins was observed (Fig. 1C, overlay channels). This reorganization was evident in the fluorescence intensity profiles for both proteins (Fig. 1C, right panels). In contrast POPC did not alter cytoskeletal distribution.

NO₂-POPC releases NO *in vitro* and in cell culture. Nitrated fatty acids have been reported to increase NO bioavailability in cell culture models and to suffer various decompositions in biomimetic systems leading to NO release [33, 34]. In accordance with these evidences, we observed that incubation of NO₂-POPC in buffer resulted in the time- and concentration-dependent release of NO, as estimated by the determination of nitrite (Fig. 2A and B). An

increase in nitrite concentration was not detected upon incubation of POPC in aqueous media, but could also be measured in solutions of nitrated FA, namely, 9-NO₂-oleic acid (9-NO₂-OA) and of the known NO donors nitrosoglutathione (GSNO) or SNP (Fig. 2B). In cultured cells, addition of the various agents produced changes in the levels of nitrite in the cell culture supernatant, similar to those observed *in vitro*, indicative of NO release, with NO₂-POPC, 9-NO₂-OA and the NO donors inducing an increase in the detection of nitrite, which was not observed with POPC (Fig. 2C). Moreover, incubation in the presence of NO₂-POPC induced a time-dependent increase in the levels of intracellular NO, as detected with the fluorescent probe DAF-2 DA (Fig. 2D). Again, POPC had no effect on the levels of intracellular NO, whereas the NO donor SNP, used as a positive control, also increased DAF fluorescence (Fig. 2E). Representative images of cells incubated with DAF-2 DA are depicted in Figure 2F, illustrating the intracellular distribution of the fluorescence increase elicited by NO₂-POPC.

Effect of NO₂-POPC on cell adhesion. Given the morphological alterations observed in cells we assessed the effect of NO₂-POPC on their capacity to adhere to the substrate, using two approaches intended to monitor initial cell adhesion and cell detachment, respectively. We observed that plating in the presence of NO₂-POPC at 30 μM reduced the capacity of cells to adhere to the dish (Fig. 3A). In addition, treatment of adherent cells with 30 μM NO₂-POPC induced cell detachment (Fig. 3B). These effects were mimicked by 9-NO₂-OA but not by the NO donor SNP or by POPC. In addition, the lower concentration of NO₂-POPC (10 μM) did not induce appreciable changes in cell adhesion under these conditions, suggesting that there may be a threshold for this effect. Monitoring actin distribution showed that both NO₂-POPC and 9-NO₂-OA elicited cell shrinking with actin condensation at the cell periphery (Fig. 3C). Staining of focal adhesion kinase (FAK) revealed a pattern highly coincident with that of actin in control and POPC-treated cells, showing spiculated structures regularly distributed at the cell edge, compatible with focal adhesions. Interestingly, the reorganization induced by NO₂-POPC and 9-NO₂-OA was accompanied by a reduction in structures positive for FAK and f-actin staining (Fig. 3C), suggesting a negative effect on focal adhesions. Further studies are needed to ascertain this point.

Effect of NO₂-POPC on vimentin organization. The organization of the intermediate filament protein vimentin is highly sensitive to electrophilic stress. Given the parallelism between the effects of NO₂-FA and NO₂-PL in cell adhesion, we decided to study the impact of NO₂-POPC on vimentin organization in more detail. For this, we employed cells stably expressing a previously characterized GFP-vimentin construct [27], which does not form full filaments in vimentin-deficient cells and assembles in short fibrils or “squiggles” distributed in a quite homogeneous

lattice in the cytoplasm (Fig. 4A). Moreover, GFP-vimentin displays an increased sensitivity to oxidants and electrophiles by reorganizing into dots or small aggregates [27]. Using this experimental model we observed that NO₂-POPC, but not the parent POPC, induced cell rounding (Fig. 4A and B), consistent with the above observations. Moreover, NO₂-POPC selectively induced the condensation of GFP-vimentin into dots or small aggregates in a majority of cells (quantitated in Fig. 4C). Notably, although 9-NO₂-OA also induced cell shrinking and squiggle aggregation, the resulting cell morphology was more irregular, and the pattern of GFP-vimentin was more heterogeneous than upon NO₂-POPC treatment, with frequent appearance of condensed squiggles and/or formation of bigger and more irregular aggregates (Fig. 4A). Also, some cells showed diffuse fluorescence, indicative of vimentin disassembly (not shown). Interestingly, neither GSNO nor SNP elicited cell rounding or formation of vimentin dots, suggesting that the effects of NO₂-POPC and 9-NO₂-OA are not solely due to the release of NO and pointing to additional mechanisms of action.

Effect of NO₂-POPC on cells expressing the C328S vim mutant. The response of vimentin to oxidative or electrophilic stress is highly dependent on the presence of the cysteine residue, C328 [12]. Therefore, we next assessed the effect of NO₂-POPC in cells expressing a vimentin C328S mutant. For this we chose conditions under which we could observe vimentin remodeling without loss of cell adhesion. As shown above, although incubation with 30 μM NO₂-POPC induced significant loss of adhesion, no changes were observed with 10 μM. Therefore, we first run a time course assay with the lower concentration of NO₂-POPC. We observed that cells could be incubated with 10 μM NO₂-POPC at least for 6 h without suffering cell detachment (Fig. 5A). Moreover, this concentration of NO₂-POPC did not induce major alterations in the distribution of actin (Fig. 5B, insets). Interestingly, at this time point, a marked reorganization of vimentin, consisting in juxtanuclear condensation was apparent in a significant proportion of cells (Fig. 5A). This reorganization is reflected by filament retraction from the cell periphery, which results in a decrease of the cellular area occupied by vimentin (Fig. 5B and C). Therefore, we compared the behavior of wt and C328S vimentin treated under these conditions. As shown in figure 5B, vimentin remodeling is attenuated in cells expressing the C328S mutant, in which the area covered by vimentin filaments is not significantly reduced (Fig. 5C). These results indicate that direct or indirect modification of this residue may be important for the effect of NO₂-POPC.

Effect of NO₂-POPC on the binding of biotinylated iodoacetamide to recombinant proteins.

Given its electrophilic nature, NO₂-POPC could interact with proteins either in a covalent or non-covalent fashion. Here, we have employed a gel-based competition approach in order to

explore the interaction of NO₂-POPC with potential target proteins *in vitro*. Thus, we incubated recombinant proteins with cysteine residues well known as targets of lipoxidation, namely, vimentin and PPAR γ , with NO₂-POPC and observed if this affected their modification by biotinylated iodoacetamide (Iac-B).

C328 of vimentin has been reported to be the site of addition of various electrophiles and oxidants, as recently shown for 15d-PGJ₂ and diamide [12]. Incubation of vimentin with NO₂-POPC, but not with POPC, diminished its modification by Iac-B, a process that selectively occurs at C328 (Fig. 6A). The LBD of PPAR γ contains a cysteine residue (C285) which is the target for modification by several electrophilic lipids acting as PPAR γ agonists, including 15d-PGJ₂ [24]. Interestingly, incubation of the PPAR γ LBD in the presence of NO₂-POPC blocked its labeling with Iac-B (Fig. 6B). Remarkably, POPC partially diminished Iac-B incorporation, whereas 15d-PGJ₂, consistent with its role as agonist of PPAR through interaction with C285, also markedly reduced modification by Iac-B. Thus, these results suggest that NO₂-POPC, but not POPC, effectively shields certain cysteine residues which are known targets for lipoxidation.

Whereas *in vitro* experiments suggest an interaction of NO₂-POPC with PPAR, we did not observe a higher nuclear content of PPAR γ in cells treated with this lipid (Suppl. Fig. 2A), or a significant increase in DNA binding activity in EMSA (Suppl. Fig. 2B). Thus, our current results support an interaction of NO₂-POPC with PPAR, but whether this lipid can behave as PPAR agonist requires further investigation.

Discussion

The number of structurally diverse reactive lipid species identified in biological systems continues to increase. Electrophilic lipids and their derivatives, known for a long time as deleterious products of lipid peroxidation, are beginning to be considered as important signaling molecules relevant in physiology and pathophysiology [1, 6, 18]. Their biological actions depend on their structure, levels and place of generation, which can influence the type of targets that are present and on which they can act. Moreover, cellular antioxidant and detoxifying mechanisms are important determinants of the action of electrophilic lipid mediators [35-37]. Importantly, the fact that they seem to target certain proteins selectively depending on their structure determines that they are being held as potential therapeutic agents [38]. In fact, certain electrophilic lipids such as cyclopentenone prostaglandins or NO₂-FA have been the subject of clinical trials for diverse diseases, including cardiovascular diseases

and cancer [39-41]. Moreover, some electrophilic lipids are considered to contribute to the beneficial effects of some food components [6]. Nevertheless, their pleiotropic effects and the difficulties to control their levels may pose limitations to their use.

Here we have assessed the effects of NO₂-PL, recently identified electrophilic lipids, on basic cellular features, including cell morphology, apoptosis, cytoskeletal organization and cell adhesion. We have observed that NO₂-POPC, but not its non-modified counterpart, induces changes consisting in vimentin reorganization at low concentrations (10 μM), and cell rounding and detachment, and a minor increase in early apoptotic cells and marked reorganization of actin and vimentin, at higher concentrations (30 μM). Although NO₂-POPC releases NO, its biological effects on cell morphology and cytoskeletal rearrangement are not mimicked by NO donors, pointing to the participation of additional mechanisms of action. The requirement of vimentin C328 to observe the full effect of NO₂-POPC on the remodeling of the vimentin network and the observation that this lipid, but not the non-nitrated POPC, precludes subsequent alkylation of cysteine residues in both vimentin and PPARγ LBD, point to an electrophilic mechanism of action.

Both actin and vimentin can be targets for several electrophilic modifications, as well as for thiolation and nitrosylation [42, 43]. Actin has been shown to be nitrosylated *in vitro* [44] in isolated neutrophils [45] and in Alzheimer disease [46]. S-nitrosylation of actin alters polymerization leading to short filaments, and affects integrin clustering [45]. In addition, actin has been extensively studied as a target for lipoxidation [47-49], and is considered an important “hot spot” for modification by electrophilic lipids, and potentially an electrophile “scavenger” protein that could contribute to cell defense [3]. Based on previous evidence, the electrophilic modification of actin is expected to induce alterations in its polymerization. Moreover, in cells, indirect mechanisms may operate through the modification of actin-regulatory proteins, including small GTPases [50]. Our observations indicate that actin is remodeled in response to high concentrations (30 μM) of NO₂-POPC. The marked cell rounding observed is accompanied by a loss of peripheral actin structures positive also for FAK, which may indicate a negative effect of focal adhesions. Nitroalkenes have been shown to inhibit TNF-α-induced VCAM-1 and monocyte rolling and adhesion [19, 51]. However, to the best of our knowledge, the effect of NO₂-FA on adhesion to substrate, actin organization or FAK complexes has not been reported. Nonetheless, the potential contribution of this and other mechanisms to the observed loss of adhesion capacity by NO₂-POPC requires further study.

Vimentin is an intermediate filament protein expressed in cells of mesenchymal origin and in epithelial tumors that have undergone epithelial-mesenchymal transition. Vimentin plays a key role in cell architecture and organelle positioning, as well as in many fundamental processes such as cell migration, response to stress and division [27, 52-54]. The vimentin network suffers extensive reorganization in response to several types of stress. The vimentin monomer possesses a single cysteine residue (C328), which has been shown to be the target for nitrosylation [55] and lipoxidation [56-58]. Moreover, this residue appears to act as a “hinge” for vimentin assembly, since several electrophilic agents induce vimentin remodeling in cells or alterations of vimentin filament assembly *in vitro* in a manner dependent on the presence of C328 [12]. Nevertheless, although vimentin is particularly susceptible to electrophilic species, many other protein targets can act as hot spots for reactive lipids [3].

Our results indicate that NO₂-POPC release NO *in vitro* and bring about an increase in intracellular NO when added to cells in culture. The effects of NO on several forms of cell adhesion are well known. NO inhibits cell adhesion in endothelial cells by interfering with the phosphorylation status of FAK and reducing the recruitment of paxillin [59]. Moreover, inhibition of mesangial cell adhesion to collagen by NO has been reported [60]. In addition, NO has been shown to induce ADP-ribosylation of actin in macrophages, reducing adhesion to a laminin substratum [61]. Interestingly, under our conditions, NO₂-POPC reduced cell adhesion to the substrate but this effect was not mimicked by the NO donors GSNO and SNP, which also release NO *in vitro*. This could be related to a different kinetics and/or location of NO release by the various compounds or to the involvement of additional mechanisms in the effect of NO₂-POPC. The effect of NO₂-POPC shares some features with that of NO₂-OA. Both agents induce loss of cell adhesion and cytoskeletal reorganization, although the morphological changes elicited are somewhat different, with NO₂-POPC inducing a more regular cell rounding and NO₂-OA treatment resulting in a more irregular cellular shape with cell shrinking and membrane blebbing. Moreover, the pattern of organization of GFP-vimentin, which forms fine squiggles under control conditions, is also more uniform after NO₂-POPC treatment, with appearance of dots and small aggregates, whereas the structures observed upon treatment with NO₂-OA are highly polymorphic and comprise dots, large aggregates and condensed squiggles. Therefore, the effects of NO₂-POPC and NO₂-OA, do not appear to be completely superimposable.

Additionally, other mechanisms of action of NO₂-PL can be considered. Increased intracellular NO availability could lead to various protein modifications, including cysteine S-nitrosylation or, if coincident with superoxide anion generation it could result in the formation of

peroxynitrite [62], and nitration of aromatic residues tyrosine and tryptophan [63]. The possibility of occurrence of additional modifications upon treatment with NO₂-POPC will be the subject of further studies. Moreover, lipid peroxidation can induce alterations in membrane permeability [64]. An important component of NO₂-FA actions, and potentially NO₂-PL, relies in their electrophilic nature. Therefore, similar to NO₂-FA, NO₂-PL could modify cysteine or histidine residues through S-nitroalkylation or Michael addition, respectively [1]. Additionally, their spontaneous decay could give rise to other reactive species [1]. Recently, the formation of adducts of NO₂-POPC with glutathione (GSH) has been shown by mass spectrometry (MS) approaches [65], although the extent of formation is low. To the best of our knowledge, NO₂-PL adducts with proteins have not yet been detected. Therefore, the characterization of the GSH adduct and the typical product ions observed under MS2 spectra may aid in the identification of NO₂-PL-peptide adducts in biological samples.

Our results suggest that the presence of C328 of vimentin is important for vimentin reorganization in response to NO₂-POPC. Nevertheless, it should be taken into account that this requirement could respond to several reasons. On one hand, C328 could be a direct target for modification by nitrated species, as indicated above. On the other hand, “indirect modifications” of the cysteine residue due to the exposure of cells to NO₂-POPC, including (lip)oxidation by species generated in a chain reaction, could also be involved in vimentin remodeling and thus, explain the protective effect of the mutation. Lastly, the presence of the cysteine residue could be important for protein-protein interactions that could be affected by modifications of other proteins.

Given the current technical limitations for the detection of NO₂-PL-protein adducts, we have explored the potential interaction of NO₂-POPC with two lipoxidation targets, namely, vimentin and PPAR γ , by an indirect competition strategy widely used to assess the interaction of various ligands with cysteine residues [24, 26]. Under our conditions we observe a reduction in iodoacetamide labeling of the cysteine residues of the targets studied that depends on the presence of the NO₂ moiety in the PL molecule. These observations strongly suggest that NO₂-POPC somehow interacts with these targets shielding the cysteine residue from modification. Nevertheless, at this point, a direct covalent modification cannot be confirmed. Moreover, the reduction in the modification in the presence of NO₂-POPC could also be due to the occurrence of other modifications of the cysteine, including nitrosylation or oxidation. These possibilities will be the subject of further studies.

In summary, we have observed clear cellular effects of NO₂-POPC which are not superimposable to those of NO donors and differ also from those of NO₂-OA. This broadens the panoply of electrophilic lipid mediators with potential biological actions and paves the way for future studies on their site of formation in biological systems and interacting targets.

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References

- [1] M.R. Domingues, P. Domingues, T. Melo, D. Pérez-Sala, A. Reis, C. Spickett, Lipoxidation adducts with peptides and proteins: deleterious modifications or signalling mechanisms?, *J. Proteomics* 92 (2013) 110-131.
- [2] Y.V. Vasil'ev, S.C. Tzeng, L. Huang, C.S. Maier, Protein modifications by electrophilic lipoxidation products: adduct formation, chemical strategies and tandem mass spectrometry for their detection and identification, *Mass Spectrom Rev* 33 (2014) 157-82.
- [3] G. Aldini, M.R. Domingues, C.M. Spickett, P. Domingues, A. Altomare, F.J. Sánchez-Gómez, C.L. Oeste, D. Pérez-Sala, Protein lipoxidation: detection strategies and challenges, *Redox Biol* 5 (2015) 253-266.
- [4] E.K. Ceaser, D.R. Moellering, S. Shiva, A. Ramachandran, A. Landar, A. Venkartraman, J. Crawford, R. Patel, D.A. Dickinson, E. Ulasova, S. Ji, V.M. Darley-Usmar, Mechanisms of signal transduction mediated by oxidized lipids: the role of the electrophile-responsive proteome, *Biochem Soc Trans* 32 (2004) 151-155.

- [5] D. Pérez-Sala, Electrophilic eicosanoids: signaling and targets, *Chem Biol Interact* 192 (2011) 96-100.
- [6] F.J. Schopfer, C. Cipollina, B.A. Freeman, Formation and signaling actions of electrophilic lipids, *Chem Rev* 111 (2011) 5997-6021.
- [7] T. Patinen, S. Adinolfi, C.C. Cortes, J. Harkonen, A. Jawahar Deen, A.L. Levonen, Regulation of stress signaling pathways by protein lipoxidation, *Redox Biol* (2019) 101114.
- [8] M. Jove, I. Pradas, M. Dominguez-Gonzalez, I. Ferrer, R. Pamplona, Lipids and lipoxidation in human brain aging. Mitochondrial ATP-synthase as a key lipoxidation target, *Redox Biol* (2018) 101082.
- [9] C.L. Oeste, D. Pérez-Sala, Modification of cysteine residues by cyclopentenone prostaglandins: interplay with redox regulation of protein function, *Mass Spectrom Rev* 33 (2014) 110-125.
- [10] G. Subbanagounder, J.W. Wong, H. Lee, K.F. Faull, E. Miller, J.L. Witztum, J.A. Berliner, Epoxyisoprostane and epoxycyclopentenone phospholipids regulate monocyte chemotactic protein-1 and interleukin-8 synthesis. Formation of these oxidized phospholipids in response to interleukin-1 β , *J Biol Chem* 277 (2002) 7271-81.
- [11] S.K. Srivastava, K.V. Ramana, D. Chandra, S. Srivastava, A. Bhatnagar, Regulation of aldose reductase and the polyol pathway activity by nitric oxide, *Chem Biol Interact* 143-144 (2003) 333-40.
- [12] A. Mónico, S. Duarte, M.A. Pajares, D. Pérez-Sala, Vimentin disruption by lipoxidation and electrophiles: role of the cysteine residue and filament dynamics, *Redox Biol* (2019) 101098.
- [13] T. Melo, P. Domingues, R. Ferreira, I. Milic, M. Fedorova, S.M. Santos, M.A. Segundo, M.R. Domingues, Recent Advances on Mass Spectrometry Analysis of Nitrated Phospholipids, *Analytical Chem* 88 (2016) 2622-9.
- [14] T. Melo, P. Domingues, T.M. Ribeiro-Rodrigues, H. Girao, M.A. Segundo, M.R.M. Domingues, Characterization of phospholipid nitrooxidation by LC-MS in biomimetic models and in H9c2 Myoblast using a lipidomic approach, *Free Radic Biol Med* 106 (2017) 219-227.
- [15] B. Neves, P. Domingues, M.M. Oliveira, M.D.R. Domingues, T. Melo, Profile of Phosphatidylserine Modifications under Nitroxidative Stress Conditions Using a Liquid Chromatography-Mass Spectrometry Based Approach, *Molecules* 24 (2018) E107.
- [16] T. Melo, S.S. Marques, I. Ferreira, M.T. Cruz, P. Domingues, M.A. Segundo, M.R.M. Domingues, New Insights into the Anti-Inflammatory and Antioxidant Properties of Nitrated Phospholipids, *Lipids* 53 (2018) 117-131.
- [17] B. Coles, A. Bloodsworth, S.R. Clark, M.J. Lewis, A.R. Cross, B.A. Freeman, V.B. O'Donnell, Nitrolinoleate inhibits superoxide generation, degranulation, and integrin expression by

human neutrophils: novel antiinflammatory properties of nitric oxide-derived reactive species in vascular cells, *Circ Res* 91 (2002) 375-81.

[18] E. Kansanen, H.K. Jyrkkanen, A.L. Levonen, Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids, *Free Radic Biol Med* 52 (2012) 973-82.

[19] T. Cui, F.J. Schopfer, J. Zhang, K. Chen, T. Ichikawa, P.R. Baker, C. Batthyany, B.K. Chacko, X. Feng, R.P. Patel, A. Agarwal, B.A. Freeman, Y.E. Chen, Nitrated fatty acids: Endogenous anti-inflammatory signaling mediators, *J Biol Chem* 281 (2006) 35686-98.

[20] F.J. Schopfer, M.P. Cole, A.L. Groeger, C.S. Chen, N.K. Khoo, S.R. Woodcock, F. Golin-Bisello, U.N. Motanya, Y. Li, J. Zhang, M.T. Garcia-Barrio, T.K. Rudolph, V. Rudolph, G. Bonacci, P.R. Baker, H.E. Xu, C.I. Batthyany, Y.E. Chen, T.M. Hallis, B.A. Freeman, Covalent peroxisome proliferator-activated receptor gamma adduction by nitro-fatty acids: selective ligand activity and anti-diabetic signaling actions, *J Biol Chem* 285 (2010) 12321-33.

[21] E. Kansanen, G. Bonacci, F.J. Schopfer, S.M. Kuosmanen, K.I. Tong, H. Leinonen, S.R. Woodcock, M. Yamamoto, C. Carlberg, S. Yla-Herttuala, B.A. Freeman, A.L. Levonen, Electrophilic nitro-fatty acids activate NRF2 by a KEAP1 cysteine 151-independent mechanism, *J Biol Chem* 286 (2011) 14019-27.

[22] A. Rossi, G. Elia, G. Santoro, Inhibition of nuclear factor κ B by prostaglandin A_1 : an effect associated with heat shock transcription factor activation., *Proc. Natl. Acad. Sci. USA* 94 (1997) 746-750.

[23] E. Cernuda-Morollón, E. Pineda-Molina, F.J. Cañada, D. Pérez-Sala, 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J_2 inhibition of NF- κ B DNA binding through covalent modification of the p50 subunit, *J. Biol. Chem.* 276 (2001) 35530-35536.

[24] T. Shiraki, N. Kamiya, S. Shiki, T.S. Kodama, A. Kakizuka, H. Jingami, α,β -Unsaturated Ketone is a Core Moiety of Natural Ligands for Covalent Binding to Peroxisome proliferator-activated receptor, *J Biol Chem* 280 (2005) 14145-14153.

[25] K. Itoh, M. Mochizuki, Y. Ishii, T. Ishii, T. Shibata, Y. Kawamoto, V. Kelly, K. Sekizawa, K. Uchida, M. Yamamoto, Transcription Factor Nrf2 Regulates Inflammation by Mediating the Effect of 15-Deoxy-Delta(12,14)-Prostaglandin J(2), *Mol. Cell. Biol.* 24 (2004) 36-45.

[26] S. Zorrilla, B. Garzón, D. Pérez-Sala, Selective binding of the fluorescent dye 8-anilinonaphthalene-1-sulfonic acid to PPAR γ allows ligand identification and characterization, *Anal Biochem* 399 (2010) 84-92.

[27] D. Pérez-Sala, C.L. Oeste, A.E. Martínez, B. Garzón, M.J. Carrasco, F.J. Cañada, Vimentin filament organization and stress sensing depend on its single cysteine residue and zinc binding, *Nat Commun* 6 (2015) 7287.

- [28] Á. Viedma-Poyatos, Y.d. Pablo, M. Pekny, D. Pérez-Sala, The cysteine residue of glial fibrillary acidic protein is a critical target for lipoxidation and required for efficient network organization, *Free Rad Biol Med* 120 (2018) 380-394.
- [29] F.J. Sánchez-Gómez, B. Díez-Dacal, M.A. Pajares, O. Llorca, D. Pérez-Sala, Cyclopentenone prostaglandins with dienone structure promote cross-linking of the chemoresistance-inducing enzyme Glutathione Transferase P1-1, *Mol Pharmacol* 78 (2010) 723-733.
- [30] D. Pérez-Sala, D. Collado-Escobar, F. Mollinedo, Intracellular Alkalinization Suppresses Lovastatin-induced Apoptosis in HL-60 Cells through the Inactivation of a pH-dependent Endonuclease, *J Biol Chem* 270 (1995) 6235-6242.
- [31] E. Cernuda-Morollón, F. Rodríguez-Pascual, P. Klatt, S. Lamas, D. Pérez-Sala, PPAR agonists amplify iNOS expression while inhibiting NF- κ B: implications for mesangial cell activation by cytokines, *J Am Soc Nephrol* 13 (2002) 2223-2231.
- [32] M. Díaz-Cazorla, D. Pérez-Sala, S. Lamas, Dual effect of nitric oxide donors on cyclooxygenase-2 expression in human mesangial cells, *J Am Soc Nephrol* 10 (1999) 943-952.
- [33] P. Manini, L. Capelli, S. Reale, M. Arzillo, O. Crescenzi, A. Napolitano, V. Barone, M. d'Ischia, Chemistry of nitrated lipids: remarkable instability of 9-nitrolinoleic acid in neutral aqueous medium and a novel nitronitrate ester product by concurrent autoxidation/nitric oxide-release pathways, *J Org Chem* 73 (2008) 7517-25.
- [34] F.J. Schopfer, P.R. Baker, G. Giles, P. Chumley, C. Batthyany, J. Crawford, R.P. Patel, N. Hogg, B.P. Branchaud, J.R. Lancaster, Jr., B.A. Freeman, Fatty acid transduction of nitric oxide signaling. Nitrolinoleic acid is a hydrophobically stabilized nitric oxide donor, *J Biol Chem* 280 (2005) 19289-97.
- [35] X. Yu, P.A. Egner, J. Wakabayashi, N. Wakabayashi, M. Yamamoto, T.W. Kensler, Nrf2-mediated induction of cytoprotective enzymes by 15-deoxy-Delta12,14-prostaglandin J2 is attenuated by alkenal/one oxidoreductase, *J Biol Chem* 281 (2006) 26245-52.
- [36] B. Díez-Dacal, D. Pérez-Sala, A-class prostaglandins: early findings and new perspectives for overcoming tumor chemoresistance, *Cancer Lett* 320 (2012) 150-157.
- [37] F.J. Sánchez-Gómez, B. Díez-Dacal, E. García-Martín, J.A.G. Agúndez, M.A. Pajares, D. Pérez-Sala, Detoxifying enzymes at the cross-roads of inflammation, oxidative stress and drug hypersensitivity: Role of glutathione transferase P1-1 and aldose reductase, *Front Pharmacol* 7 (2016) 237.
- [38] B. Díez-Dacal, J. Gayarre, S. Gharbi, J.F. Timms, C. Coderch, F. Gago, D. Pérez-Sala, Identification of aldo-keto reductase AKR1B10 as a selective target for modification and inhibition by PGA1: Implications for anti-tumoral activity, *Cancer Res* 71 (2011) 4161-4171.

- [39] S. Fukushima, Y. Takeuchi, S. Kishimoto, S. Yamashita, K. Uetsuki, S. Shirakawa, M. Suzuki, K. Furuta, R. Noyori, H. Sasaki, Y. Kikuchi, T. Kita, T. Yamori, J. Sawada, M. Kojima, A. Hazato, S. Kurozumi, M. Fukushima, Antitumor activity, optimum administration method and pharmacokinetics of 13,14-dihydro-15-deoxy-deoxy-Delta⁷-prostaglandin A1 methyl ester (TEI-9826) integrated in lipid microspheres (Lipo TEI-9826), *Anticancer Drugs* 12 (2001) 221-34.
- [40] P.I. Homem de Bittencourt, Jr., D.J. Lagranha, A. Maslinkiewicz, S.M. Senna, A.M. Tavares, L.P. Baldissera, D.R. Janner, J.S. Peralta, P.M. Bock, L.L. Gutierrez, G. Scola, T.G. Heck, M.S. Krause, L.A. Cruz, D.S. Abdalla, C.J. Lagranha, T. Lima, R. Curi, LipoCardium: endothelium-directed cyclopentenone prostaglandin-based liposome formulation that completely reverses atherosclerotic lesions, *Atherosclerosis* 193 (2007) 245-58.
- [41] M. Fazzari, D.A. Vitturi, S.R. Woodcock, S.R. Salvatore, B.A. Freeman, F.J. Schopfer, Electrophilic fatty acid nitroalkenes are systemically transported and distributed upon esterification to complex lipids, *J Lipid Res* 60 (2019) 388-399.
- [42] I. Dalle-Donne, D. Giustarini, R. Rossi, R. Colombo, A. Milzani, Reversible S-glutathionylation of Cys374 regulates actin filament formation by inducing structural changes in the actin molecule, *Free Rad Biol Med* 34 (2003) 23-32.
- [43] M. Fratelli, H. Demol, M. Puype, S. Casagrande, I. Eberini, M. Salmona, V. Bonetto, M. Mengozzi, F. Duffieux, E. Miclet, A. Bachi, J. Vandekerckhove, E. Gianazza, P. Ghezzi, Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes, *Proc Natl Acad Sci U S A* 99 (2002) 3505-3510.
- [44] I. Dalle-Donne, A. Milzani, D. Giustarini, P. Di Simplicio, R. Colombo, R. Rossi, S-NO-actin: S-nitrosylation kinetics and the effect on isolated vascular smooth muscle, *Journal of muscle research and cell motility* 21 (2000) 171-81.
- [45] S.R. Thom, V.M. Bhopale, D.J. Mancini, T.N. Milovanova, Actin S-nitrosylation inhibits neutrophil beta2 integrin function, *J Biol Chem* 283 (2008) 10822-34.
- [46] S. Zahid, R. Khan, M. Oellerich, N. Ahmed, A.R. Asif, Differential S-nitrosylation of proteins in Alzheimer's disease, *Neuroscience* 256 (2014) 126-36.
- [47] G. Aldini, I. Dalle-Donne, G. Vistoli, R. Maffei Facino, M. Carini, Covalent modification of actin by 4-hydroxy-trans-2-nonenal (HNE): LC-ESI-MS/MS evidence for Cys374 Michael adduction, *J Mass Spectrom* 40 (2005) 946-54.
- [48] G. Aldini, M. Carini, G. Vistoli, T. Shibata, Y. Kusano, L. Gamberoni, I. Dalle-Donne, A. Milzani, K. Uchida, Identification of Actin as a 15-Deoxy-Delta(12,14)-prostaglandin J(2) Target in Neuroblastoma Cells: Mass Spectrometric, Computational, and Functional Approaches To Investigate the Effect on Cytoskeletal Derangement, *Biochemistry* 46 (2007) 2707-2718.

- [49] J. Gayarre, D. Sánchez, F.J. Sánchez-Gómez, C. Terrón, O. Llorca, D. Pérez-Sala, Addition of electrophilic lipids to actin alters filament structure, *Biochem Biophys Res Commun* 349 (2006) 1387-1393.
- [50] S.B. Wall, J.Y. Oh, L. Mitchell, A.H. Laube, S.L. Campbell, M.B. Renfrow, A. Landar, Rac1 modification by an electrophilic 15-deoxy $\Delta^{12,14}$ -prostaglandin J2 analog, *Redox Biol* 4 (2015) 346-54.
- [51] J. Hwang, K.E. Lee, J.Y. Lim, S.I. Park, Nitrated fatty acids prevent TNF α -stimulated inflammatory and atherogenic responses in endothelial cells, *Biochem Biophys Res Commun* 387 (2009) 633-40.
- [52] J. Ivaska, H.M. Pallari, J. Nevo, J.E. Eriksson, Novel functions of vimentin in cell adhesion, migration, and signaling, *Exp Cell Res* 313 (2007) 2050-62.
- [53] M. Pekny, E.B. Lane, Intermediate filaments and stress, *Exp Cell Res* 313 (2007) 2244-54.
- [54] S. Duarte, Á. Viedma-Poyatos, E. Navarro, A.E. Martínez, M.A. Pajares, D. Pérez-Sala, Vimentin filaments interact with the mitotic cortex allowing normal cell division, *BioRxiv* (2018) <https://doi.org/10.1101/356642>.
- [55] J. Jia, A. Arif, F. Terenzi, B. Willard, E.F. Plow, S.L. Hazen, P.L. Fox, Target-selective protein S-nitrosylation by sequence motif recognition, *Cell* 159 (2014) 623-34.
- [56] J. Chavez, W.G. Chung, C.L. Miranda, M. Singhal, J.F. Stevens, C.S. Maier, Site-specific protein adducts of 4-hydroxy-2(E)-nonenal in human THP-1 monocytic cells: protein carbonylation is diminished by ascorbic acid, *Chem Res Toxicol* 23 (2010) 37-47.
- [57] K. Stamatakis, F.J. Sánchez-Gómez, D. Pérez-Sala, Identification of novel protein targets for modification by 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ in mesangial cells reveals multiple interactions with the cytoskeleton., *J Am Soc Nephrol* 17 (2006) 89-98.
- [58] S. Gharbi, B. Garzón, J. Gayarre, J. Timms, D. Pérez-Sala, Study of protein targets for covalent modification by the antitumoral and anti-inflammatory prostaglandin PGA₁: focus on vimentin, *J Mass Spectrom* 42 (2007) 1474-1484.
- [59] M.S. Goligorsky, H. Abedi, E. Noiri, A. Takhtajan, S. Lense, V. Romanov, I. Zachary, Nitric oxide modulation of focal adhesions in endothelial cells, *Am J Physiol* 276 (1999) C1271-81.
- [60] J. Yao, H.O. Schoecklmann, F. Prols, S. Gauer, R.B. Sterzel, Exogenous nitric oxide inhibits mesangial cell adhesion to extracellular matrix components, *Kidney Int* 53 (1998) 598-608.
- [61] C.D. Jun, M.K. Han, U.H. Kim, H.T. Chung, Nitric oxide induces ADP-ribosylation of actin in murine macrophages: association with the inhibition of pseudopodia formation, phagocytic activity, and adherence on a laminin substratum, *Cellular Immunol* 174 (1996) 25-34.
- [62] R. Radi, G. Peluffo, M.N. Alvarez, M. Naviliat, A. Cayota, Unraveling peroxynitrite formation in biological systems, *Free Radic Biol Med* 30 (2001) 463-88.

- [63] F.J. Schopfer, P.R. Baker, B.A. Freeman, NO-dependent protein nitration: a cell signaling event or an oxidative inflammatory response?, *Trends Biochem Sci* 28 (2003) 646-54.
- [64] J. Wong-Ekkabut, Z. Xu, W. Triampo, I.M. Tang, D.P. Tieleman, L. Monticelli, Effect of lipid peroxidation on the properties of lipid bilayers: a molecular dynamics study, *Biophys J* 93 (2007) 4225-36.
- [65] J.F. Montero-Bullón, T. Melo, M.R. Domingues, P. Domingues, Characterization of Nitrophospholipid-Peptide Covalent Adducts by Electrospray Tandem Mass Spectrometry: A First Screening Analysis Using Different Instrumental Platforms, *European Journal of Lipid Science and Technology* 120 (2018) 1800101.

Figure legends

Fig. 1. Effect of NO₂-POPC on cell morphology and on cytoskeletal proteins. (A) SW13/cl.2 and U-251 MG cells were treated with vehicle (DMSO, control), POPC or NO₂-POPC 30 μ M in serum free medium for 1h 15 min, fixed and observed by differential interference contrast microscopy (DIC). The graphs depict the cell area upon treatment. Results are average values $\pm$ SEM of three experiments totaling at least 40 cells per experimental condition (* p < 0.001 vs control by Student's t -test). (B) Detection of apoptotic cells by flow cytometry analysis. Results are average values $\pm$ SEM of five experiments (* p < 0.05 vs control by Student's t -test; ns, non-significant). (C) SW13/cl.2 cells stably transfected with a plasmid expressing full-length vimentin (RFP//vim wt) were treated in the same conditions as described above. The distribution of the cytoskeletal proteins vimentin (green) and f-actin (red) was assessed by immunofluorescence and phalloidin staining, respectively, and confocal microscopy. Images of overall projections and overlay sections, as indicated are shown. Scale bars, 20 μ m. The panels on the right depict the fluorescence intensity profiles of fluorescence and actin along the dotted lines shown in the overlay sections in order to illustrate the relative distributions of both proteins.

Fig. 2. Release of NO by various nitrated lipids and NO donors in culture medium in the presence or absence of cells. (A) NO₂-POPC at 30 μ M was incubated in serum-free cell culture medium at 37°C for the indicated times. Release of NO was estimated as the concentration of nitrite. (B) The indicated compounds were incubated in serum free medium at 37°C for 1 h 15 min and nitrite concentration was measured as in (A). 9-NO₂-OA, 9-NO₂ oleic acid; GSNO, nitrosoglutathione; SNP, sodium nitroprusside. Results are average values $\pm$ SEM of three experiments (* p < 0.01 vs control by Student's t -test). (C) Nitrite concentration was measured in the cell culture supernatant of SW13/cl.2 cells treated with 30 μ M POPC, 10 μ M and 30 μ M NO₂-POPC or GSNO, 30 μ M 9-NO₂-OA, or 300 μ M SNP at for 1 h 15 min in serum free medium. Results are average values $\pm$ SEM of three experiments (* p < 0.01 vs control by Student's t -test). Finally, SW13/cl.2 cells were treated with 30 μ M NO₂-POPC for the indicated times (D), or with 30 μ M POPC, 30 μ M NO₂-POPC or 300 μ M SNP for 1 h 15 min (E), and intracellular NO generation was quantified by measuring DAF-2 fluorescence as described in Materials and Methods. The fluorescence intensity versus cell area of the various conditions is given in arbitrary units. At least 50 cells were monitored per experimental condition. In (D), * p < 0.05 and vs control and # p < 0.05 vs the previous time point; in (E), * p < 0.001 vs control by Student's t -test. (F) Representative fluorescence microscopy images of cells treated as in (E). Bar, 20 μ m.

Fig. 3. Alteration of cell adhesion by NO₂-POPC. (A) SW13/cl.2 cells plated in the presence of POPC 30 μ M, NO₂-POPC at 10 μ M and 30 μ M, 9-NO₂-OA at 30 μ M or SNP at 300 μ M in serum free medium. After 2 h, not adhered cells were washed with PBS. (B) Fully adhered SW13/cl.2 cells were treated with the same agents as in (A) for 1 h 15 min, after which, cells that had lost adhesion to the substrate were removed by washing with PBS. Cells remaining in the plate in (A) and (B) were fixed, stained with crystal violet and absorbance at 540 nm was measured. Results are average values $\pm$ SEM of three experiments (* p < 0.01 vs control by Student's t -test). (C) SW13/cl.2 cells treated with POPC, NO₂-POPC and 9-NO₂-OA at 30 μ M as in (B) were fixed and the distribution of f-actin (red) and focal adhesion kinase (FAK, green) was visualized by phalloidin staining and immunofluorescence, respectively. Scale bars, 20 μ m.

Fig. 4. Effect of NO₂-POPC on the organization GFP-vimentin short filaments. SW13/cl.2 stably transfected with GFP-vimentin wt (GFP-vim wt) were treated POPC 30 μ M, NO₂-POPC at 10 μ M or 30 μ M, 9-NO₂-OA at 30 μ M or with the NO donors GSNO at 30 μ M or SNP at 300 μ M for 1 h 15 min in serum free medium. The organization of GFP-vimentin was observed by direct visualization by confocal microscopy. Overall projections are shown. Scale bars, 20 μ m. The graphs depict the percentage of rounded cells and the percentage of cells with vimentin dots/aggregates after the different treatments. Results are average values of three experiments $\pm$ SEM, * p <0.001 by Student's t -test.

Fig. 5. Effect of NO₂-POPC on the organization of the vimentin network. (A) SW13/cl.2 stably expressing vimentin wt were treated with vehicle (0.5% (v/v) DMSO) or NO₂-POPC at 10 or 30 μ M in serum free medium for the indicated times. (B) SW13/cl.2 stably transfected with plasmids expressing full-length vimentin wt or C328S mutant (RFP//vim wt or RFP//vim C328S, respectively) were treated with vehicle (DMSO) or NO₂-POPC at 10 μ M in serum free medium for 6 h. Cells were fixed and the distribution of vimentin and f-actin were visualized by immunofluorescence. Scale bars, 20 μ m. (C) Vimentin area was quantified in relation to the total cell area. At least 100 cells were monitored per experimental condition (* p < 0.001 vs control by Student's t -test).

Fig. 6. Effect of NO₂-POPC on the binding of biotinylated iodoacetamide to recombinant proteins. Recombinant vimentin (A) or PPAR γ LBD (B) were pre-incubated with 100 μ M non-modified POPC or NO₂-POPC, 10 μ M 15d-PGJ₂ or vehicle (DMSO), as indicated, for 2 h at room temperature before the addition of 20 μ M biotinylated iodoacetamide (Iac-B), and the incubation was continued for 30 min. Incorporation of Iac-B as well as vimentin or PPAR γ LBD levels were assessed by blot and detection of the biotin signal with HRP-streptavidin or by

western blot with anti-vimentin antibody, respectively. In (A), the dotted line shows where lanes from the same gel have been cropped. The graph presents the results of three independent experiments, expressed as percentage of the biotin signal obtained upon incubation with lac-B in the absence of lipids (mean $\pm$ SEM, * $p < 0.01$ by Student's t-test). In (B) the graph presents the result of a representative assay out of three with similar results.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Nitrated phospholipids induce concentration-dependent cytoskeletal rearrangements

NO donors do not mimic the effects of nitrated phospholipids

Nitrated fatty acids recapitulate some effects of nitrated phospholipids

Nitrated phospholipids could act through electrophilic mechanisms