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Macrophage activation induces formation of the anti-inflammatory lipid cholesteryl-nitrolinoleate

Ana M. FERREIRA*, Mariana I. FERRARI*, Andrés TROSTCHANSKY†, Carlos BATTHYANY†§, José M. SOUZA†, María N. ALVAREZ†, Gloria V. LÓPEZ‡, Paul R. S. BAKER§, Francisco J. SCHOPFER§, Valerie O'DONNELL‖, Bruce A. FREEMAN§[1] and Homero RUBBO†[1]

*Department of Immunology, Faculty of Sciences, University of the Republic, Montevideo 11400, Uruguay, †Department of Biochemistry, Center for Free Radical and Biomedical Research, Faculty of Medicine, University of the Republic, Montevideo 11800, Uruguay, ‡Department of Organic Chemistry, Faculty of Sciences, University of the Republic, Montevideo 11800, Uruguay, §Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, U.S.A., and ‖Department of Medical Biochemistry, University of Wales College of Medicine, Cardiff CF14 4XN, U.K.

Nitroalkene derivatives of fatty acids act as adaptive, anti-inflammatory signalling mediators, based on their high-affinity PPARγ (peroxisome-proliferator-activated receptor γ) ligand activity and electrophilic reactivity with proteins, including transcription factors. Although free or esterified lipid nitroalkene derivatives have been detected in human plasma and urine, their generation by inflammatory stimuli has not been reported. In the present study, we show increased nitration of cholesteryl-linoleate by activated murine J774.1 macrophages, yielding the mononitrated nitroalkene $CLNO_2$ (cholesteryl-nitrolinoleate). $CLNO_2$ levels were found to increase ~20-fold 24 h after macrophage activation with *Escherichia coli* lipopolysaccharide plus interferon-γ; this response was concurrent with an increase in the expression of NOS2 (inducible nitric oxide synthase) and was inhibited by the •NO (nitric oxide) inhibitor L-NAME (N^G-nitro-L-arginine methyl ester). Macrophage (J774.1 and bone-marrow-derived cells) inflammatory responses were suppressed when activated in the presence of $CLNO_2$ or LNO_2 (nitrolinoleate). This included: (i) inhibition of NOS2 expression and cytokine secretion through PPARγ and •NO-independent mechanisms; (ii) induction of haem oxygenase-1 expression; and (iii) inhibition of NF-κB (nuclear factor κB) activation. Overall, these results suggest that lipid nitration occurs as part of the response of macrophages to inflammatory stimuli involving NOS2 induction and that these by-products of nitro-oxidative reactions may act as novel adaptive down-regulators of inflammatory responses.

Key words: cholesteryl-nitrolinoleate, haem oxygenase-1, inducible nitric oxide synthase, inflammation, lipid nitration, macrophage.

INTRODUCTION

Nitro-derivatives of unsaturated fatty acids have been structurally characterized and quantified in plasma, red blood cells and urine of both healthy and hypercholesterolaemic humans [1–3]. Although dietary sources may contribute to tissue nitro-fatty acid levels, these species can also be formed by oxidative and nitrative reactions associated with inflammation. Mechanisms known to mediate fatty acid nitration to nitroalkene derivatives include nucleophilic (nitronium group, NO_2^+) and/or radical (nitrogen dioxide, $^\bullet NO_2$) addition reactions with olefinic carbons [2,4,5]. Nitroalkene fatty acid derivatives display pluripotent cell signalling actions that limit inflammatory responses. In particular, LNO_2 (nitrolinoleate; 9, 10, 12 and 13-nitro-9,12-*cis*-octadecadienoic acid) and $OANO_2$ (nitro-oleate; 9 and 10-nitro-9-*cis*-octadecenoic acid) activate PPARγ (peroxisome-proliferator-activated receptor γ), representing more potent or higher-affinity ligands for PPARγ than various putative endogenous ligands (i.e. lysophosphatidic acid, conjugated linoleate and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2) and rivalling the potency of synthetic thiazolidinedione agonists [3,6]. Nitroalkene signalling via PPARγ activation modulates expression of metabolic- and inflammatory-related genes [7–13]. In particular, LNO_2 induces pre-adipocyte differentiation and aP2 (adipocyte P2) expression, and increases glucose uptake by adipocytes [6]. Fatty acid nitroalkene derivatives also display PPARγ-independent actions on various cell types; for example, they decay in aqueous milieu to •NO (nitric oxide) [14,15], thus promoting vessel relaxation via endothelial-independent, cGMP-dependent pathways [16]. This latter property is readily inhibited by intercalation of nitroalkenes into organized lipid micelles or membranes [14]. Other anti-inflammatory actions of LNO_2 include modulation of cytokine secretion by macrophages via a mechanism involving interference with NF-κB (nuclear factor κB) activation [17], as well as cAMP-mediated inhibition of platelet and neutrophil function [18,19]. In human aortic endothelial cells, LNO_2 also potently induces tissue-protective HO-1 (haem oxygenase-1) expression by •NO-independent mechanisms [20].

The nitration of the principal oxidizable lipid in human blood, CL (cholesteryl-linoleate) has been detected [21]. $CLNO_2$ (cholesteryl-nitrolinoleate) is present in healthy humans at plasma concentrations of 77 ± 11 nM [22], representing one component of net esterified LNO_2 [2]. $CLNO_2$ behaves similarly to LNO_2, with respect to an ability to release •NO in aqueous milieu and promote endothelium-independent vasorelaxation [15]. To date, oxidative and nitro-oxidative inflammatory reactions have not been reported to generate free or esterified nitrated fatty acids in the biological milieu. Moreover, the biological properties of the

Abbreviations used: BCA, bicinchoninic acid; BMDM, bone-marrow-derived macrophage; CL, cholesteryl-linoleate; $CLNO_2$, cholesteryl-nitrolinoleate; DAF-2DA, 4,5-diaminofluorescein diacetate; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; EPI, enhanced product ion; ESI–MS, electrospray ionization MS; ESI–MS/MS, ESI–tandem MS; FCS, fetal calf serum; HO-1, haem oxygenase 1; IFNγ, interferon γ; IL, interleukin; LA, linoleate; L-NAME, N^G-nitro-L-arginine methyl ester; LNO_2, nitrolinoleate; LPS, lipopolysaccharide; MRM, multiple reaction monitoring; NF-κB, nuclear factor κB; NOS2, inducible nitric oxide synthase; $OANO_2$, nitro-oleate; PPARγ, peroxisome-proliferator-activated receptor γ; Rf, retention factor; TNF, tumour necrosis factor.

[1] Correspondence may be addressed to either of these authors (email hrubbo@fmed.edu.uy or freerad@pitt.edu).

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predominant reservoir of oxidized and nitrated membrane and lipoprotein derivatives, esterified lipids, have not been studied. In the present study, we reveal significant increases in the formation of $CLNO_2$ during macrophage activation and an ability of this nitrated esterified lipid to down-regulate monocyte/macrophage activation.

EXPERIMENTAL

Reagents

Inorganic salts, culture medium [RPMI 1640 and DMEM (Dulbecco's modified Eagle's medium)], chloroquine, cycloheximide, DAPI (4′,6-diamidino-2-phenylindole), mouse recombinant IFNγ (interferon γ), LPS (lipopolysaccharide; *Escherichia coli* serotype O127:B8), L-NAME (N^G-nitro-L-arginine methyl ester) and PMA were purchased from Sigma Chemicals. LA (linoleate) and CL were from Nu-Check Prep. Silica gel HF TLC plates were from Uniplate, Analtech. Solvents (HPLC grade or better) were purchased from Fisher Scientific or Pharmco. Alexa Fluor® 488 was from Invitrogen, Molecular Probes. DAF-2DA (4,5-diaminofluorescein diacetate) was from Alexis. FCS (fetal calf serum) was from Bio Whittaker. NOC-18 (an $^{\bullet}NO$ donor) was from Dojindo Laboratories. Rosiglitazone was from Cayman Chemicals. SuperSignal chemiluminescent substrate was from Pierce. Affinity-purified rabbit anti-NOS2 (NOS2 is inducible NO synthase) IgG was from Sigma. Monoclonal antibodies (anti-human CD36 and anti-human CD45), as well as FITC-conjugated immunoglobulins used for flow cytometry were from BD Biosciences. A BCA (bicinchoninic acid) protein assay kit, polyclonal rabbit anti-(NF-κB p65) and peroxidase-conjugated anti-rabbit IgG were from Calbiochem. Rabbit anti-mouse HO-1 was from Stressgen Biotechnologies.

Cell culture

J774.1 murine macrophage-like cells (American Type Culture Collection) were maintained by passage in DMEM containing 4 mM L-glutamine, and supplemented with 10 % heat-inactivated FCS. THP-1 human monocyte-like cells (American Type Culture Collection) were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 1 % sodium pyruvate, 10 mM Hepes and supplemented with 10 % heat-inactivated FCS. BMDM (bone-marrow-derived macrophages) were obtained as described previously [23]. Briefly, cells were harvested from femur and tibias of 6–10-week-old Balb/c mice [DILAVE (Veterinary Laboratories Division of the General Direction of Livestock Services, Uruguay)], cultured in DMEM supplemented with 10 % heat-inactivated FCS and 15 % L929 conditioned medium as a source of M-CSF (macrophages colony-stimulating factor). On day 7, cells were detached, purified by centrifugation (150 ***g*** for 10 min at 20 °C) and plated at 5×10^5 cells/well. Animal experimentation was carried out in accordance with the legal requirements of the Honorary Commission on Animal Experimentation (CHEA), University of the Republic, Uruguay.

Synthesis and characterization of $CLNO_2$ and LNO_2

$CLNO_2$ was synthesized by the reaction of CL with nitrite at acid pH as described in [22]. Briefly, CL (1.5 mmol) was added to a degassed mixture of hexane and 1 % sulfuric acid (1:1, v/v, 4 ml final vol.) followed by the addition of sodium nitrite in two portions at 15 min intervals (7.5 mmol total). The biphasic reaction system was vigorously stirred using a stoppered round-bottom flask and a stir bar (30 min at 25 °C). Following this reaction, the organic layer was separated and dried under a stream of nitrogen. The reaction mixture was diluted with methylene chloride and lipids were separated by preparative TLC on silica gel using a mixture of hexane/ether (80:20, v/v) as the solvent. The separated lipid components were detected by UV absorption and by charring following the reaction with concentrated sulfuric acid. Regions of silica containing nitrated lipids were extracted, dried under vacuum, dissolved in propan-2-ol and stored at − 20 °C [24]. The obtained products were characterized by MS on a 2000 Q-Trap (Applied Biosystems/MDS Sciex) using ESI–MS (electrospray ionization MS) and ESI–MS/MS (ESI–tandem MS; positive mode, direct injection in propan-2-ol containing 4 mM ammonium acetate, and detection of $[M + NH_4]^+$ ammonium adducts). NMR analysis of $CLNO_2$ involved ^1H, ^{13}C and bi-dimensional studies recorded in $CDCl_3$ (Bruker 400 MHz). Quantitation of $CLNO_2$ was done by gravimetry as well as by determining the nitrogen content using chemiluminescent nitrogen analysis (Antek Instruments) as described in [3]. LNO_2 and $[^{13}C]LNO_2$ were synthesized, purified and quantified as described previously [2,3].

Quantification of $CLNO_2$ in macrophages

Confluent monolayers of J774.1 cells were stimulated with LPS/IFNγ or PBS, pH 7.2 (control). The NOS inhibitor L-NAME (5 mM) was included in some assays to assess whether the $CLNO_2$ generation was influenced by $^{\bullet}NO$ generation during macrophage activation. After 6 or 24 h incubation, nitrite was measured in cell supernatants using Griess reagent [25] and cells were recovered for lipid extraction [24]. A $[^{13}C]CLNO_2$ internal standard could not be prepared, owing to loss of the nitro group during $[^{13}C]LNO_2$-cholesterol esterification reactions. Alternatively, during the monophase stage of lipid extraction, a known amount of $[^{13}C]LNO_2$ was added as an internal standard to correct for sample-to-sample differences in extraction and handling efficiency. $CLNO_2$ was determined by HPLC ESI–MS/MS. Both $CLNO_2$ and $[^{13}C]LNO_2$ were eluted from a 150 mm × 2.1 mm C_{18} GraceVydac columm (5 μm particle size) using an isocratic solvent consisting of 60 mM propan-2-ol/40 mM acetonitrile/4 mM ammonium acetate. For quantitative analysis, two MRM (multiple reaction monitoring) transitions were monitored: m/z 711 → 369 for the ammonium adduct of $CLNO_2$ and m/z 342 → 297 for $[^{13}C]LNO_2$ in the positive and negative ion mode respectively [3,22]. The relative amount of $CLNO_2$ present in samples was determined by comparing the peak areas of $CLNO_2$ and $[^{13}C]LNO_2$ standards in control and treated samples. The identity of $CLNO_2$ was confirmed by both monitoring fragmentation of $CLNO_2$ to LNO_2 (m/z 711 → 326) and generating EPI (enhanced product ion) spectra of the m/z 711 product. Owing to the lack of the $[^{13}C]CLNO_2$ standard, absolute quantification was not obtained by this method; rather, a relative increase with respect to paired controls is reported (the ratio of the peak areas of $CLNO_2$ relative to the internal standard). In addition, to better estimate the $CLNO_2$ content in macrophages, we constructed an external $CLNO_2$ calibration curve using known concentrations of synthetic $CLNO_2$. This procedure, although revealing, on occasion may not yield entirely accurate values of $CLNO_2$ concentrations in biological samples, because of the potential loss of $CLNO_2$ during sample lipid extraction.

Analysis of $CLNO_2$ effects on NOS2 induction

Macrophages (confluent monolayers of J774.1 or BMDM as indicated) were exposed to LPS (0.5–1 μg/ml) in the presence or absence of IFNγ (400 units/ml). Reactions were performed in DMEM containing 10 % (v/v) FCS with addition of vehicle or indicated concentrations of CL, $CLNO_2$ or LNO_2. Nitroalkenes were added prior to (6 and 20 h), or concurrently

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with, the stimulus. After 4–5 h activation, supernatants were discarded, cells were washed and NOS2 expression was assessed immunocytochemically using anti-NOS2 antibodies or by monitoring •NO generation using the cell-permeant fluorescence indicator DAF-2DA [26]. Assays using rosiglitazone (10–50 μM) and NOC-18 (0.1–50 μM) were also performed to evaluate potential mitigation by PPARγ ligand and •NO actions respectively. Examination of the nitroalkene effect on NOS2 activity was performed using cycloheximide, an inhibitor of protein translation; cells were pre-activated with LPS for 6 h to induce NOS2, and then cycloheximide (5 μM) was added together with nitroalkenes ($CLNO_2$ or LNO_2) or vehicle (control), and NOS2 expression was measured 3 h later. Cell viability after treatment with the different agents under study was examined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide].

$CLNO_2$ modulation of IL (interleukin)-1β and TNF (tumour necrosis factor) secretion

In order to quantify in parallel cytokine and CD36 expression (see below) we used human THP-1 macrophages, since previous studies showed that cytokine secretion by murine and human macrophages were similarly affected by nitroalkenes [17]. Briefly, cells were seeded at 5×10^5 cells/well and stimulated with 50 ng/ml PMA in the presence of 10–50 μM CL, $CLNO_2$ or vehicle. Cells were also activated in the presence of LA, LNO_2 or rosiglitazone. After 20 h of culture, cell supernatants were collected and analysed for IL-1β and TNF levels by capture ELISA (OptEIA sets; BD Biosciences) using antibody pairs and protocols as recommended by the manufacturer.

Analysis of CD36 induction by $CLNO_2$

THP-1 cells (5×10^5 cells/well) were stimulated with 50 ng/ml PMA in the presence of nitroalkenes ($CLNO_2$ or LNO_2), their lipid precursors (CL, LA), rosiglitazone or vehicle (control). After 20–22 h stimulation, the expression of CD36 and CD45 was analysed by flow cytometry (FACSCalibur system; BD Biosciences) using mouse monoclonal antibodies. The increase in CD36 or CD45 expression (relative increase) was calculated as the ratio between the fluorescence intensity (median values) obtained for treated and control cells.

HO-1 induction by $CLNO_2$

Induction of HO-1 was analysed in cells incubated with $CLNO_2$ or LNO_2. Treated cells were rinsed once with ice-cold PBS, quickly frozen and then lysed in 10 mM Hepes (pH 7.9) containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT (dithiothreitol) and a mixture of protease inhibitors. Insoluble material was removed by centrifugation (5000 ***g*** at 4 °C for 5 min). Supernatants were collected and the protein concentration was determined using a BCA protein assay. Lysates (25–30 μg of protein) were electrophoresed on SDS/PAGE (10 % gels) and electrotransferred on to nitrocellulose membranes. Western blot analysis was performed using rabbit anti-(HO-1) antibodies followed by a peroxidase-conjugated secondary antibody. SuperSignal chemiluminescent substrate was used for development.

Analysis of $CLNO_2$ effects on NF-κB activation

J774.1 cells were stimulated with LPS (1 h, 37 °C, 5 % CO_2) in the presence or absence of nitroalkenes. Afterwards, nuclear and cytoplasmic extracts were prepared as described previously [27]. Proteins (25 μg) were separated by SDS/PAGE (10 % gels) and transferred on to nitrocellulose membranes. Western blot analysis was performed using an anti-(NF-κB p65) antibody, followed by a goat anti-rabbit IgG conjugated to peroxidase. Development was performed using the SuperSignal chemiluminescent substrate.

Statistical analysis

Results are shown as means $\pm$ S.D. Differences between mean values were evaluated using a Tukey–Kramer multiple comparison test and were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Macrophage activation by inflammatory stimuli generates $CLNO_2$

To address whether nitrative inflammatory reactions occurring during macrophage activation led to nitration of sterol esters, we compared the $CLNO_2$ content between LPS/IFNγ-activated and control macrophages. For that purpose we first synthesized a $CLNO_2$ standard using methods previously described [22], by the reaction of CL with nitrite at acidic pH. From preparative TLC, two bands were separated [arrows, Figure 1A, termed $CLNO_2$ top and bottom isomers in reference to chromatographic Rf (retention factor) properties], and then analysed by positive ion ESI–MS (Figure 1B). The two bands displayed an *m*/*z* of 711, characteristic of the ammonium adduct of $CLNO_2$ ($[M + NH_4]^+$). ESI–MS/MS of the *m*/*z* 711 species yielded daughter ions of *m*/*z* 369 (cholesterol-OH)$^+$, 326 (LNO_2^+) and 279 [loss of the nitro group, (LNO_2-HNO_2)$^+$] (Figure 1B, inset). NMR analysis of these two bands (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/417/bj4170223add.htm) confirmed that nitration occurred on the acyl moiety of CL and corresponded to nitroalkene isomers (Figure 1C, termed $CLNO_2$ top and bottom isomers, in reference to chromatographic Rf properties). The synthetic $CLNO_2$ standard was then characterized by reverse-phase HPLC/MS, using MRM detection for two specific transitions: *m*/*z* 711 $\rightarrow$ 369 and *m*/*z* 711 $\rightarrow$ 326, which correspond to $CLNO_2$ fragmentation to cholesterol and LNO_2 respectively (Figure 2A). Both top and bottom $CLNO_2$ isomers eluted at the same retention time (9.5 min) and the *m*/*z* 711 $\rightarrow$ 326 transition ion intensity was lower. The identity of these species was confirmed by EPI of the *m*/*z* 711 product (Figure 2A, right-hand panel).

$CLNO_2$ was eluted from lipid fractions of either activated or control macrophages, showing the same HPLC profile as the $CLNO_2$ standard (Figures 2B and 2C respectively). The identity of these species was also confirmed by EPI of the *m*/*z* 711 product (Figure 2B). In the case of control macrophages, HPLC/MS analysis yielded weak ion intensities at these MRM transitions (detection using *m*/*z* 711 $\rightarrow$ 326 transition produced almost no signal; results not shown). For $CLNO_2$ quantification, the content of $CLNO_2$ was determined by comparing the peak areas of $CLNO_2$ (scanned using *m*/*z* 711 $\rightarrow$ 369 transition) and [^{13}C]LNO_2 (internal standard, scanned using *m*/*z* 342 $\rightarrow$ 297). Results are presented as area ratios of analyte to [^{13}C]LNO_2 (Figure 2D). Unstimulated murine J774.1 macrophages contained levels of $CLNO_2$ lower than that detected in LPS plus IFNγ-activated macrophages; the latter displayed a 3-fold and 19-fold increase in $CLNO_2$ levels following 6 and 24 h activation respectively (Figure 2D). Using an external $CLNO_2$ calibration curve, we estimated a $CLNO_2$ content of 100–300 pmol/10^6 cells following 24 h of inflammatory activation. As expected, $CLNO_2$ formation by activated macrophages was inhibited by L-NAME (Figure 2D), which also inhibited 50–60 % of nitrite formation in the culture medium (results not shown), supporting the contribution of •NO-derived species towards CL nitration. Furthermore, the increase of $CLNO_2$ in activated macrophages occurred in concert with increased NOS2 activity (Figure 2E). At longer activation times

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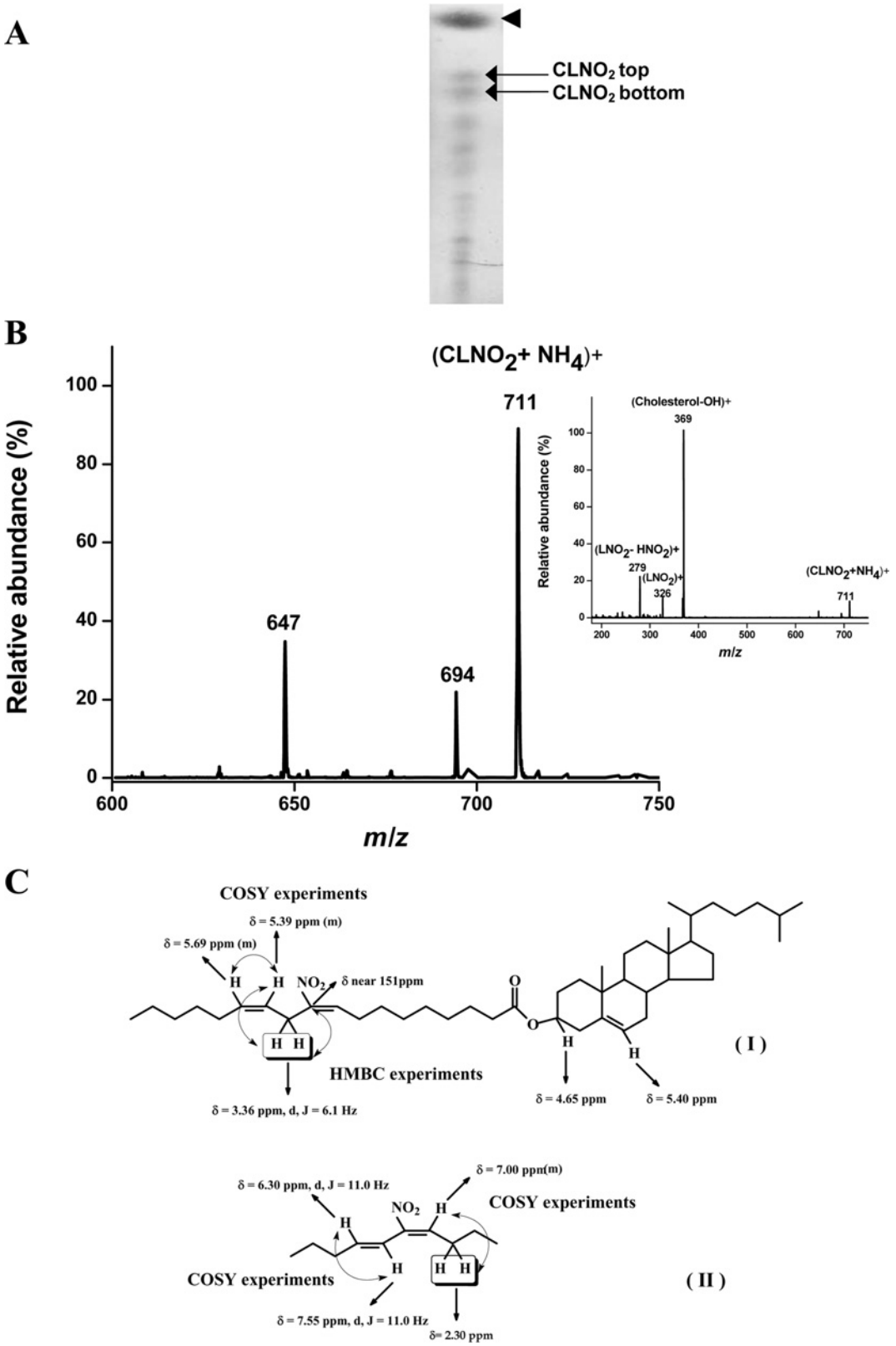

Figure 1 Characterization of $CLNO_2$

(**A**) TLC analysis of products obtained from nitrite treatment of CL, showing two main nitrated products (arrows) and non-reacted CL (arrowhead). These products were extracted from the silica, dried under vacuum and analysed by ion-trap MS (positive mode, direct injection, propan-2-ol containing 4 mM ammonium acetate). (**B**) MS spectra of the ammonium adducts of $CLNO_2$; insert: MS/MS of parent molecule showing LNO_2 as a daughter ion. (**C**) ^{1}H-, ^{13}C-NMR spectrometry and two-dimensional COSY, HMBC (heteronuclear multiple bond correlation) and HMQC (heteronuclear single quantum correlation) analysis. $CLNO_2$ bottom data correspond to cholesteryl-10-nitrolinoleate (I) and its conjugated isomer (II). $CLNO_2$ top data correspond to a mixture of cholesteryl-9-nitrolinoleate and (I).

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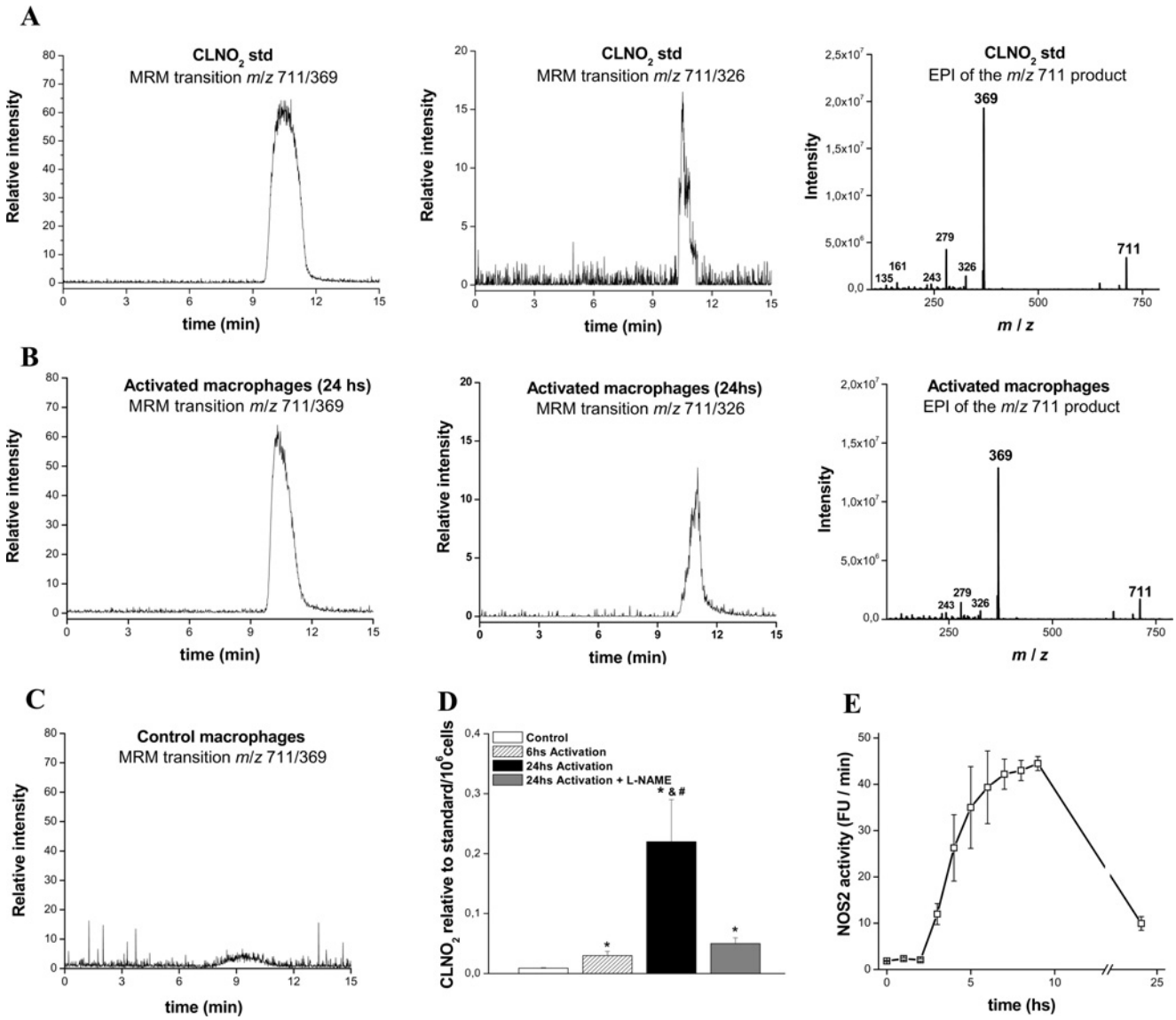

Figure 2 CL nitration generated $CLNO_2$ in activated macrophages

J774.1 cells were stimulated with LPS (1 μg/ml) plus IFNγ (400 units/ml). After 6 or 24 h incubation, cells were recovered, lipids extracted and the presence of $CLNO_2$ was analysed by HPLC/MS using the MRM scan mode (*m*/*z* 711 $\rightarrow$ 369 and *m*/*z* 711 $\rightarrow$ 326). HPLC elution profiles of the $CLNO_2$ standard (**A**) and lipid extractions from activated (**B**) and non-activated (**C**) macrophages are shown. Representative EPI of the *m*/*z* 711 product present in eluted $CLNO_2$ from the standard (**A**, right-hand panel) and activated macrophage (**B**, right-hand panel) lipid fractions. (**D**) Quantification of $CLNO_2$ relative to the internal standard [(^{13}C)LNO_2] in control and activated cells as well as in the presence of L-NAME (5 mM). Results are expressed as the means $\pm$ S.E.M. ($n = 4$). $^{*}P < 0.05$ compared with controls, $^{\&}P < 0.05$ compared with 6 h activation and $^{\#}P < 0.05$ compared with L-NAME. (**E**) Time course of NOS2 expression in cells stimulated with LPS/IFNγ; NOS2 activity was followed by fluorimetry using DAF-2DA. Results are expressed as means $\pm$ S.D. ($n = 4$).

(24 h) when induced levels of NOS2 activity had significantly decreased, the cumulative content of $CLNO_2$ was 19-fold greater than controls (Figure 2E). A concomitant increase in nitrite levels in the medium was observed over time, with levels 10-fold greater after 24 h compared with 6 h stimulation. In summary, macrophage activation by LPS and IFNγ led to acyl chain nitration of CL, revealing that nitration of esterified unsaturated fatty acids takes place in a biological milieu and, particularly, in an inflammatory condition where NOS2 is induced.

$CLNO_2$ and LNO_2 inhibit the generation of inflammatory mediators by monocytes/macrophages

As already mentioned, fatty-acid-derived nitroalkenes are capable of modulating the expression of inflammatory genes. Thus the formation of $CLNO_2$ during macrophage activation may influence cell functions through LNO_2 release by cell cholesteryl ester hydrolases. Moreover, in the case of macrophages, activation by inflammatory stimulus could increase neutral cholesteryl esterase activity [28], potentially favouring LNO_2 release. Thus we studied the effect of $CLNO_2$ and LNO_2 on the generation of inflammatory mediators by monocytes/macrophages. First, the effect of these nitroalkenes on $^{\bullet}$NO production was investigated in murine macrophages (BMDM and J774.1 cells); cells were treated for various time intervals relative to the addition of LPS (alone or plus IFNγ) with $CLNO_2$ (bottom and top isomers), LNO_2 and their corresponding precursors (CL and LA). Nitroalkene effects on $^{\bullet}$NO generation by BMDM and J774.1-activated macrophages were comparable: $CLNO_2$ (both isomers) and LNO_2 caused a dose-dependent reduction of $^{\bullet}$NO generation, whereas CL

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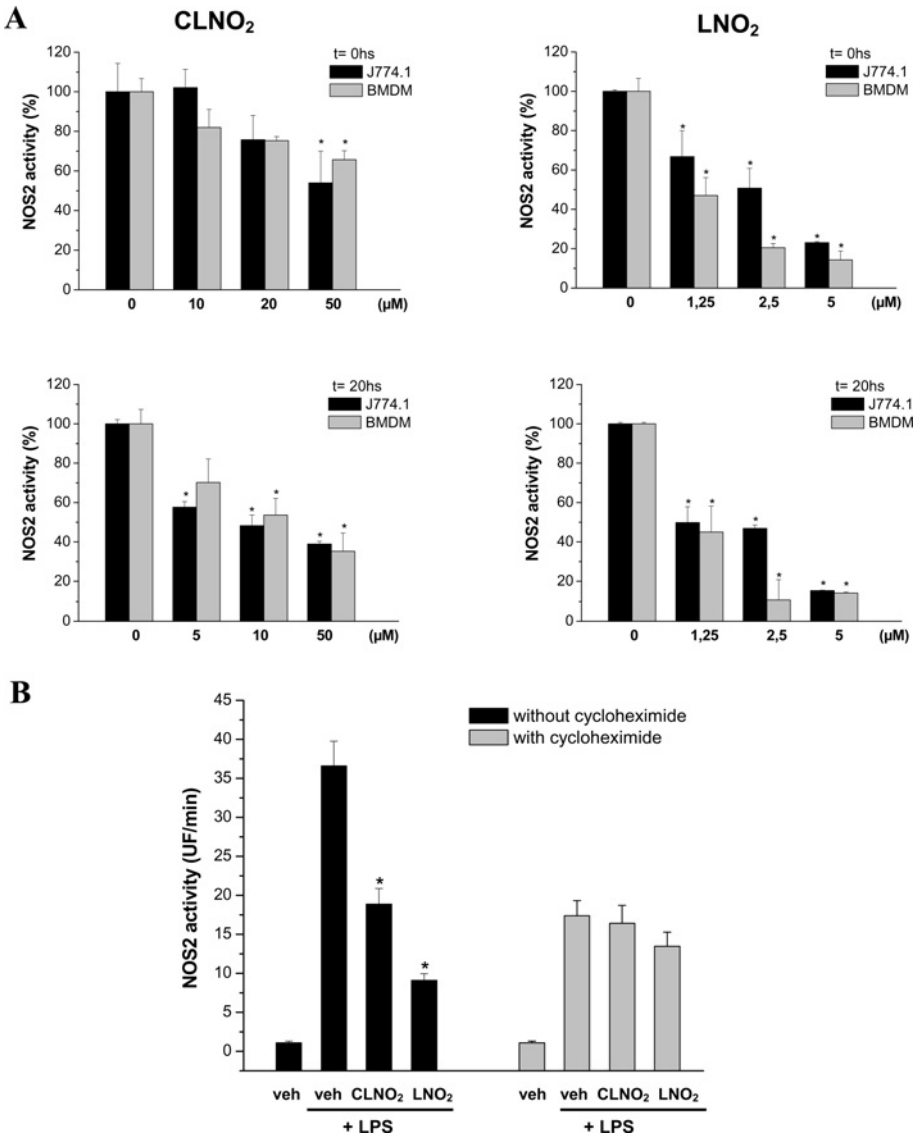

Figure 3 $CLNO_2$ inhibited •NO generation by activated macrophages

Activation of mouse macrophages (J774.1 cells and BMDM) was performed in the presence or absence of the indicated molecules, and after 5 h incubation •NO was measured by fluorimetry. (**A**) Nitroalkenes were added concurrently ($t = 0$hs) or 20 h ($t = 20$hs) prior to LPS stimulation. NOS2 activity is expressed relative to the control (vehicle) and values correspond to means ± S.D. ($n = 3$). (**B**) Cells were activated with LPS and nitroalkenes (20 μM LNO_2 or 50 μM $CLNO_2$) added together with the activating molecules (black bars) or at 6 h post-activation in the presence of cycloheximide (grey bars). NOS2 activity was measured at 9 h post-stimulation by fluorimetry. Results are expressed as the means ± S.D. ($n = 3$). In all cases * indicates a statistical significance ($P < 0.05$) compared with the control [vehicle (veh)].

and LA had no effect (Figures 3A and Supplementary Figure S2 at http://www.BiochemJ.org/bj/417/bj4170223add.htm). The inhibitory effect of $CLNO_2$ on •NO generation was more pronounced when added to macrophages 6 h (results not shown) and 20 h prior to activation with LPS alone (Figure 3A). In contrast, LNO_2 inhibition was independent of the time interval at which it was added in relation to LPS, where LNO_2 was the strongest inhibitor of •NO generation (1.25 μM LNO_2 caused a reduction comparable with 5 μM $CLNO_2$). To address whether these inhibitory effects on •NO generation were linked to modulation of NOS2 expression, we performed experiments in which cells were pre-activated for 6 h to induce NOS2 expression and then nitroalkenes were added together with the protein translation inhibitor cycloheximide. An inhibitory effect of $CLNO_2$ and LNO_2 on •NO generation was not observed in these conditions (Figure 3B), suggesting that nitroalkene actions were dependent on inhibiting NOS2 gene expression, rather than inhibiting NOS2 catalytic activity. Immunohistochemical analysis of NOS2 expression by activated macrophages confirmed that $CLNO_2$ down-regulated NOS2 expression and that LNO_2 had more potent inhibitory effects (Figure 4). Under these conditions, $CLNO_2$ or LNO_2 did not induce changes in cell viability (Supplementary Figure S3 at http://www.BiochemJ.org/bj/417/bj4170223add.htm) or protein levels.

It has recently been shown that nitroalkenes, particularly LNO_2 and $OANO_2$, are capable of modulating the production of inflammatory cytokines (IL-6 and TNFα) by human, as well as murine, macrophages [17]. Thus we also explored whether $CLNO_2$ was

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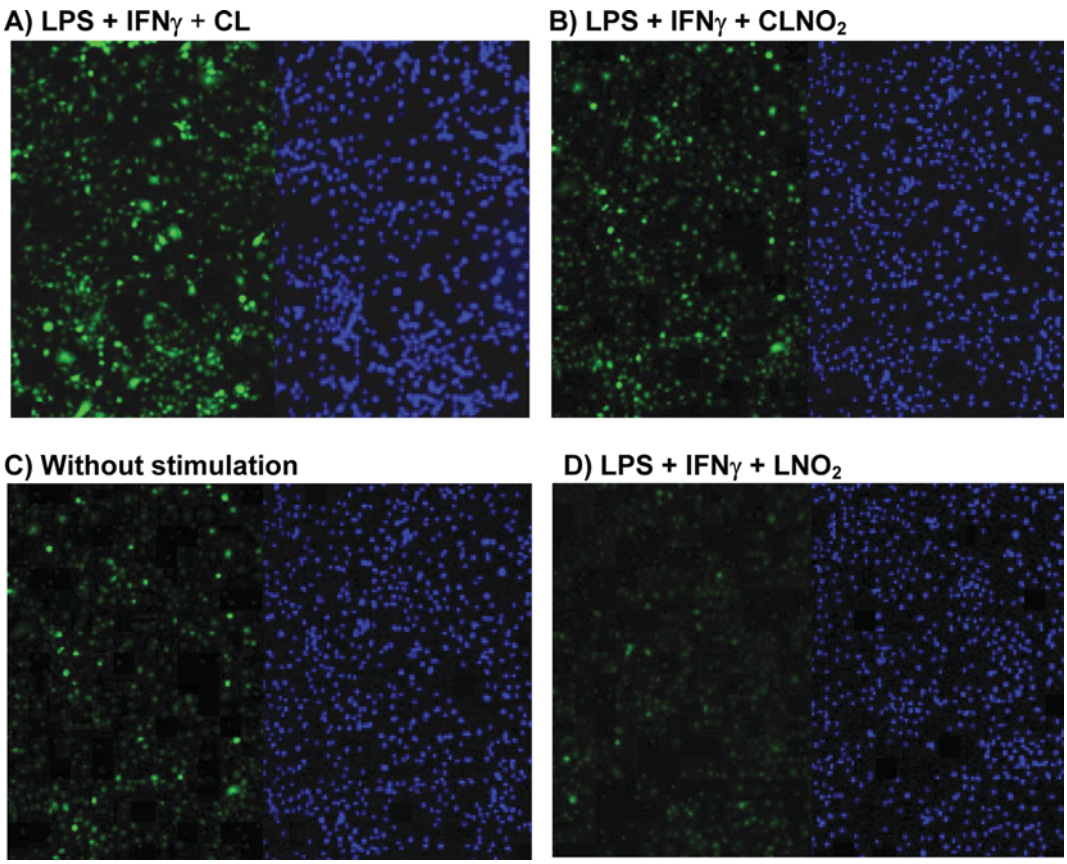

Figure 4 $CLNO_2$ modulation of NOS2 expression

Mouse J774.1 cells at confluence were stimulated for 6 h with LPS plus IFNγ in the presence or absence of 50 μM $CLNO_2$ (top and bottom isomers) and analysed by immunofluorescence for NOS2 expression (left-hand panels). Cell nuclei were stained using DAPI as a control (right-hand panels).

able to modulate cytokine secretion by monocytes/macrophages, determining the extent of IL-1β and TNF secretion by human THP-1 cells stimulated with PMA. $CLNO_2$ and LNO_2 induced a significant decrease in pro-inflammatory cytokine secretion ($P < 0.05$; Figure 5) under conditions where nitroalkenes were added concurrently with the stimulus. This inhibition required greater nitroalkene concentrations than those previously reported [17]. Among other reasons, discrepancies may be due to the presence in our assays of a 10-fold higher concentration of FCS during cell stimulation; this may have caused a reduction in the level of available nitroalkene for interacting with cells.

LNO_2 was a stronger modulator of NOS2 and cytokine expression than $CLNO_2$. Since nitroalkenes have the capability to signal by directly interacting with cellular targets (e.g. receptors or nucleophilic reaction centres), the differences in potency observed may be explained by a more limited access of $CLNO_2$ to those targets, as the cholesterol moiety can be expected to decrease solubility and diffusional properties in cellular compartments. Alternatively, as mentioned above, $CLNO_2$ may express activity only upon hydrolytic release of fatty acid nitro-derivatives by cell lipases and esterases; if this were the case, this sterol ester derivative may be relevant as a cellular reservoir of LNO_2 in hydrophobic compartments. In our macrophage studies, it is plausible that $CLNO_2$ may traffic to lysosomes, and LNO_2 released by acid cholesteryl ester hydrolase may contribute to the observed effects. To address the possible contribution of hydrolytically released LNO_2 to the signalling actions of $CLNO_2$ we performed studies in the presence of chloroquine, an inhibitor of lysosome acidification, which is necessary for the activity of acid cholesteryl ester hydrolases. When $CLNO_2$ was added concurrently with LPS, $CLNO_2$-mediated NOS2 down-regulation was not affected by chloroquine, suggesting that the release of LNO_2 from $CLNO_2$ was not required (Supplementary Figure S3). Long-term chloroquine treatment of macrophages resulted in increased cell susceptibility to LPS activation, as shown by higher percentages of cell death after 4–5 h of LPS stimulation. Thus definitive assessment of the contribution of LNO_2 release to the inhibitory effects of $CLNO_2$ requires further study (e.g. silencing of cholesteryl ester hydrolases).

Overall, these results showed that $CLNO_2$ and LNO_2 suppressed NOS2 and cytokine expression by macrophages in response to inflammatory stimuli, suggesting that $CLNO_2$ formation upon macrophage activation, directly or indirectly via LNO_2, may serve an adaptive role by contributing to the attenuation of inflammatory gene expression.

PPARγ and •NO are not involved in $CLNO_2$- and LNO_2-dependent inhibition of the production of inflammatory mediators by monocytes/macrophages

Nitroalkenes are potent PPARγ ligands [3,6] with PPARγ a critical mediator of the regulation of macrophage inflammatory responses. For exploring whether the inhibitory effects of $CLNO_2$ and LNO_2 on NOS2 induction in LPS-activated macrophages

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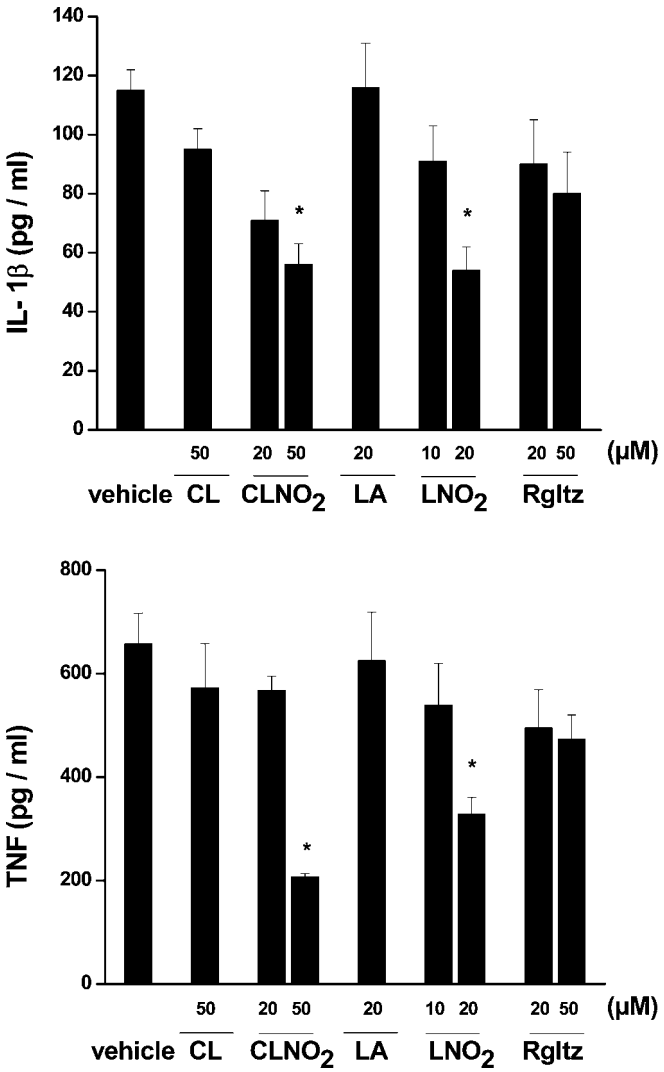

Figure 5 Effects of $CLNO_2$, LNO_2 and rosiglitazone on cytokine secretion

THP-1 cells were differentiated with 50 ng/ml PMA in the presence of the molecules indicated. After 20 h incubation, supernatants were assessed for IL-1β (**A**) and TNF (**B**) by ELISA. Results are expressed as means $\pm$ S.D. ($n = 3$). * indicates a statistical significance ($P < 0.05$) compared with control.

occurred via PPARγ, we first evaluated, using THP-1 cells, the ability of nitroalkenes to induce the expression of CD36, a PPARγ-regulated gene product in monocytes/macrophages. $CLNO_2$ showed no influences on CD36 expression, even at 20–50 μM (Figure 6A). In contrast, LNO_2 and the high-affinity PPARγ ligand rosiglitazone (10–50 μM) induced a significant increase in CD36 expression ($P < 0.05$; Figures 6B and 6C). In these assays, CD45 expression was analysed in parallel as a control, and showed no alteration by either nitroalkene or rosiglitazone treatment. These results confirmed that $CLNO_2$ does not display significant PPARγ ligand activity, and is consistent with the fact that nitroalkenes, which are high-affinity PPARγ ligands [3], lose receptor-activating capability upon ester derivatization of the carboxylic acid. Furthermore, the well-known PPARγ activator rosiglitazone was tested to address the contribution of PPARγ to the observed anti-inflammatory actions of $CLNO_2$ and LNO_2. Rosiglitazone did not inhibit cytokine production (Figure 5) or $^{\bullet}$NO generation (results not shown) by activated monocytes/macrophages, confirming that the modulatory actions of nitroalkenes on these cells were not PPARγ-dependent. These results are consistent with previous observations [17], and with the fact that the anti-inflammatory effects of electrophilic PPARγ agonists such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 predominantly occur via PPARγ-independent mechanisms [29,30].

The potential contribution of nitroalkene-derived $^{\bullet}$NO to inhibition of NOS2 expression was also examined. LPS- and LPS/IFNγ-induced generation of $^{\bullet}$NO by macrophages was not affected by the $^{\bullet}$NO donor NOC-18 (1–50 μM), even at concentrations that far exceed any possible $^{\bullet}$NO concentration that might be derived from nitroalkene decay in assay systems (results not shown). Therefore $CLNO_2$-mediated inhibition of macrophage activation could not be explained by an ability to release $^{\bullet}$NO, as described previously [17,31,32]. Of note, $^{\bullet}$NO-mediated attenuation of NOS2 expression frequently requires high, non-biological concentrations of $^{\bullet}$NO [32] that may be transduced by mechanisms shown in the present study. Thus additional pathways appear to be involved in signalling actions linked to nitroalkene-mediated control of NOS2 expression.

$CLNO_2$ and LNO_2 mediate induction of HO-1 expression in macrophages

The induction of HO-1 is an endogenous cytoprotective pathway triggered by a variety of stress-related signals and electrophilic species [33,34]. HO-1 catalyses the oxidative degradation of haem to biliverdin, releasing carbon monoxide (CO) and the chelated Fe^{2+}. These products exert anti-oxidant and anti-inflammatory actions; thus attention has been recently devoted to CO, which possesses intriguing signalling properties affecting numerous critical cellular functions including proliferation and apoptosis, as well as the generation of inflammatory mediators [35]. Fatty acid nitroalkene derivatives (LNO_2, $OANO_2$) induce HO-1 expression in endothelial cells [20] and RAW264.7 macrophages [17]. Thus we explored whether $CLNO_2$, as well as LNO_2, were capable of driving HO-1 expression in macrophages. As shown in Figure 7(A), HO-1 was induced in J774.1 macrophages by $CLNO_2$ and LNO_2, but not by their respective native fatty acid precursors, with $CLNO_2$ less potent than LNO_2 in up-regulating HO-1 expression. Of relevance, the time course of NOS2 and HO-1 expression diverged in macrophages activated with LPS in the presence or absence of $CLNO_2$. HO-1 was induced after approx. 9 h macrophage activation with LPS, in parallel with the decrease in NOS2 expression (Figure 7B). In contrast, addition of $CLNO_2$ during LPS-induced macrophage activation caused an earlier induction of HO-1 compared with controls (3 compared with 9 h respectively), and resulted in inhibition of NOS2 expression (Figure 7B, compare lanes 2 and 7 of the bottom gel, indicated with arrows). Thus the well-known capacity of HO-1 to modulate NOS2 expression in macrophages [27], together with the fact that nitroalkenes robustly induce HO-1 at low concentrations ([17,20], and the present study), suggest potential HO-1 mitigation of the macrophage NOS2 responses observed in the present study. Two observations might support this contention: first, LNO_2 was more potent in inducing HO-1 expression and inhibiting NOS2 expression than $CLNO_2$. Secondly, at low micromolar concentrations (5 μM) $CLNO_2$ inhibited NOS2 expression only when pre-incubated with cells for 20 h, a condition also required for detecting HO-1 induction (results not shown). Mechanisms associated with the induction of HO-1 by nitroalkenes in macrophages are unknown; they might involve Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) activation and/or require synergy between the cAMP-dependent response element

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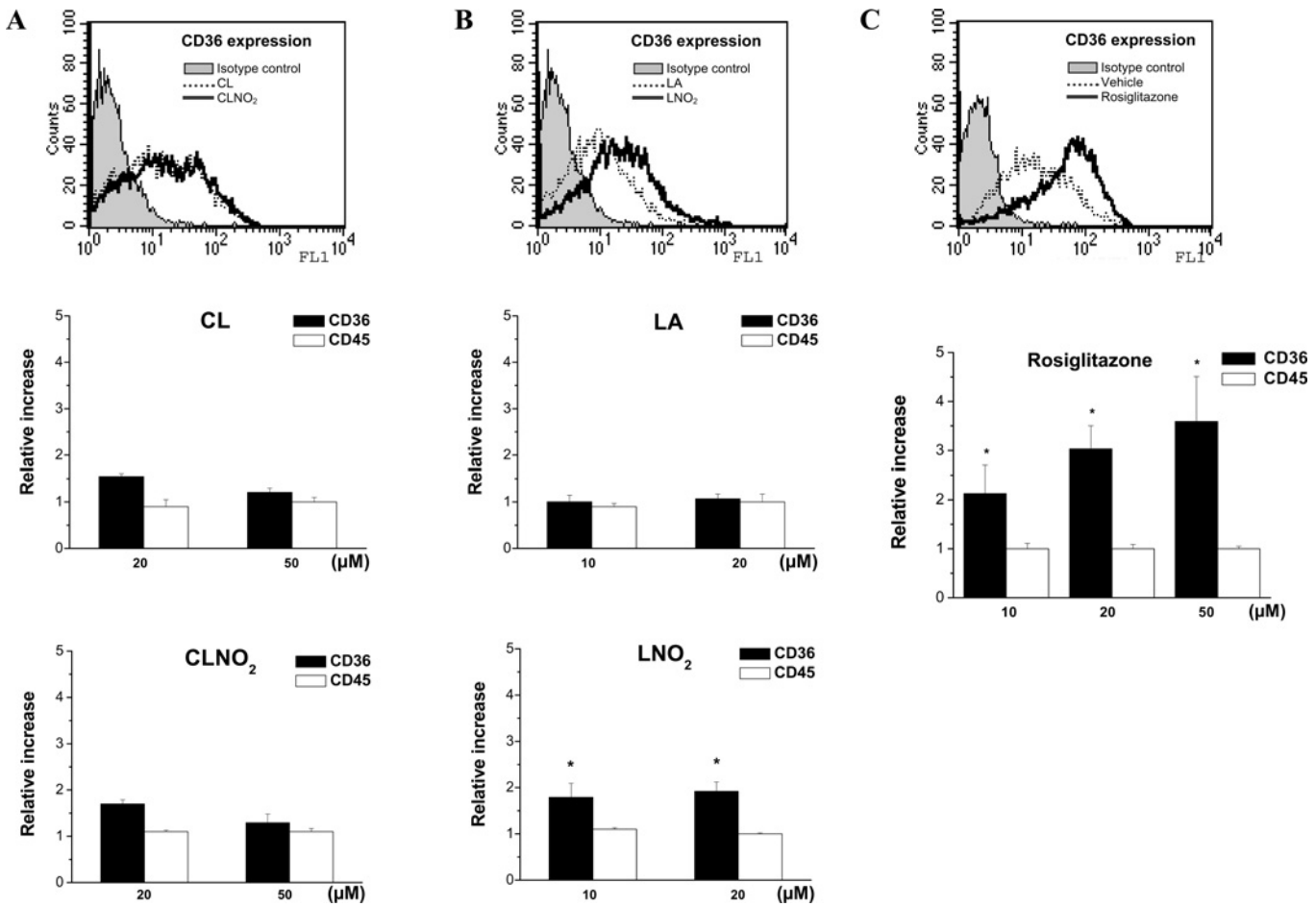

Figure 6 Effects of $CLNO_2$ (A), LNO_2 (B) and rosiglitazone (C) on CD36 expression by monocytes

THP-1 cells were differentiated with 50 ng/ml PMA in the presence of the molecules indicated. After 20 h incubation, cells were stained for CD36 or CD45 and analysed by flow cytometry. Top panels showed a representative histogram plot obtained for CD36 analysis. Bottom panels show CD36 and CD45 expression as relative increases (fluorescence of treated cells/fluorescence of control cells); results are expressed as the means ± S.D. ($n = 3$). * indicates a statistical significance ($P < 0.05$) compared with control.

CRE and AP-1 (activator protein 1) sequences in the HO-1 promoter region [36,37].

$CLNO_2$ and LNO_2 inhibit LPS-induced NF-κB signalling in macrophages

An additional mechanism that could contribute to $CLNO_2$ and LNO_2-induced inhibition of NOS2 and cytokine expression is a Michael addition reaction of this electrophilic species with transcriptional factors, preventing expression of downstream gene products [38]. In this regard, the inhibition of macrophage cytokine secretion by nitro-fatty acids via post-translational electrophilic alkylation of critical thiols of the NF-κB p65 subunit has been observed [17]. Since the transcription factor NF-κB contributes to LPS-induced NOS2 gene expression [39], we explored whether $CLNO_2$ and LNO_2 were capable of inhibiting NF-κB activation under the cell activation conditions used for studying NOS2 expression. Both $CLNO_2$ and LNO_2 did not inhibit NF-κB activation when added concurrently to cells with LPS (Figure 8A). In contrast, a marked inhibition of NF-κB activation was observed when macrophages were pretreated with both nitroalkene derivatives for 20 h prior to LPS stimulation (Figure 8B), supporting that nitroalkene effects on NF-κB activation are not an immediate/early event. It has been shown that CO, one of the products resulting from HO-1 activity, inhibits LPS-induced activation of NF-κB by preventing the phosphorylation and degradation of the regulatory subunit IκBα (inhibitor of NF-κB) [27,35]. Therefore the contribution of HO-1 to nitroalkene inhibition of NOS2 expression might occur via CO-mediated inhibition of NF-κB.

Concluding remarks

Overall, the results of the present study support the concept that $^{\bullet}$NO increases the breadth of reactions that transduce 'redox signalling', thus linking the metabolic and immune status of cells with information transfer reactions. The nitration of CL to yield $CLNO_2$ upon activation of macrophages with pro-inflammatory stimuli affirms that the oxidative and nitrative reactions characteristic of inflammation also induce lipid nitration. In particular, the generation of $CLNO_2$ may be of relevance considering that this molecule would concentrate in hydrophobic environments, where Nef-like decay reactions are limited, to potentially serve as a hydrolysable reserve of esterified LNO_2. Thus $CLNO_2$ may mediate cell signalling actions directly or indirectly, by serving as a precursor for subsequent LNO_2 release. These indirect $CLNO_2$

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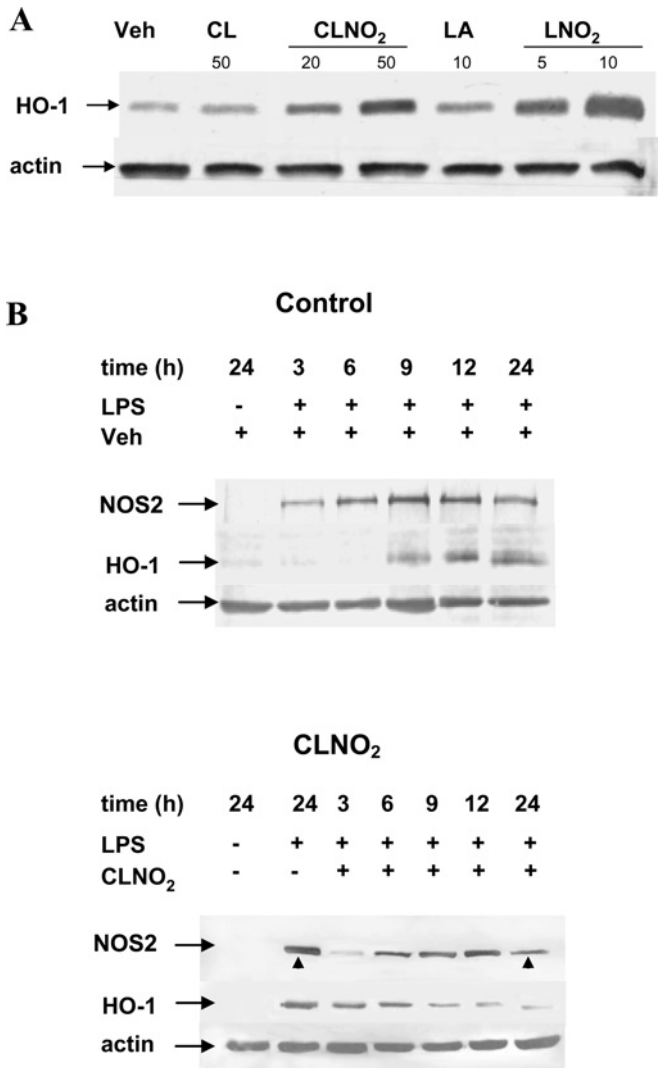

Figure 7 $CLNO_2$ induced HO-1 expression in macrophages

(**A**) Mouse J774.1 cells were stimulated with $CLNO_2$, LNO_2 or their respective fatty acid precursors at the indicated concentrations. After 20 h stimulation, the presence of HO-1 and actin (control) was analysed in cell lysates by Western blot. (**B**) Time course of NOS2 and HO-1 expression in LPS-activated macrophages in the absence and presence of $CLNO_2$. Mouse J774.1 cells at confluence were stimulated with LPS (1 μg/ml) in the presence or absence of $CLNO_2$ (25 μM). At different times post-stimulation (3, 6, 9, 12 and 24 h), the presence of NOS2, HO-1 and actin was examined by Western blot. Veh, vehicle.

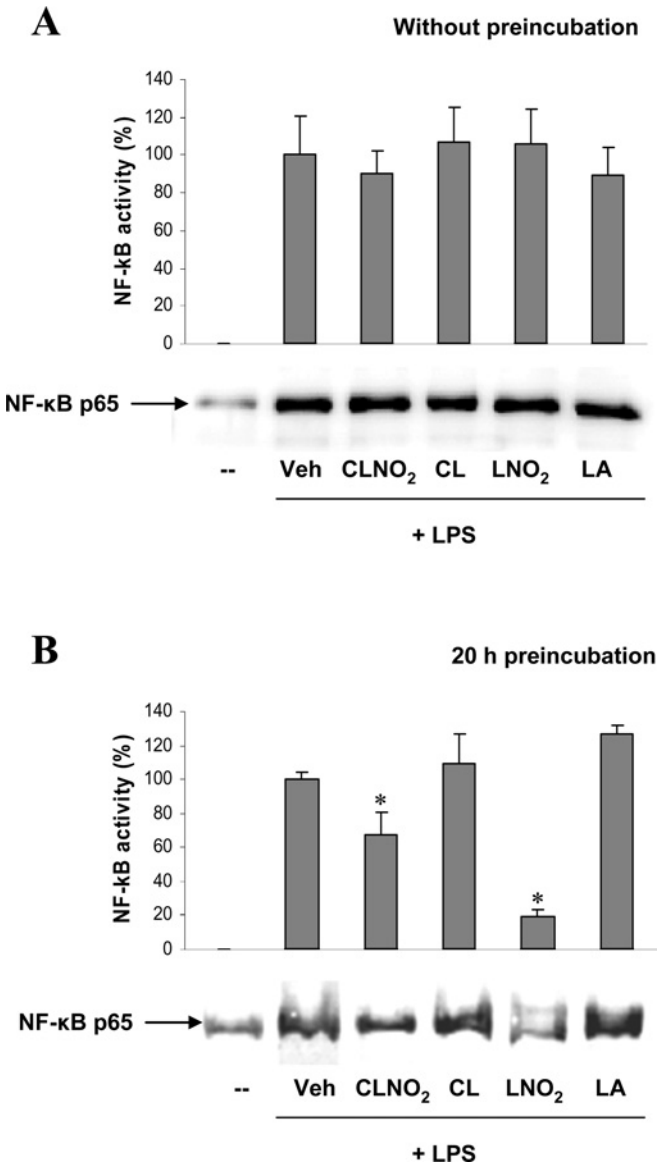

Figure 8 $CLNO_2$ and LNO_2 inhibited NF-κB activation

LPS-induced activation of NF-κB was analysed in nuclear extracts of activated cells in comparison with controls. Mouse J774.1 cells were stimulated with LPS (1 μg/ml) in the presence of $CLNO_2$ (25 μM), CL (25 μM), LNO_2 (10 μM), LA (10 μM) or vehicle. The addition of nitroalkenes was made concurrently (**A**) or prior (20 h; **B**) to LPS stimulation. Cells were also incubated in the presence of vehicle. After 1 h LPS-stimulation, nuclear extracts were prepared and levels of NF-κB in extracts were determined by Western blot analysis. Top panels correspond to the densitometric analysis of bands and correspond to means $\pm$ S.D. *indicates a statistical significance ($P < 0.05$, $n = 3$) compared with control. Veh, vehicle.

effects may be significant, inasmuch as LNO_2 was shown to be a more potent modulator of macrophage activation than $CLNO_2$, and a robust PPARγ ligand [40]. It remains to be established what specific concentrations of $CLNO_2$ and LNO_2 are generated during macrophage activation, an insight that will shed light on the physiological relevance of fatty acid nitration during inflammatory responses. We determined that $CLNO_2$ content in activated macrophages reached $\sim$100–300 nM per 10^6 cells/ml, but this value probably underestimates net cellular content as it was obtained using a method in which losses derived from sample preparation, as well as from nitroalkene decay reactions and Michael addition reactions with cell components, would not be compensated for. It was observed that the concentrations of $CLNO_2$ required to effectively induce modulatory effects on macrophage function were in the low micromolar range. This is consistent with previous observations that *in vitro* functional responses to lipophilic stimuli (e.g. prostaglandins and other eicosanoid derivatives) do not reproduce the *in vivo* responses to much lower concentrations of endogenously formed mediators; a significant percentage of which are not expected to efficiently reach critical intracellular targets *in vitro* because of solubility and diffusional limitations and an expectation of alternative decomposition/electrophilic reactions with medium components [38,41]. Thus further studies will be focused on determining the

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net concentration of nitrated fatty acid derivatives in activated macrophages, and improving functional assays (i.e. by using phospholipid vesicles for nitroalkene administration). With these caveats in mind, and taking into account the ability of $CLNO_2$ and LNO_2 to inhibit pro-inflammatory gene expression and induce the expression of cytoprotective enzymes such as HO-1, the formation of lipid nitration derivatives during macrophage activation is viewed to act as an endogenous adaptative signalling mechanism that down-regulates cell oxidative responses and contributes to the control of inflammation.

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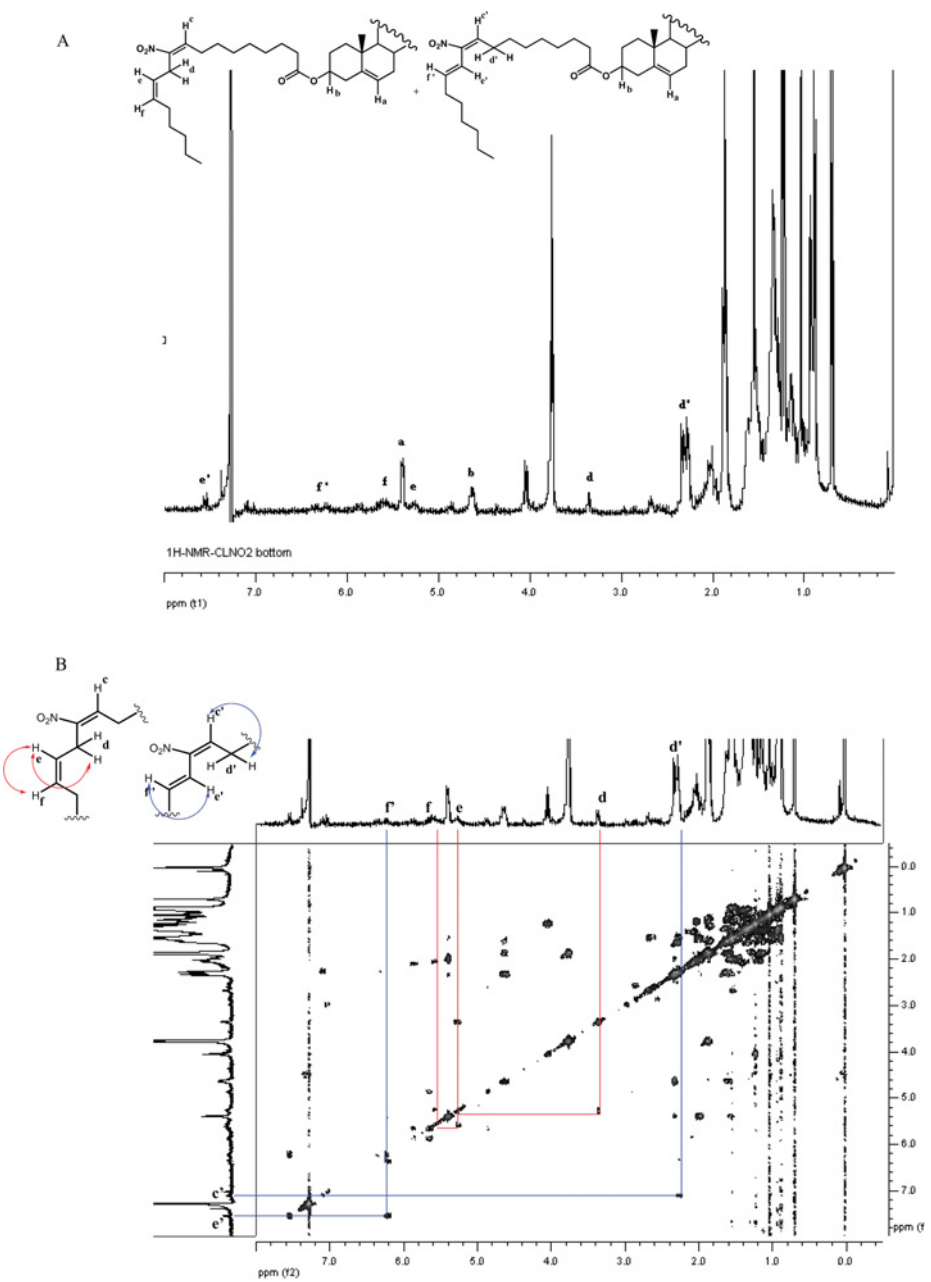
online data

SUPPLEMENTARY ONLINE DATA

Macrophage activation induces formation of the anti-inflammatory lipid cholesteryl-nitrolinoleate

Ana M. FERREIRA*, Mariana I. FERRARI*, Andrés TROSTCHANSKY†§, Carlos BATTHYANY†§, José M. SOUZA†, María N. ALVAREZ†, Gloria V. LÓPEZ‡, Paul R. S. BAKER§, Francisco J. SCHOPFER§, Valerie O'DONNELL‖, Bruce A. FREEMAN§[1] and Homero RUBBO†[1]

*Department of Immunology, Faculty of Sciences, University of the Republic, Montevideo 11400, Uruguay, †Department of Biochemistry, Center for Free Radical and Biomedical Research, Faculty of Medicine, University of the Republic, Montevideo 11800, Uruguay, ‡Department of Organic Chemistry, Faculty of Sciences, University of the Republic, Montevideo 11800, Uruguay, §Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, U.S.A., and ‖Department of Medical Biochemistry, University of Wales College of Medicine, Cardiff CF14 4XN, U.K.



[1] Correspondence may be addressed to either of these authors (email hrubbo@fmed.edu.uy or freerad@pitt.edu).

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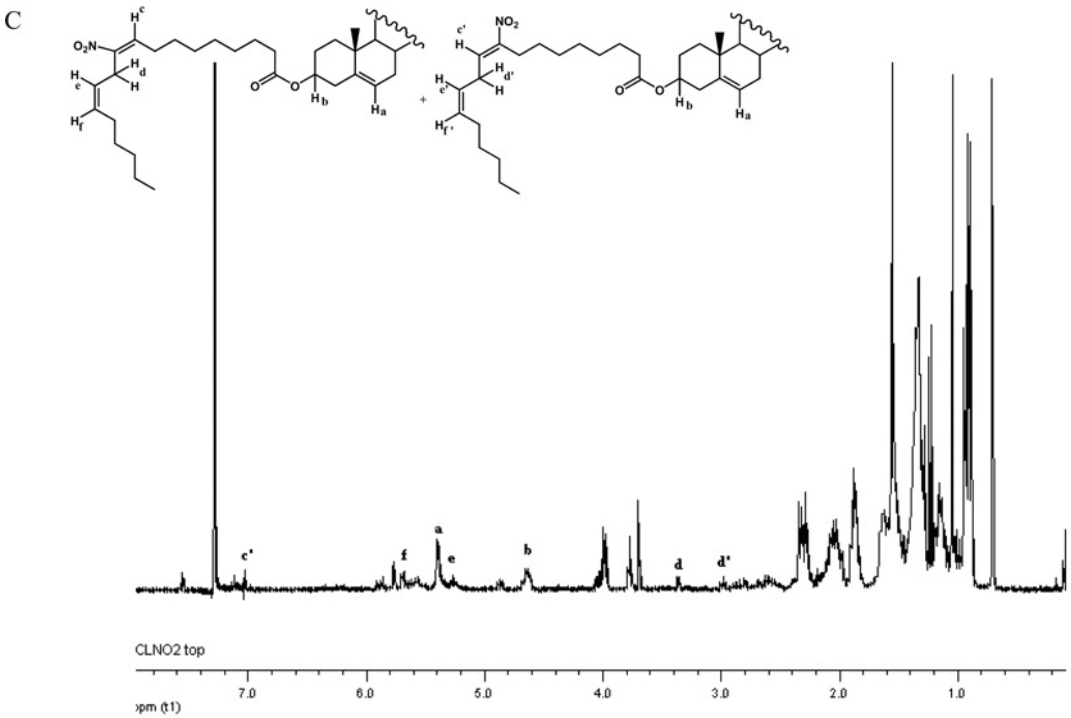

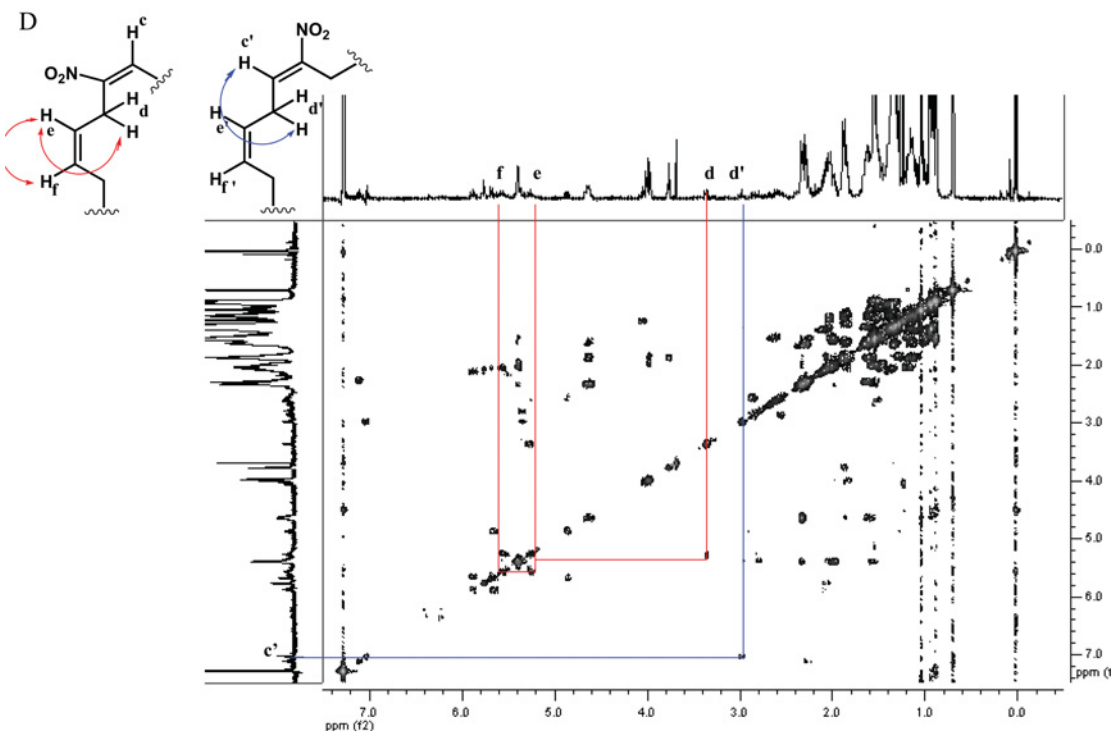

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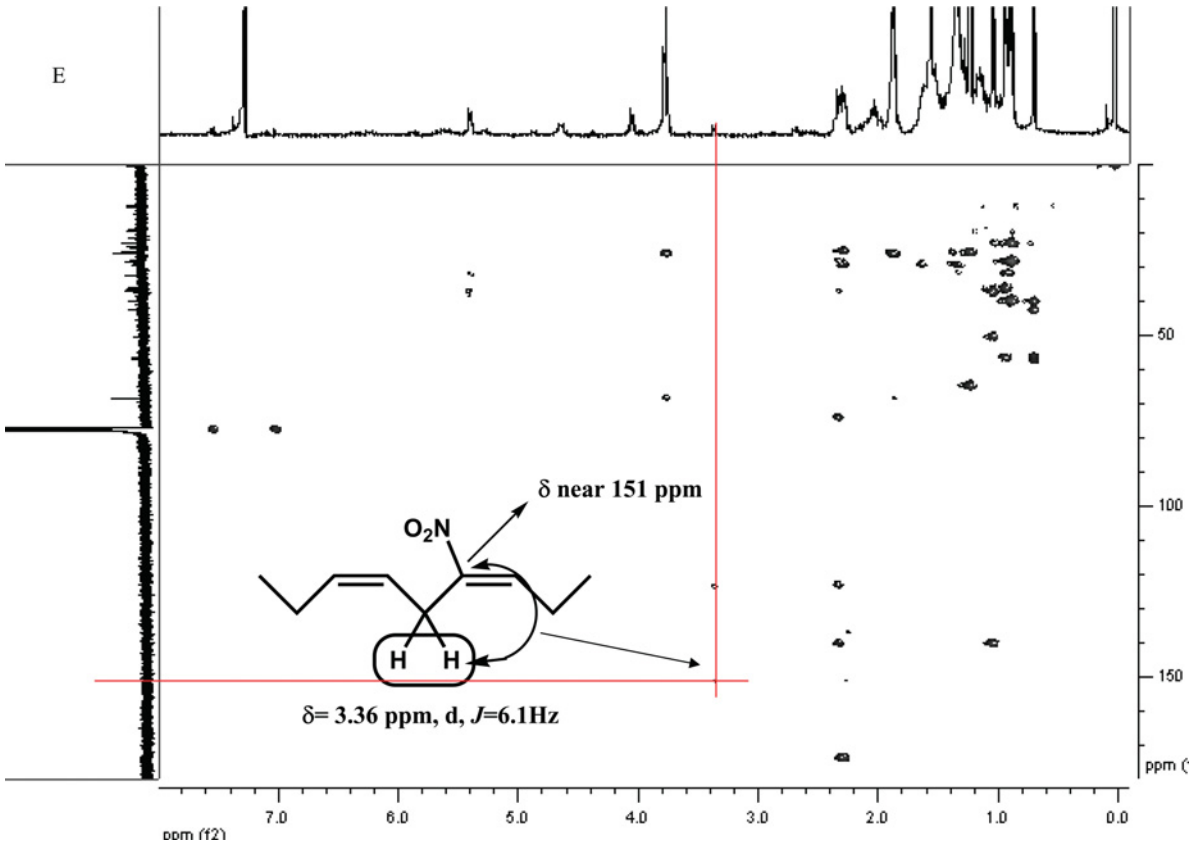

Figure S1 Spectroscopic data for $CLNO_2$bottom and $CLNO_2$top

The labelled chemical shift values assigned confirm that nitration occurred on the acyl moiety of CL and that $CLNO_2$bottom and $CLNO_2$top correspond to the nitroalkene isomers shown.

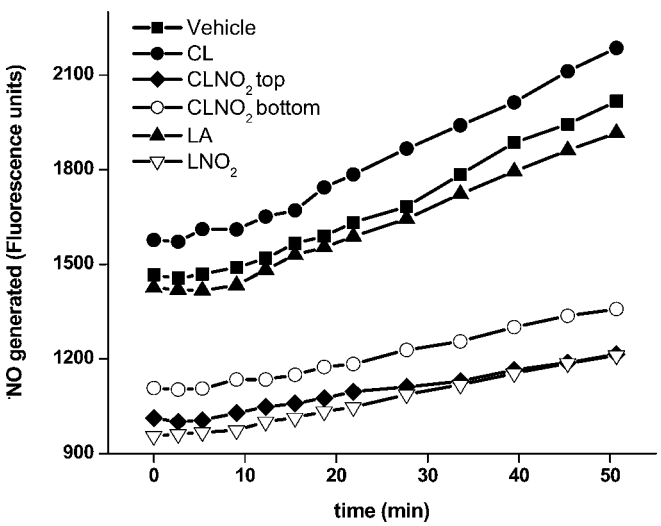

Figure S2 Effect of $CLNO_2$ isomers (top and bottom) on •NO generation by activated macrophages

Mouse J774.1 macrophages were activated with LPS in the presence of the indicated molecules. After 5 h incubation, •NO was measured by fluorimetry to evaluate NOS2 activity. The •NO generation (fluorescence units) was plotted against time ($CLNO_2$ and CL, 50 μM; LNO_2 and LA, 10 μM). Results are representative of three independent experiments.

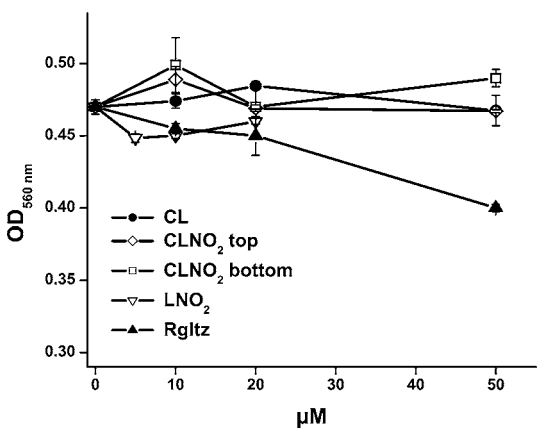

Figure S3 Effect of nitrated lipids on cell viability

THP-1 cells were activated with PMA in the presence of the indicated molecules as described in the Materials and methods section of the main paper. After 20 h incubation, cells were analysed for viability by measuring the mitochondrial-dependent reduction of MTT to formazan. Briefly, MTT was added to cells (final concentration 0.2 mg/ml) and cells were then incubated at 37 °C for 4 h. After removing the medium, formazan crystals were dissolved in DMSO and the absorbance at 570 nm was read using a microplate spectrophotometer. Results are expressed as the percentage of the control (stimulated cells in the presence of the vehicle), and correspond to the mean ± S.D. ($n = 3$).

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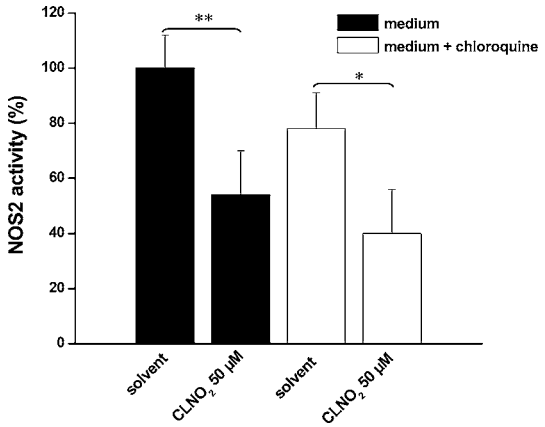

Figure S4 Effect of chloroquine on the ability of $CLNO_2$ to inhibit NOS2 expression by macrophages

Mouse J774.1 cells were pretreated (2 h) with chloroquine (20 μg/ml) and then stimulated with LPS (1 μg/ml) in the presence of CLNO2 (50 μM) or vehicle. After 5 h stimulation, NOS2 activity was measured following $^{\bullet}$NO generation by fluorimetry. Results are expressed as the mean $\pm$ S.D. ($n = 3$; $^{**}P < 0.01$, $^{*}P < 0.05$).

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