Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 125 – 132
Journal of Nutritional Biochemistry

Bioactivity of nitrolinoleate: effects on adhesion molecules and CD40–CD40L system☆

Luciane A. Faine[a,b], Danielle M.H. Cavalcanti[a], Martina Rudnicki[a], Simone Ferderbar[a], Sandra M.D. Macedo[a], Heraldo P. Souza[c], Sandra H.P. Farsky[a], Lisardo Boscá[b], Dulcineia Saes Parra Abdalla[a,*]

[a]Faculty of Pharmaceutical Sciences, Department of Clinical and Toxicological Analyses, University of São Paulo, Sao Paulo 05508-900, Brazil
[b]Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Madrid 28029, Spain
[c]Emergency Medicine Research Laboratory, University of São Paulo, Sao Paulo 01246-903, Brazil

Received 12 August 2008; received in revised form 3 November 2008; accepted 1 December 2008

Abstract

The vascular effects of nitrolinoleate (LNO_2), an endogenous product of linoleic acid (LA) nitration by nitric oxide-derived species and a potential nitrosating agent, were investigated on rat endothelial-leukocyte interactions. Confocal microscopy analysis demonstrated that LNO_2 was capable to deliver free radical nitric oxide ($^{\cdot}NO$) into cells, 5 min after its administration to cultured cells, with a peak of liberation at 30 min. THP-1 monocytes incubated with LNO_2 for 5 min presented nitrosation of CD40, leading to its inactivation. Other anti-inflammatory actions of LNO_2 were observed in vivo by intravital microscopy assays. LNO_2 decreased the number of adhered leukocytes in postcapillary venules of the mesentery network. In addition to this, LNO_2 reduced mRNA and protein expression of β2-integrin in circulating leukocytes, as well as VCAM-1 in endothelial cells isolated from postcapillary venules, confirming its antiadhesive effects on both cell types. Moreover, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, a nitric oxide scavenger, partially abolished the inhibitory action of LNO_2 on leukocyte-endothelium interaction, suggesting that the antiadhesion effects of LNO_2 involve a dual role in leukocyte adhesion, acting as a nitric oxide donor as well as through nitric oxide-independent mechanisms. In conclusion, LNO_2 inhibited adhesion molecules expression and promoted $^{\cdot}NO$ inactivation of the CD40–CD40L system, both important processes of the inflammatory response.

Keywords: Intravital microscopy; Adhesion molecules; CD40–CD40L system; Nitrated lipids; Linoleic acid

1. Introduction

Increasing evidence indicates that the CD40–CD40L interaction plays a pivotal role in inflammation [1,2]. Adhesion molecules also play an important role as effectors of the inflammatory reaction. Previous studies demonstrated that the CD40–CD40L interaction can induce a proinflammatory and prothrombotic response, demonstrated by liberation of proinflammatory cytokines and expression of adhesion molecules [3,4]. Costimulation with CD40L and interleukin 4 selectively induces expression of the vascular cell adhesion molecule VCAM-1 and P-selectin, resulting in preferential adhesion of lymphocytes [5]. Thus, the CD40–CD40L signaling system is gaining recognition as an important contributor in inflammation and cardiovascular diseases.

The CD40–CD40L blockade has been demonstrated to protect against radiation-induced pulmonary fibrosis [6] and other inflammatory diseases, such as atherosclerosis. Treatment with anti-CD40L antibodies reduced atherosclerotic lesions and adhesion molecules expression [7].

The adhesion molecules expression and leukocyte migration to inflammatory sites may be regulated by nitric oxide [8,9]. The free radical nitric oxide ($^{\cdot}NO$) is a chemical mediator released from the vascular endothelium known to control vascular smooth muscle tone and to regulate the inflammatory

☆ This study was supported by Fundação de Apoio à Pesquisa do Estado de São Paulo and Conselho Nacional de Desenvolvimento Científico e Tecnológico/Instituto do Milênio-Redoxoma. We thank Dr. Rui Curi and Mr. Marco Aurélio Ramirez for the flow cytometry analysis.

* Corresponding author. Tel.: +55 11 30913637; fax: +55 11 38132197.
E-mail address: dspa@usp.br (D.S.P. Abdalla).

0955-2863/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2008.12.004

response [10]. In inflammatory conditions, ·NO may be generated at elevated levels by inducible ·NO synthase, leading to the formation of potent nitrating and nitrosating species. These ·NO-derived reactive species may cause the nitration of endogenous lipids [11,12]. Nitrolinoleate (LNO_2) is an endogenous product of the nitration of linoleic acid, and previous studies demonstrated that LNO_2 plays a role in vasorelaxation and that this effect was related to its property as an ·NO donor [13]. However, other in vitro studies have considered the possibility that the effects of LNO_2 on cell signaling are due ·NO-independent mechanisms [14,15]. Moreover, there is no information on the in vivo biological effects of LNO_2, and it is still unclear whether LNO_2 combines both ·NO-dependent and ·NO-independent mechanisms.

Many studies on the therapeutic action of ·NO donors have been developed for their use as anti-inflammatory and anti-atherogenic drugs [16,17]. However, none of these compounds is endogenously produced as it is LNO_2. Recent investigations demonstrated that LNO_2 is physiologically stabilized in membrane lipid bilayers. Under inflammatory conditions, it is redistributed to the aqueous environment where this compound exerts its biological effects [18].

The biological actions of LNO_2 have been mainly demonstrated by in vitro studies. The in vivo approaches to study its anti-inflammatory properties are important to establish the mechanisms of action, as well as the possible clinical intervention strategies. In view of the lack of in vivo studies regarding the effects of LNO_2 on endothelium–leukocyte interactions in the inflammatory process and the importance of clarifying the mechanisms by which this lipid mediator exerts its action, the purpose of this study was to evaluate the in vivo LNO_2 activity on rat leukocyte–endothelium interactions and to analyze its ability to promote the nitrosation of key inflammatory proteins, such as the CD40–CD40L system.

2. Material and methods

2.1. Materials

The LNO_2 was synthesized in our lab according to LIMA et al. [12]. Briefly, LNO_2 was obtained by the reaction of linoleic acid with NO_2BF_4. The products were extracted with ether and purified by high-performance liquid chromatography (HPLC), as previously described [12]. The eluted fractions were analyzed by mass spectrometry and that presenting the highest concentration of purified LNO_2 was evaporated under nitrogen, diluted in ethanol and frozen at −80°C. A mixture of LNO_2 isomers was used in the experiments. All solvents were HPLC grade purchased from Merck, São Paulo, Brazil. The linoleic acid and the anti-rabbit CD40 antibody were from Sigma, St Louis, MO, USA. The monoclonal antibodies for β2 integrin and VCAM were purchased from BD Pharmingen, San Jose, CA, USA. The polymerase chain reaction (PCR) kit assay was purchased from Promega, Madison, WI, USA. All other reagents were purchased from Sigma.

2.2. In vitro experiments

The human leukemia cell line THP-1 monocytes were grown in RPMI media containing 10% FCS supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°.

2.2.1. Confocal microscopy

The ·NO liberation from LNO_2 molecule was assessed by confocal microscopy (Carl Zeiss). Human THP-1 cells, which have low nitric oxide synthase (NOS) expression [19], were differentiated along the monocytic lineage following exposure to phorbol-12-myristate-13-acetate (10^{-8} M), pretreated with N^G-monomethyl-L-arginine (500 μM) for 1 hour and incubated with specific green fluorescent probe, the 4,5-diaminofluorescein diacetate (DAF-2DA), which is a cell membrane-permeable precursor of DAF-2 (4,5-diaminofluorescein), utilized to measure intracellular NO concentration. THP-1 cells were loaded with DAF-2DA for 30 minutes in the absence of light and were washed three times with phosphate-buffered saline (PBS) before the assay. Once inside the cells, the diacetate group is cleaved by intracellular esterases, and *DAF-2* is trapped inside the cells. In the presence of NO and oxygen, a relatively nonfluorescent DAF-2 is transformed into the highly green fluorescent triazole form, diaminofluorescein-2 (DAF-2T). Thus, the increases in triazole form of DAF-2T fluorescence is a reliable indicator of the increases of intracellular NO concentration inside the cytoplasm of cells that received LNO_2 [20]. The excitation and emission wavelength were 488 and 510 nm, respectively. Multiple exposure images were acquired at different times after the addition of LNO_2 (10 μM) or vehicle to the cells. Image analysis was performed by LSM Image Examiner (Carl Zeiss). The concentration of ·NO released from LNO_2 was also determined by chemiluminescence in the culture medium in the absence of cells treated with LNO_2 (10 μM) at different time intervals.

2.2.2. Biotin switch

THP-1 cells were incubated with the vehicle ethanol, LA (10 μM) and LNO_2 (10 μM) for 5 min and then collected for biotin switch assay, where the *S*-nitrosylated proteins are biotinylated, as described by Jaffrey and Snyder [21]. Biotin switch controls without 3-*N*-(maleimidopropionil)-biocytin or ascorbate were performed to eliminate interference with artifacts dependent of these substances. The biotinylated proteins were detected by immunoprecipitation with streptavidin agarose and Western blot for CD40 receptor (anti-rabbit CD40). Densities were analyzed by Image J software.

2.3. In vivo experiments

The Ethics Committee for Conduction of Animal Studies of the Faculty of Pharmaceutical Sciences, University of São Paulo, approved the experimental protocol. Adult male Wistar rats (250–300 g) were allowed a standard pellet diet and water ad libitum.

The doses of the LNO_2 (0.3 mg/kg) and LA (0.24 mg/kg) administered subcutaneously to the rats were estimated corresponding to an elevation of 10 μM in the total blood concentration. The final volume of injection was set at 40 μL. Since LNO_2 has higher stability in organic solvents, ethanol was chosen as vehicle. The nitric oxide scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) was subcutaneously injected corresponding to a final concentration of 100 μM in total blood.

2.3.1. Intravital microscopy assay

Animals were divided into 6 groups which received the following treatments: Control group (vehicle), LNO_2 (10 μM), LA (10 μM), LNO_2 (3 μM), LA (3 μM) and a final group which received LNO_2 (10 μM) and carboxy-PTIO (100 μM). Carboxy-PTIO was injected 30 min before the administration of LNO_2 (10 μM). All substances used for treatment were subcutaneously injected in the rats 1 h before the intravital microscopy assay. Rats were anesthetized (sodium pentobarbital, 65 mg/kg i.p.) and the mesentery was exteriorized. The animals were kept on a special board, which included a transparent platform on which the tissue to be transilluminated was placed. The preparation was kept moist and warmed by irrigating the tissue with Ringer-Locke solution (pH 7.4; 154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 6 mM $NaHCO_3$ and 5 mM glucose) containing 1% gelatin. Transilluminated images were obtained by optical microscopy (Axyoplan II, Carl-Zeiss equipped with x5.0/0.30 plan Neofluar or 10.0/0.25 Achroplan longitudinal distance objectives/numeric aperture and ×1.0, ×1.25 or ×1.60 optovar). The images were captured by a video camera (ZVS, 3C75DE, Carl-Zeiss) and were transmitted simultaneously to a TV monitor and a computer. Digitalized images on the computer monitor were subsequently analyzed by image-analyzing software (KS300, Kontron).

2.3.2. Assessment of leukocyte-endothelial interactions

The interaction of leukocytes and vessel walls was evaluated by determining the number of rolling and adhered leukocytes of the postcapillary venule wall (diameter, 20-30 μm; length, 200 μm) of the mesentery at 10-min intervals before and after topical administration of a proinflammatory stimulus, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP 10^{-8} M), a chemoatractant that acts directly on leukocytes to induce activation and adhesion. The result was expressed as the difference between the numbers of leukocyte rollers or leukocytes adhered after and before fMLP application. Three fields of each animal were evaluated. Leukocytes moving in the periphery of the axial stream, in contact with the endothelium, were considered to be rollers. The number of leukocytes adhering to the endothelium (stopped at the vessel wall) was determined in the same vascular segment [22,23].

2.3.3. Leukocyte β_2-integrin expression by flow cytometry

Blood was collected from the abdominal aorta (EDTA 100 mg/mL) 1 h after the following treatments: vehicle, 10 μM LNO_2 or 10 μM LA. Blood was immediately submitted to erythrocyte lysis using an ammonium chloride solution (0.13 M). Leukocytes were recovered after washing with Hanks Balanced Salt Solution. Cells (1×10^{-6}) were incubated with fMLP (10^{-9}M for 30 min). After washing, leukocytes were further incubated for 30 min at 4°C in the dark with 10 μL monoclonal antibody against β_2-integrin (BD Pharmingen, cat. 554979). Immediately after incubation, cells were analyzed with a FACScalibur flow cytometer (Becton & Dickinson, San Jose, CA, USA). Data from 10,000 cells were obtained and only morphologically viable leukocytes were considered for analysis. Results were expressed as fluorescence units.

2.3.4. VCAM-1 expression by immunohistochemistry

Animals were subcutaneously injected with vehicle, 10 μM LNO_2 or 10 μM LA, 1 h before i.p. injection of lipopolysaccharide (LPS; 5 mg/kg). Twelve hours after LPS injection, animals were anesthetized by i.p. injection of ketamine/xylazine (90/13 mg/kg) and testes were collected for quantification of VCAM-1 expression in postcapillary venules. Sections of 8 μm were obtained for subsequent immunohistochemistry assay. The slides were warmed at room temperature for 2 h and immunohistochemistry steps were performed as follows: primary antibody biotin-conjugated mouse anti-rat CD106 (VCAM-1; BD PharMingen, cat. 559229) at 4°C overnight, streptavidin-peroxidase complex for 1 hour at room temperature, chromogen diaminobenzidine (DAB) (1 mL/100 mL PBS-H_2O_2) for 40 s, and counterstain (hematoxylin) for 15 s, followed by ethanol and xylol for 5 min. Negative controls had the primary antibody replaced by goat IgG. Immunohistochemical analysis was performed by microscopy and digitized images on the computer monitor were subsequently analyzed by image-analyzing software (KS300, Kontron).

2.3.5. Quantification of leukocyte β_2-integrin or VCAM-1 mRNA levels

2.3.5.1. Microvascular endothelial primary cultures. Primary cultures of endothelial cells were obtained from the rat cremaster muscle using the method described by Chen et al. [24]. The cells were subcultured and used for VCAM-1 mRNA level assay. The endothelial identification was performed by adding monoclonal antibody against platelet endothelial cell adhesion molecule, PECAM-1.

For the mRNA level analysis, cells received the stimulation treatments: vehicle, 10 μM LNO_2, 10 μM LA, 10 μM LNO_2 plus C-PTIO 100 μM for 1 h before LPS (2 mg/mL). After 12 hours of LPS administration, the total RNA was extracted following Trizol method for VCAM-1 assay. RNA quantification was done by reading the absorbance at 260 nm.

2.3.5.2. Polymorphonuclear cells (PMN) cell-enriched leukocyte preparation. Blood was collected from the abdominal aorta of rats previously treated (1 h before,

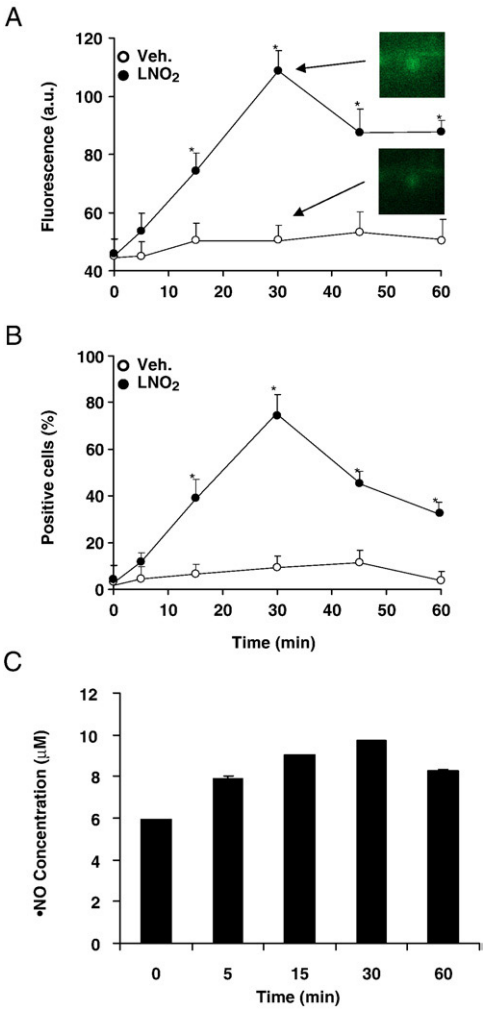

Fig. 1. Concentration of ˙NO in THP-1 monocytes treated with LNO_2 and ˙NO liberation from LNO_2 in culture medium. Cells were pre-incubated with the probe DAF-2 and were treated with 10 μM LNO_2. The images were obtained at the indicated times after LNO_2 or vehicle administration. Results are presented by units of fluorescence (A) or as percentage of positive cells considering 45 U of fluorescence as negative cells (B). LNO_2 10 μM was administered to culture medium without cells and the NO concentration was measured at different time intervals by chemiluminescence (C). Results show the mean±S.D. of four experiments; *P<.01 vs. the vehicle condition.

subcutaneously) with vehicle, LA (10 μM) or LNO_2 (10 μM) using 2% EDTA. Cell separation was performed by adding 3 mL Percoll (56% in sterile PBS) to 5-mL blood samples. After centrifugation (1000×g, 40 min), the interface containing mainly PMN cells was collected, and erythrocyte lysis was promoted by adding lysis solution (8.02 g/L NH_4Cl, 0.84 g/L $NaHCO_3$).

For β_2-integrin analysis, neutrophils from treated animals were stimulated with fMLP (10^{-9} M for 30 min), and total RNA was extracted with Trizol in RNAse-free environment.

2.3.5.3. Reverse transcriptase-polymerase chain reaction. cDNA synthesis and chain polymerase reaction were performed according to a previous study [25]. The cDNA amplification for β2-integrin, VCAM-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed at 92°C for denaturation; 54°C, 57°C and 55°C for annealing, respectively; and 72°C for extension, performed in 30 cycles. The used primers were GAPDH sense 5′TATGATGACATCAAGAAGGTGG'3, GAPDH antisense 5′CACCACCCTGGTGCTGTA'3; β_2-integrin sense 5′TGCGCCCCTCACTGCTGCTTG 3′ β_2-integrin antisense 5′GAGATCCAT GAGGTAGTACAGATC 3′ and VCAM-1 antisense 5′ ACCGTGCAGTTGACATGAC 3′. PCR products were detected on 2% agarose electrophoresis gel stained with ethidium bromide using 100- or 50-bp ladders as nucleotide size markers. A FLA-3000 scanner (Fuji Film) scanned the gels, and the densitometric analyses were performed using the Image J Software. The level of each expression was normalized by GAPDH.

2.4. Statistical analysis

Means and standard deviation are presented and were compared using analysis of variance with Tukey's test. The level of significance was set at .05.

3. Results

3.1. ˙NO release from LNO2 molecule and nitrosation of CD40

Five min after LNO_2 addition to the culture medium it was possible to detect ˙NO into the cells by confocal microscopy analysis (Fig. 1A,B). The maximum liberation was achieved at 30 min and more than 75% of the cells exhibited an NO increase. The NO liberation was also performed in the culture medium without cells by chemilu-

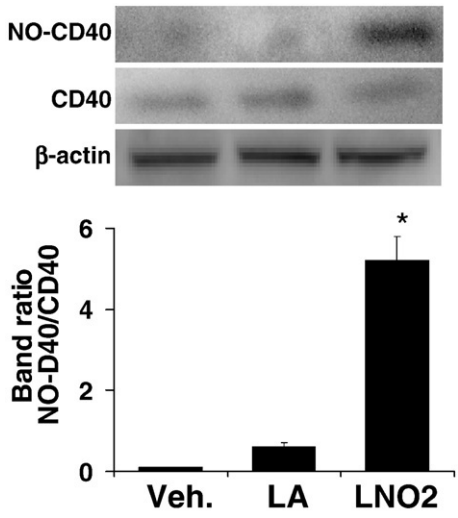

Fig. 2. Nitrosation of CD40 by LNO_2 in THP-1 cells. Cells were incubated with 10 μM LNO_2 10 μM LA or vehicle for 5 min and recovered for biotin switch assay. Data are presented as mean±S.D. of three experiments. *P<.001 vs. vehicle and LA groups.

minescence and the liberation of ·NO exhibited the same pattern with a maximum concentration achieved at 30 min (Fig. 1C). The nitrosation of CD40 was measured after 5 min of LNO_2 administration to THP-1 cells by the biotin switch method. The results obtained demonstrated that LNO_2 induced CD40 nitrosation, which was not observed when LA or vehicle were used (Fig. 2).

3.2. Leukocyte-endothelial interactions in vivo

Topical application of fMLP increased the number of rolling leukocytes measured by intravital microscopy. Treatments with LNO_2 (10 or 3 μM), LA (10 or 3 μM) or LNO_2 + C-PTIO significantly reduced the number of these cells with respect to values found in control animals (Fig. 3A). However, the number of adhered leukocytes after fMLP application was decreased by LNO_2 (10 or 3 μM)

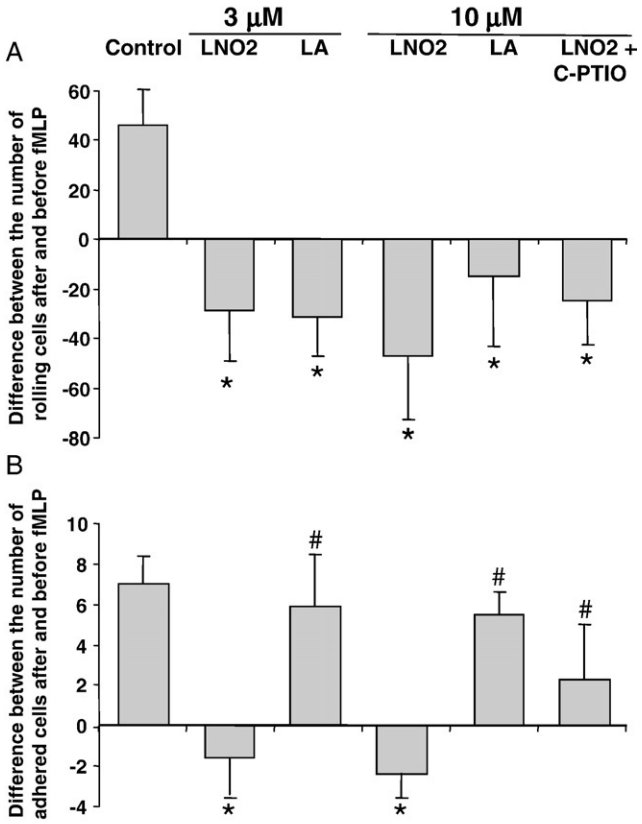

Fig. 3. Inhibitory effect of LNO_2 on leukocyte-endothelial interactions in vivo. Wistar rats were divided into six groups which received the following treatments: control group (vehicle), LNO_2 (10 μM), LA (10 μM), LNO_2 (3 μM), LA (3 μM) and a final group which received LNO_2 (10 μM) and carboxy-PTIO (100 μM). Carboxy-PTIO was injected 30 min before the administration of LNO_2 (10 μM). All substances used for treatment were subcutaneously injected in the rats 1 hour before the intravital microscopy assay. Results are expressed as the difference between the number of leukocyte rollers or adhered after and before fMLP application. Three fields of each animal were evaluated. (A) Effect of treatments on the number of rollers in the rat mesentery. (B) Effect of treatment on the number of adhered leukocytes in the rat mesentery. Results are expressed as means±S.D. of six animals in each group.*P<.05 vs. control group; #P<.05 vs. respective LNO_2 doses.

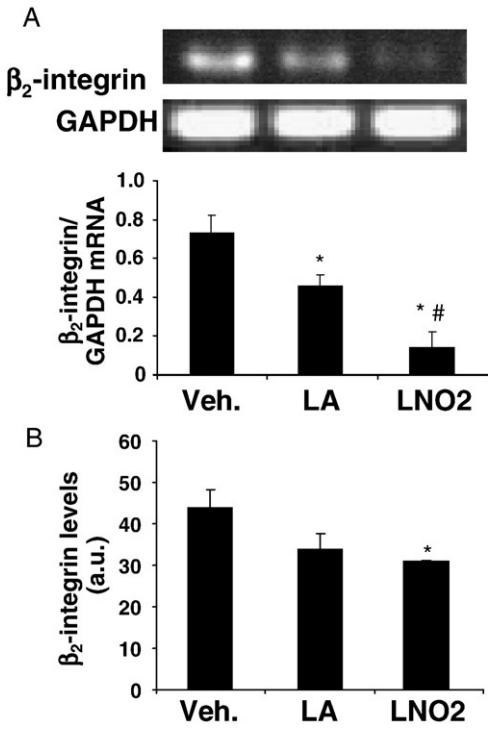

Fig. 4. Inhibitory effect of LNO_2 on β2 integrin mRNA and protein levels. Cells were collected from vehicle, LNO_2 (10 μM) and LA (10 μM)-pretreated animals. (A) Diminished expression of β2 integrin mRNA levels on stimulated leukocytes from animals treated with LNO_2. Representative gel of PCR products corresponding to β_2 integrin and GAPDH levels. Neutrophils from treated animals were separated by Percoll method and stimulated with fMLP, (10^{-9} M for 30 min). Agarose gel was visualized under ultraviolet light using the fluorescent properties of ethidium bromide and scanned using FLA 3000 scanner (Fuji film). (B) Diminished expression of β2 integrin on stimulated leukocytes from animals treated with LNO_2. Blood was collected from the abdominal aorta 1 hour after the treatments. Then, after erythrocytes lysis the leukocytes were recovered and incubated with fMLP; 10^{-9} M for 30 min. After washing, leukocytes were further incubated for 30 min at 4°C in the dark with the respective monoclonal antibodies. Immediately after incubation, cells were analyzed with a FACScalibur flow cytometer. Experiments were performed in triplicate. Results are expressed as means±S.D.*P<.05 vs. vehicle group; #P<.05 vs. LA group.

but not by LA (10 or 3 μM). A stronger antiadhesive effect was observed in LNO_2 groups (LNO_2 10 μM; 120% compared with control group). C-PTIO partially inhibited the anti-adhesive action of LNO_2 (Fig. 3B).

3.3. Expression of leukocyte and endothelial adhesion molecules

Reverse transcriptase-PCR assays showed impairment of β_2-integrin expression, as the levels of the corresponding mRNA were reduced in leukocytes collected from LNO_2-treated rats (Fig. 4A). This result was reflected in a lower membrane expression of β_2-integrin (22% vs. vehicle group) in LNO_2-treated animals (Fig. 4B).

VCAM-1 was quantified in cultured cells obtained form naïve rats and incubated for 1 h with LPS, vehicle, LNO_2,

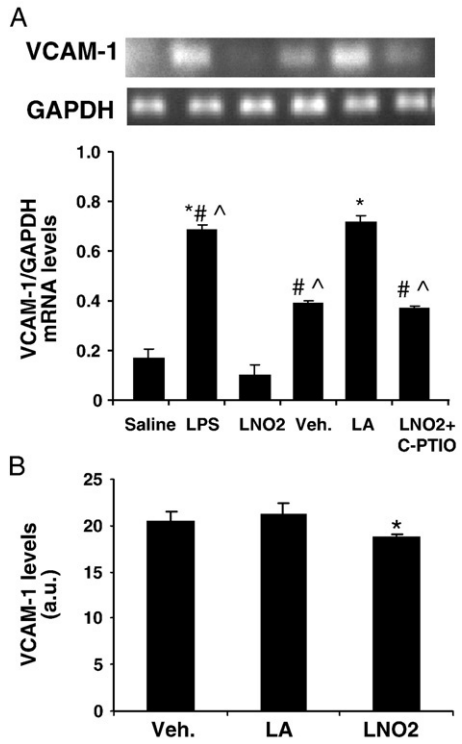

Fig. 5. Inhibitory action of LNO_2 on VCAM-1 expression on endothelial cells of treated animals and synthesis on primary culture of treated endothelial cells. (A) Representative gel of PCR products showing the VCAM-1: GAPDH expression ratio in primary culture of treated endothelial cells. The endothelial cells were treated with vehicle, LNO_2 and LA, and 1 h later, LPS (2 mg/ml) was added to the medium. The RNA extraction was performed after 12 h of LPS stimulus. Agarose gel was visualized under ultraviolet light using the fluorescent properties of ethidium bromide and scanned using FLA 3000 scanner (Fuji film). Experiments were performed in triplicate. Results are expressed as means±S.D. *P<.05 vs. vehicle group; #P<.05 vs. LA group; ^P<.05 vs. LPS group. (B) DAB-immunohistochemistry for VCAM-1 of testis microvasculature endothelium of treated rats. LNO_2 and LA were used at 10 μM and LPS (5 mg/kg) was injected 1 h after vehicle, LNO_2 and LA administration. Twelve hours after LPS injection, animals were anesthetized, and testis were collected, frozen and processed for immunohistochemistry assay. *P<.05 vs. vehicle and LA groups.

LA or LNO_2 plus C-PTIO. At the end of the treatment, cells were stimulated with LPS, and the expression of VCAM-1 was quantified at 12 h. The expression of VCAM-1 mRNA was reduced by the administration of LNO_2. Interestingly, cells stimulated with LNO_2 + C-PTIO exhibited a suppression of the inhibitory effect elicited by LNO_2 but failed to show VCAM-1 mRNA levels in the range of LPS (Fig. 5A). Expression of VCAM-1 on endothelial cells from postcapillary venules was quantified in the cremaster muscle from treated animals and locally stimulated with LPS. These results demonstrated equivalent expressions in cells from vehicle or LA-treated rats. According to the observed diminished mRNA expression, VCAM-1 from LNO_2-treated rats was significantly lower (18% compared with vehicle) than that detected in other groups of animals (Fig. 5B).

4. Discussion

Nitrolinoleate is a novel lipid derivative identified in blood plasma and cells that has cell signaling properties [18,26]. Some of the biological effects of LNO_2 include inhibition of platelet activation, superoxide generation and degranulation by human neutrophils in vitro [14]. Although these studies have not considered a role for ˙NO in the effects of LNO_2, the present work demonstrates that this lipid derivative may act also as a ˙NO donor. Confocal microscopy showed ˙NO liberation from LNO_2 molecule after 5 min of administration, with a peak of liberation after 30 min (Fig. 1A).

The readiness of the LNO_2 action was observed in the biotin switch assay for CD40, a key protein of the inflammatory process. Within 5 min of incubation, it was possible to observe the nitrosation of CD40 in cells that received LNO_2. These observations are important for future therapeutic applications for LNO_2, since CD40 nitrosation leads to its inactivation and blockade of inflammation. Moreover, CD40 signaling system inactivation has been associated with decreased adhesion molecules and leukocyte migration [7,27].

Recent studies revealed other approaches for the anti-inflammatory action of LNO_2. LNO_2 has been shown as a specific ligand for peroxisome proliferator-activated receptor γ (PPARγ) [18]. PPARs are members of the nuclear hormone receptor superfamily of transcription factors that have anti-inflammatory properties. Diminished levels for intercellular adhesion molecule (ICAM-1), VCAM-1 and α4β7 integrin were reported in endothelial cells treated with the PPARγ agonist troglitazone [28]. Thus, PPARγ agonists were shown to inhibit several events in inflammation, particularly leukocyte-endothelial interactions mediated by nuclear factor κB (NF-κB)-dependent adhesion molecule expression [29,30]. Furthermore, recent investigations have demonstrated that LNO_2 induced nitroalkylation of the recombinant NF-κB p65 protein [31]. Thus, NF-κB-dependent target gene expression, such as adhesion molecules, may be inhibited by LNO_2.

Since these anti-inflammatory effects observed for LNO_2 were not tested in vivo, intravital microscopy assays were performed in rats and demonstrated that administration of LNO_2 (3 and 10 μM) for a period of 1 h had specific and major effects on leukocyte adhesion. Both concentrations of LNO_2 decreased leukocyte adherence to the mesenteric postcapillary venule wall compared to vehicle and LA. Although LA showed a decreased number of adhered leukocytes compared to the vehicle, LNO_2 presented the most potent action, suggesting that this effect of LNO_2 could be due to an intrinsic property of this molecule and/or its ability to act as a nitric oxide donor. In order to clarify this issue, a group of animals received the ˙NO scavenger carboxy-PTIO and LNO_2 (10 μM). The effects of LNO_2 on rolling were not altered by carboxy-PTIO, revealing that these LNO_2 effects may be ˙NO-independent. However, the anti–cell adhesion effects of LNO_2 were partially inhibited

by carboxy-PTIO, suggesting the contribution of both ˙NO-dependent and ˙NO-independent mechanisms to modulate this process. This dual role of LNO_2 in the regulation of neutrophil adhesion has not been described previously in in vitro experiments. In a previous study, the expression of the integrin CD11b on neutrophils was decreased by LNO_2 through activation of adenylate cyclase, suggesting no participation of ˙NO [14]. Notwithstanding, in vivo experiments are characterized by the concurrence of many biological variables, and therefore, it is reasonable to assume that LNO_2 may be acting also as a nitric oxide donor, as described in the present study. There is considerable evidence that ˙NO is an endogenous modulator of leukocyte adhesion, since incubation of neutrophils with ˙NO inhibitors showed up-regulated expression of β_2-integrin [32]. Banick et al. [33] demonstrated that ˙NO decreased neutrophil β_2-integrin function by inhibiting membrane associated cyclic guanosine 3′,5′-phosphate (GMP) synthesis. Integrins are a family of membrane glycoproteins which mediate cell adhesion and a wide variety of biological functions. The firm adhesion of leukocytes to the endothelium occurs via binding of neutrophil β_2-integrins to endothelial ICAM-1 [34,35]. In the present study, it was observed that LNO_2 diminished the leukocyte adhesiveness by decreasing β_2-integrin in leukocytes of treated animals. Although LNO_2 has completely inhibited β_2-integrin synthesis during the period of 1 h, the flow cytometry assay indicated that the protein was already expressed before LNO_2 action. Furthermore, LNO_2 inhibited in vivo VCAM-1 expression in LPS-treated animals. Inhibition of VCAM-1 mRNA levels was observed in primary culture of nitrolipid treated endothelial cells, suggesting that LNO_2 may inhibit VCAM-1 protein expression through protein synthesis modulation. In addition to this, it is interesting to note that the effect of LNO_2 on VCAM-1 was partially delayed when LNO_2 was administered in the presence of the ˙NO scavenger, confirming the contribution of ˙NO to the LNO_2 antiadhesive effects as observed by intravital microscopy. Moreover, the β_2-integrin and VCAM-1 modulation by LNO_2 resulted from specific characteristics of the LNO_2 molecule, since LA was unable to cause a similar inhibition on these adhesion molecules.

Our results clearly demonstrate that dorsal subcutaneous injection of LNO_2 had a systemic effect 1 h after injection, observed in the mesentery network. The intravital microscopy assay performed in the mesentery microcirculation revealed effects that were dose-dependent (3 and 10 μM). These results indirectly demonstrated that the administration of LNO_2 into rats increased its basal concentrations in the plasma, since a broad spectrum of anti-inflammatory effects, including decrease of mRNA and protein expression of adhesion molecules, was observed following LNO_2 administration.

The mechanisms whereby LNO_2 acts to inhibit leukocyte-endothelial interactions are likely to be multifactorial, involving interference with a wide range of transcription factors to modulate cell adhesion molecule synthesis and activity. Moreover, LNO_2 mechanisms included nitrosation and subsequent inactivation of CD40–CD40L system. This broad spectrum of action of this lipid derivative opens new approaches for clinical interventions. Notwithstanding, the lack of information concerning LNO_2 biological properties make further investigations on this emerging compound necessary.

References

[1] Lip GYH, Patel JV, Hughes E, Hart RG. High-sensitivity C-Reactive protein and soluble CD40 ligand as indices of inflammation and platelet activation in 880 patients with nonvalvular atrial fibrillation. Stroke 2007;38(4):1229–37.

[2] Phipps RP. Atherosclerosis: the emerging role of inflammation and the CD40–CD40 ligand system. PNAS 2000;97:6030–2.

[3] Aukrust P, Muller F, Ueland T, Berget T, Aaser E, Brunsvig A, et al. Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Circulation 1999;100:614–20.

[4] Urbich C, Dernbach E, Aicher A, Zeiher AM, Dimmeler S. CD40 ligand inhibits endothelial cell migration by increasing production of endothelial reactive oxygen species. Circulation 2002;106:981–6.

[5] Kotowicz K, Dixon GLJ, Klein NJ, Peters MJ, Callard RE. Biological function of CD40 on human endothelial cells: costimulation with CD40 ligand and interleukin-4 selectively induces expression of vascular cell adhesion molecule-1 and P-selectin resulting in preferential adhesion of lymphocytes. Immunology 2000;100:441–8.

[6] Adawi A, Zhanga Y, Baggs R, Rubind P, Williamsd J, Finkelstein J, et al. Blockade of CD40–CD40 ligand interactions protects against radiation-induced pulmonary inflammation and fibrosis. Clin Immunol Immunopathol 1998;89(3):222–30.

[7] Mach F, Schoenbeck U, Sukhova G, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signaling. Nature 1998; 394:200–3.

[8] Jiang MZ, Tsukahara H, Ohshima Y, Todoroki Y, Hiraoka M, Maeda M, et al. Effects of antioxidants and nitric oxide on TNF-alpha induced adhesion molecule expression and NF-kappa B activation in human dermal microvascular endothelial cells. Life Sci 2004;75(10): 1159–70.

[9] Moreno SE, Alves-Filho JC, Bertozi G, Alfaya TM, Theze J, Ferreira SH, et al. Systemic administration of interleukin-2 inhibits inflammatory neutrophil migration: role of nitric oxide. Brit J Pharmacol 2006; 148(8):1060–6.

[10] Rekka EA, Chrysselis MC. Nitric oxide in atherosclerosis. Mini Rev Med Chem 2002;2(6):585–93.

[11] O'Donnell VB, Freeman BA. Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease. Circ Res 2001;88(1):12–21.

[12] Lima ES, Di Mascio P, Rubbo H, Abdalla DS. Characterization of linoleic acid nitration in human blood plasma by mass spectrometry. Biochem 2002;41(34):10717–22.

[13] Lima ES, Bonini MG, Augusto O, Barbeiro HV, Souza HP, Abdalla DS. Nitrated lipids decompose to nitric oxide and lipid radicals and cause vasorelaxation. Free Rad Biol Med 2005;39(4):532–9.

[14] Coles B, Bloodsworth A, Clark SR, Lewis MJ, Cross AR, Freeman BA, et al. 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 2002;91(5):375–81.

[15] Schopfer FJ, Lin Y, Baker PR, Cui T, Garcia-Barrio M, Zhang J, et al. Nitrolinoleic acid: an endogenous peroxisome proliferator-activated receptor gamma ligand. Proc Natl Acad Sci U S A 2005;102(7): 2340–5.

[16] Napoli C, Cirino G, Del SP, Sorrentino R, Sica V, Condorelli M, et al. Effects of nitric oxide-releasing aspirin versus aspirin on restenosis in

hypercholesterolemic mice. Proc Natl Acad Sci U S A 2001;98(5): 2860–4.
[17] Krieger MH, Santos KF, Shishido SM, Wanschel AC, Estrela HF, Santos L, et al. Antiatherogenic effects of *S*-nitroso-*N*-acetylcysteine in hypercholesterolemic LDL receptor knockout mice. Nitric Oxide 2006; 14(1):12–20.
[18] Shopfer FJ, Baker PRS, Giles G, Chumley P, Batthyany C, Crawford J, et al. Fatty acid transduction of nitric oxide signaling: nitrolinoleic acid is hidrofobically stabilized nitric oxide donor. J Biol Chem 2005;280 (19):19289–97.
[19] Albina JE. On the expression of nitric oxide synthase by human macrophages. Why no NO? J Leukoc Biol 1995;58(6):643–9.
[20] Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, et al. Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. Anal Chem 1998;70(13): 2446–53.
[21] Jaffrey SR, Snyder SH. The biotin switch method for the detection of *S*-nitrosylated proteins. Sci STKE 2001;86:PL1.
[22] Farsky SP, Sannomiya P, Garcia-Leme J. Secreted glucocorticoids regulate leukocyte-endothelial interactions in inflammation. A direct vital microscopic study. J Leukoc Biol 1995;57(3):379–86.
[23] Farsky SH, Borelli P, Fock RA, Proto SZ, Ferreira JM, Mello SB. Chronic blockade of nitric oxide biosynthesis in rats: effect on leukocyte endothelial interaction and on leukocyte recruitment. Inflam Res 2004;53(9):442–52.
[24] Chen SF, Fei X, Li SH. A simple method for isolation of microvascular endothelial cells avoiding both chemical and mechanical injuries. Microvasc Res 1995;50(1):119–28.
[25] Cavalcanti DMH, Lotufo CMC, Borelli P, Tavassi AMC, Pereira ALM, Markus RP, et al. Adrenal deficiency alters mechanisms of neutrophils mobilization. Mol Cell Endocrinol 2006;249(1-2):32–9.
[26] Lima ES, Di Mascio P, Abdalla DS. Cholesteryl nitrolinoleate, a nitrated lipid present in human blood plasma and lipoproteins: Synthesis, characterization, and detection by HPLC coupled to mass spectrometry. J Lipid Res 2003;44(9):1660–6.
[27] Lei XF, Ohkawara Y, Stämpfli MR, Mastruzzo C, Marr RA, Snider D, et al. Disruption of antigen-induced inflammatory responses in CD40 ligand knockout mice. J ClinInvest 1998;101(6):1342–53.
[28] Sasaki M, Jordan P, Welbourne T, Minagar A, Joh T, Itoh M, et al. Troglitazone, a PPARγ activator prevents endothelial cell adhesion molecule expression and lymphocyte adhesion mediated by TNF-α. Biomed Central Physiol 2005;5(1):1–12.
[29] Jackson SM, Parhami F, Xi XP, Berliner JA, Hsueh WA, Law RE, et al. Peroxisome proliferator-activated receptor activators target human endothelial cells to inhibit leukocyte-endothelial cell interaction. Arterioscler Thromb Vasc Biol 1999;19:2094–104.
[30] Chen NG, Han X. Dual function of troglitazone in ICAM-1 gene expression in human vascular endothelium. Biochem Biophys Res Comm 2001;282(1):217–21.
[31] Cui T, Schopfer FJ, Zhang J, Chen K, Ichikawa T, Baker PR, et al. Nitrated fatty acids: Endogenous anti-inflammatory signaling mediators. J Biol Chem 2006;281(47):35686–98.
[32] Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci U S A 1991;88 (11):4651–5.
[33] Banick PD, Chen Q, Xu YA, Thom SR. Nitric oxide inhibits neutrophil beta 2 integrin function by inhibiting membrane-associated cyclic GMP synthesis. J Cell Physiol 1997;172(1):12–24.
[34] Douglass WA, Hyland RH, Buckley CD, Al-Shamkhani A, Shaw JM, Scarth SL, et al. The role of the cysteine-rich region of the beta2 integrin subunit in the leukocyte function-associated antigen-1 (LFA-1, alphaLbeta2, CD11a/CD18) heterodimer formation and ligand binding. FEBS Lett 1998;440(3):414–8.
[35] Tan SM, Walters SE, Mathew EC, Robinson MK, Drbal K, Shaw JM, et al. Defining the repeating elements in the cysteine-rich region (CRR) of the CD18 integrin beta 2 subunit. FEBS Lett 2001;505(1):27–30.