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Novel Antiinflammatory Properties of Nitric Oxide–Derived Reactive Species in Vascular Cells

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Abstract—Nitration of unsaturated fatty acids such as linoleate by NO-derived reactive species forms novel derivatives (including nitrolinoleate [LNO_2]) that can stimulate smooth muscle relaxation and block platelet activation by either NO/cGMP or cAMP-dependent mechanisms. Here, LNO_2 was observed to inhibit human neutrophil function. LNO_2, but not linoleic acid or the nitrated amino acid 3-nitrotyrosine, dose-dependently (0.2 to 1 μmol/L) inhibited superoxide ($O_2^{\cdot-}$) generation, Ca^{2+} influx, elastase release, and CD11b expression in response to either phorbol 12-myristate 13-acetate or *N*-formyl-Met-Leu-Phe. LNO_2 did not elevate cGMP, and inhibition of guanylate cyclase by 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one did not restore neutrophil responses, ruling out a role for NO. In contrast, LNO_2 caused elevations in intracellular cAMP in the presence and absence of phosphodiesterase inhibition, suggesting activation of adenylate cyclase. Compared with phorbol 12-myristate 13-acetate–activated neutrophils, *N*-formyl-Met-Leu-Phe–activated neutrophils were more susceptible to the inhibitory effects of LNO_2, indicating that LNO_2 may inhibit signaling both upstream and downstream of protein kinase C. These data suggest novel signaling actions for LNO_2 in mediating its potent inhibitory actions. Thus, nitration of lipids by NO-derived reactive species yields products with antiinflammatory properties, revealing a novel mechanism by which NO-derived nitrated biomolecules can influence the progression of vascular disease. **(*Circ Res*. 2002;91:375-381.)**

Key Words: nitric oxide ■ nitrolinoleate ■ neutrophils ■ atherosclerosis ■ peroxynitrite

Nitric oxide is a free radical signaling mediator generated by the healthy endothelium to maintain vascular homeostasis through the regulation of blood pressure and leukocyte-platelet activation. When generated at elevated levels during inflammation by inducible NO synthase in a variety of cell types, NO is readily transformed into potent nitrating and nitrosating species, including peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), and nitrous acid (HONO). These species can react with unsaturated lipids, forming both oxidized and nitrated products, including nitro, nitrito, and nitroepoxy derivatives.[1–4] Several recent studies have demonstrated in vivo generation of nitrated lipids. For example, bovine cardiac muscle and human plasma contain species with chromatographic and mass spectral properties identical to those of nitrated arachidonate and linoleate, whereas plasma from rats exposed to liver ischemia/reperfusion contains nitrolinoleate[5,6] (D.G. Lim, B.A. Freeman, unpublished data, 2002). Treatment of LDL with nitrating/nitrosating and oxidizing species that are generated during myeloperoxidase oxidation of nitrite (NO_2^-) causes its modification to a form recognized by the macrophage scavenger receptor CD36.[7,8] Also, macrophage uptake of LDL treated with nitrating/nitrosating species stimulates cholesteryl ester synthesis, intracellular cholesterol and cholesteryl ester accumulation, and foam cell formation.[9,10] Finally, synthetic nitrated lipids derived from either arachidonate or linoleate inhibit multiple indices of platelet activation and cause smooth muscle relaxation[5,11] (D.G. Lim, B.A. Freeman, unpublished data, 2002). These observations raise the possibility that nitrated lipids could modulate atherogenesis and/or inflammation in vivo.

Activation of phagocytic leukocytes, including neutrophils, is a central feature of inflammatory disease.[12–16] For example, neutrophil rolling and adhesion to the endothelium are enhanced in apoE and double apoE/LDL receptor–knockout

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mice, whereas neutrophils from septic patients show alterations in integrin expression and $O_2^{\cdot-}$ generation.[13–16] Also, mice deficient in adhesion molecules or chemokines and their receptors show impaired immune responses and diminished atherosclerosis.[12,17–19] Neutrophil function in vivo can be regulated through NO- and eicosanoid-dependent mechanisms.[20–22] In this regard, nitrated unsaturated lipids formed by reaction with NO-derived reactive nitrogen species are of potential interest, inasmuch as they could influence neutrophil activation through either reactivity and may lead to the development of novel therapeutic strategies for inflammation.

A synthetic nitrated lipid, nitrolinoleate (LNO_2), which is structurally similar to that generated by $ONOO^-$- or NO_2-induced linoleate nitration, is reported in the present study to inhibit *N*-formyl-Met-Leu-Phe (fMLP)- and phorbol 12-myristate 13-acetate (PMA)-mediated activation of human neutrophils.[1] Superoxide generation, degranulation, and CD11b expression were inhibited at LNO_2 concentrations <1 μmol/L and occurred in concert with attenuated elevations in intracellular Ca^{2+} and increased intracellular cAMP. Compared with PMA-activated neutrophils, fMLP-activated neutrophils were more susceptible to LNO_2 inhibition, indicating that LNO_2 may inhibit signaling upstream and downstream of protein kinase C. In aggregate, these data reveal that nitrated lipids potently inhibit leukocyte activation and demonstrate novel mechanisms by which NO-derived nitrated biomolecules may attenuate tissue inflammatory responses.

Materials and Methods

Materials

Lymphoprep was obtained from Nycomed Pharma. Unless otherwise stated, all other reagents were obtained from Sigma-Aldrich Ltd.

Synthesis and Purification of LNO_2

The synthesis and purification of LNO_2 have recently been described elsewhere and have been adapted from previous strategies for the synthesis of conjugated nitroalkenes via nitroselenylation of alkenes.[11,23,24] Briefly, linoleic acid (LA), $HgCl_2$, PheSeBr, and $AgNO_2$ (1.0:1.3:1.0:1.0 [mol/mol]) were combined in THF-acetonitrile (1:1 [vol/vol]), with a final concentration of 0.15 mol/L LA. The mixture was degassed and stirred at 25°C in the dark for 1.5 hours, the supernatant was recovered, and solvent was removed in vacuo. Lipid was solvated again in THF, and a 10-fold molar excess of H_2O_2 was added to the lipid mixture after cooling to 0°C. After 20 minutes, the solution was warmed to ≈25°C until gas evolution was noted. The solution was cooled to 10°C, followed by stirring at ≈25°C for 30 minutes. Lipids were extracted and then chromatographed on a silica gel column (250 to 400 mesh) using a hexane-$CHCl_3$ step gradient (5% increments from 0% to 30% $CHCl_3$), with LNO_2 predominantly eluting in the 20% to 30% $CHCl_3$ fraction, as determined by negative-ion-mode mass spectroscopic monitoring of fractions. Further purification was accomplished by high pressure liquid chromatography using 0.1% acetic acid in 50% to 90% CH_3OH gradient on a 4.6×250-μm reverse-phase C18 column. LNO_2 yields were quantified by elemental analysis of nitrogen content after pyrolysis by chemiluminescent nitrogen detection (Antek Instruments), with caffeine used as a standard. After purification and quantification, LNO_2 was stored in CH_3OH under inert gas at −80°C. For all cell experiments, appropriate controls using methanol were carried out to exclude solvent effects, and methanol concentrations were always $<0.1\%$.

Neutrophil Isolation

Human neutrophils were isolated from 20 mL citrate anticoagulated whole blood as described.[25] Approval for blood donations from healthy volunteers was given by the Bro Taf Local Research Ethics Committee, and all donors gave written consent. Briefly, blood was mixed 1:1 with 0.8% trisodium citrate (wt/vol) and 2% dextran (wt/vol) in PBS (containing 137 mmol/L NaCl, 2.68 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, and 1.47 mmol/L KH_2PO_4) and allowed to sediment for 45 minutes at 20°C. After this procedure, the upper plasma layer was underlaid with ice-cold Lymphoprep (2:1 for plasma/Lymphoprep) and centrifuged (800*g*, 20 minutes, 4°C). The pellet was resuspended in ice-cold PBS and 0.4% trisodium citrate (wt/vol) and centrifuged (400*g*, 5 minutes). Contaminating erythrocytes were removed using 3 cycles of hypotonic lysis. Finally, cells were resuspended in a small volume of Krebs buffer (100 mmol/L NaCl, 50 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L NaH_2PO_4, 1 mmol/L $CaCl_2$, 2 mmol/L D-glucose, and 5 U/mL heparin, pH 7.4), counted, and kept on ice.

Neutrophil Fractionation

For the preparation of subcellular fractions, neutrophils were purified as described above with the omission of the dextran sedimentation step. Neutrophils were treated with 2.5 mmol/L diisopropyl fluorophosphate for 10 minutes at 4°C, disrupted in relaxation buffer (100 mmol/L KCl, 3 mmol/L NaCl, 3.5 mmol/L $MgCl_2$, 1 mmol/L ATP, 1.25 mmol/L EGTA, and 10 mmol/L PIPES, pH 7.3) by N_2 cavitation, and fractionated on a discontinuous Percoll gradient.[26,27] This method produces cytosolic and plasma membrane fractions whose final concentrations were adjusted to 9×10^7 and 1.25×10^9 cell equivalents per milliliter, respectively. Fractions were stored at −80°C for up to 1 year without loss of activity.

Superoxide Generation

Neutrophil $O_2^{\cdot-}$ generation was determined using superoxide dismutase–sensitive cytochrome *c* reduction.[28] Cells (10^6) were added to 2 mL Krebs buffer containing 50 μmol/L ferricytochrome *c*, with stirring, at 37°C. Cells were activated using either 1 μmol/L fMLP or 1 μg/mL PMA, and absorbance was monitored at 550 nm (ϵ 21.1 $cm^{-1} \cdot$ mmol/L for ferrocytochrome *c*). To confirm $O_2^{\cdot-}$ generation, superoxide dismutase (300 U/mL) was added in control experiments. Where used, the soluble guanylate cyclase (sGC) inhibitor 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ, 4 μmol/L) was preincubated with neutrophils 20 minutes before the assay. Superoxide generation by isolated neutrophil membranes was measured after the addition of 160 μmol/L NADPH to 0.75 mL relaxation buffer containing 5.4×10^6 cell equivalents of membrane extract per milliliter, 1.5×10^7 cell equivalents of cytosol per milliliter, 10 μmol/L GTP-γ-S, 100 μmol/L SDS, and 50 μmol/L cytochrome *c*. To facilitate the assembly of NADPH oxidase components, all constituents (excluding NADPH) were preincubated at 25° for 5 minutes before the addition of NADPH. LNO_2 (10 μmol/L) was added in ethanol (final concentration 0.1%) either during NADPH oxidase assembly or immediately before NADPH addition.

Measurement of Neutrophil $[Ca^{2+}_{ic}]$

Neutrophils prepared as described were incubated at 10^7/mL with 5 μmol/L fura 2-AM (Molecular Probes) in Ca^{2+}-free Krebs buffer at 37°C for 20 minutes. Then, cells were centrifuged at 400*g* for 10 minutes, resuspended in Ca^{2+}-free Krebs buffer at 10^7/mL, kept on ice, and studied within 90 minutes. Fluorescence of neutrophils (10^6/mL) was measured using a Perkin-Elmer LS 50B fluorescence spectrophotometer at 37°C with stirring. Excitation wavelengths were 340 and 380 nm, with an emission wavelength of 509 nm. Corrections were applied for autofluorescence (unloaded neutrophils), and calibrations were performed by adding 1 μmol/L ionomycin, 1 mmol/L $CaCl_2$ (to give maximal fluorescence ratio $[R_{max}]$ and minimum fluorescence $[S_b]$), followed by 5 mmol/L $MnCl_2$ (to give minimal fluorescence ratio $[R_{min}]$ and maximum fluorescence $[S_f]$) to fura 2-AM–loaded neutrophils. $[Ca^{2+}_{ic}]$ was calculated using the following equation: $[Ca^{2+}_{ic}]=K_d \cdot S_f \cdot (R-R_{min})/S_b \cdot (R_{max}-R)$,

where K_d is the dissociation constant of fura 2 under intracellular conditions (224 nmol/L); S_f and S_b are the maximum and minimum values of fluorescence, respectively, at 380 nm; and R_{max} and R_{min} are the maximum and minimum values of the ratio (340 nm/380 nm) under Ca^{2+}-saturating and Ca^{2+}-free conditions, respectively. Fluorescence of neutrophils was monitored on the addition of 1 μmol/L fMLP alone, 5 μmol/L LNO_2, or fMLP after a 2-minute preincubation with LNO_2 in the presence of either 1 mmol/L $CaCl_2$ or 100 μmol/L EGTA (using Ca^{2+}-free Krebs solution).

Determination of CD11b Expression

Expression of CD11b (αM integrin, MAC-1) was determined by flow cytometry.[29] Briefly, isolated neutrophils (2×10^6/mL) were incubated with and without LNO_2 (3.6 μmol/L) for 2 minutes at 37°C. After this procedure, fMLP (1 μmol/L) was added, and samples were incubated for a further 5 minutes at 37°C. Anti-human CD11b-FITC (10 μL, Serotec) or isotype control was then added, and samples were incubated for 30 minutes at 4°C. Finally, cells were washed once with ice-cold PBS and then resuspended in 0.1% paraformaldehyde-PBS until flow cytometry analysis. Neutrophils were analyzed on a FACScan flow cytometer (Becton Dickinson) and identified by forward and side scatter and FITC.

Determination of Neutrophil Degranulation by Elastase Release

Degranulation of azurophilic granules was determined by elastase release.[30] Briefly, isolated neutrophils were resuspended at 2×10^6/mL Krebs buffer containing 1 μg/mL cytochalasin B and 40 μmol/L MeO-Suc-Ala-Ala-Pro-Val-MCA at 37°C with stirring. After 5 minutes, 1 μmol/L fMLP was added, and fluorescence was monitored (excitation wavelength 380 nm, emission wavelength 460 nm). In some experiments, 3 μmol/L LNO_2 was added 2 minutes before fMLP.

Determination of cAMP and cGMP

For cAMP, neutrophils (2×10^6/mL) in Krebs buffer were prewarmed to 37°C for 3 minutes. Then, 3.6 μmol/L LNO_2 with or without 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX) was added, and samples were incubated for 1 minute at 37°C. Then, proteins were precipitated using ice-cold 6% trichloroacetic acid, which was subsequently removed by washing samples several times with water-saturated diethyl ether. For cGMP, neutrophils (4×10^6/mL) in Krebs buffer were incubated for 5 minutes at 37°C with or without 1 mmol/L IBMX, 10 μmol/L LNO_2, or 10 μmol/L 2(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEANONOate). Then, proteins were precipitated using ice-cold 66% ethanol. Cyclic nucleotides were determined by radioimmunoassay (Biotrak, Amersham).

Results

LNO_2 Inhibits fMLP- or PMA-Induced $O_2^{\cdot-}$ Generation by Intact Neutrophils but Not by Reconstituted NADPH Oxidase

Addition of LNO_2 (0.5 to 5 μmol/L) to stirred neutrophils in suspension did not stimulate $O_2^{\cdot-}$ generation (not shown). However, LNO_2 added to neutrophils 2 minutes before agonist activation using either 1 μmol/L fMLP or 1 μg/mL PMA led to concentration-dependent inhibition of $O_2^{\cdot-}$ generation (Figures 1A, 1B, and 1D). In contrast, similar concentrations of the parent fatty acid, LA, or the nitrated amino acid, 3-nitrotyrosine (3-NT), were without effect (Figure 1C). This indicates that inhibition of neutrophil activation was specific to LNO_2 and was not a feature of all unsaturated lipids or nitro-containing compounds. Neutrophil membranes were isolated and assayed for $O_2^{\cdot-}$ generation in a reconstituted system after the addition of NADPH. Preincubation of neutrophil membranes during assembly with 10 μmol/L

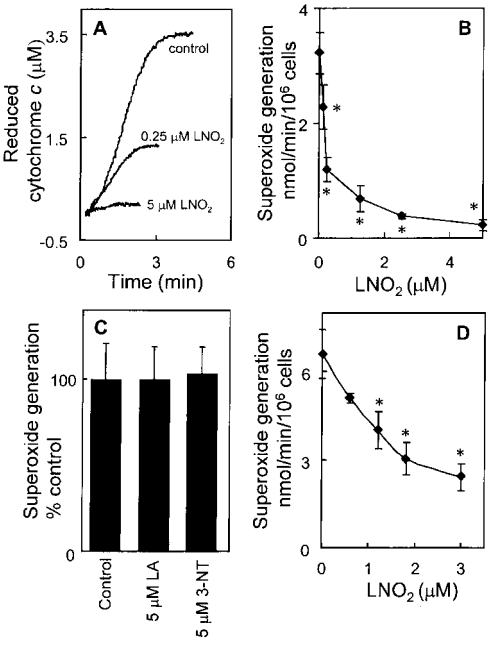

Figure 1. LNO_2 inhibits neutrophil $O_2^{\cdot-}$ generation in response to either PMA or fMLP. $O_2^{\cdot-}$ generation was measured using cytochrome *c* reduction as described in Materials and Methods. Neutrophils (10^6) in 2 mL Krebs buffer containing 50 μmol/L cytochrome *c*, with stirring at 37°C, were activated by 1 μmol/L fMLP or 1 μg/mL PMA, and absorbance (550 nm) was monitored. LNO_2, LA, or 3-NT was added 2 minutes before activation. A, Time course of $O_2^{\cdot-}$ generation by fMLP-activated neutrophils with/without LNO_2 is shown. B, Dose-dependent inhibition of fMLP-stimulated neutrophil $O_2^{\cdot-}$ generation by LNO_2 is shown. C, LA and 3-NT do not inhibit fMLP-stimulated neutrophil $O_2^{\cdot-}$ generation. D, Dose-dependent inhibition of PMA-stimulated neutrophil $O_2^{\cdot-}$ generation by LNO_2 is shown. For all data, values are mean±SD (n=3). *$P<0.05$ vs no LNO_2 after ANOVA and Bonferroni post hoc test.

LNO_2 slightly suppressed $O_2^{\cdot-}$ generation rates, although this was not significant (Figure 2A). Also, the addition of LNO_2 to the enzyme that had been allowed to preassemble in vitro had no effect on $O_2^{\cdot-}$ generation. These data indicate that LNO_2 does not inhibit $O_2^{\cdot-}$ generation through a direct effect on NADPH oxidase assembly or turnover.

cGMP Synthesis Is Not Involved in Inhibition of Neutrophil $O_2^{\cdot-}$ Generation by LNO_2

To examine whether NO was involved in the inhibitory effects of LNO_2, the sGC inhibitor ODQ was added. This did not restore neutrophil $O_2^{\cdot-}$ generation in response to fMLP (Figure 2B). Separately, cellular cGMP levels were found to be unchanged after a 5-minute incubation with LNO_2 in contrast to DEANONOate, which was used as a positive control (Figure 2C). These data rule out a role for NO activation of sGC in the inhibitory actions of LNO_2.

Effect of LNO_2 on CD11b Exposure in Response to fMLP

Basal neutrophil expression of CD11b, as evidenced by the difference in fluorescence between cells stained with either anti-CD11b antibody or isotype control, was not affected by LNO_2 (Figure 3). fMLP activation of neutrophils induced a

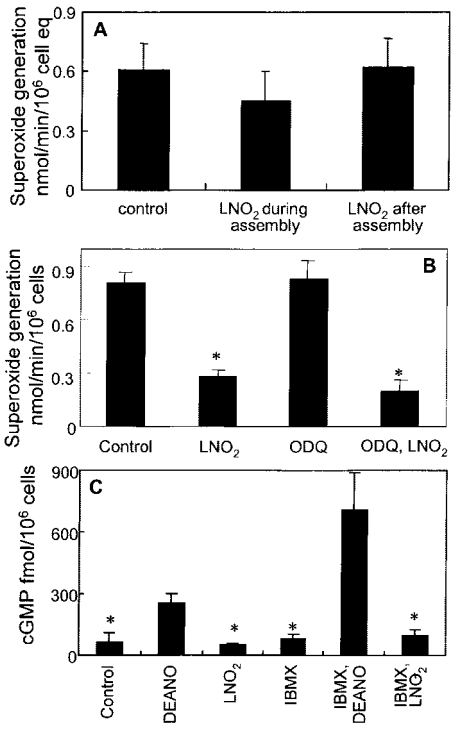

Figure 2. LNO_2 does not block $O_2^{\cdot-}$ generation in isolated neutrophil membranes, and inhibition of neutrophil $O_2^{\cdot-}$ generation does not involve sGC activation. A, O_2 generation was determined as described in Materials and Methods after the addition of 160 μmol/L NADPH to 0.75 mL relaxation buffer containing 5.4×10^6 cell equivalents (cell eq) of membrane extract per milliliter, 1.5×10^7 cell eq of cytosol per milliliter, 10 μmol/L GTP-γ-S, 100 μmol/L SDS, and 50 μmol/L cytochrome *c*. To allow assembly of NADPH oxidase components, all constituents (excluding NADPH) were preincubated at 25°C for 5 minutes before the addition of NADPH. LNO_2 (10 μmol/L) was added in ethanol (final concentration 0.1%) either during NADPH oxidase assembly (middle bar) or immediately before NADPH (right bar). B, $O_2^{\cdot-}$ generation of neutrophils in response to 1 μmol/L fMLP was determined as in Figure 1 after preincubation with/without 3 μmol/L LNO_2. Where used, 4 μmol/L ODQ was preincubated with neutrophils for 20 minutes before assay. C, cGMP synthesis by neutrophils was determined as described in Materials and Methods after 5 minutes of incubation with 10 μmol/L LNO_2 and 10 μmol/L DEANONOate (DEANO) with/without 1 mmol/L IBMX. For all data, values are mean±SD (n=3), and all experiments were repeated at least 3 times with neutrophils obtained from different donors (B). *$P<0.05$ vs control after ANOVA and Bonferroni post hoc test (C). Bars marked with asterisks are not significantly different from each other.

further significant increase in CD11b expression (Figures 3A and 3C), which was fully inhibited by preincubation of cells with 3 μmol/L LNO_2 (Figures 3B and 3C). This indicates that LNO_2 can inhibit neutrophil functions associated with adhesion.

LNO_2 Attenuates fMLP-Induced Degranulation

Pretreatment of neutrophils with 3 μmol/L LNO_2 blocked fMLP-induced azurophilic degranulation, as measured by elastase release, whereas the addition of LNO_2 to unstimulated cells had no effect (Figures 4A and 4B). This indicates that neutrophil functions associated with tissue damage and bacteriolysis can be attenuated by LNO_2.

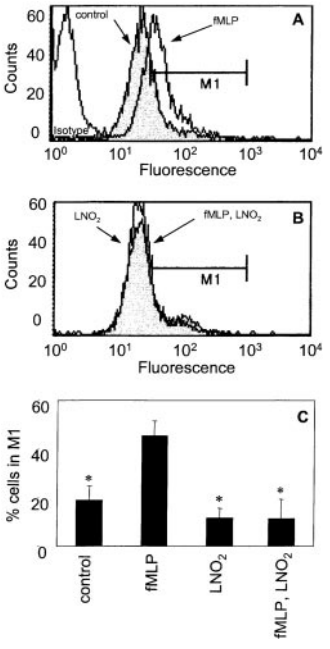

Figure 3. LNO_2 attenuates fMLP-stimulated neutrophil CD11b expression. Cells (2×10^6/mL) were incubated with/without 3.6 μmol/L LNO_2 for 2 minutes and then activated by 1 μmol/L fMLP for 5 minutes at 37°C. Anti-human CD11b-FITC or isotype control was then added, and cells were incubated for 30 minutes at 4°C. Cells were washed once with PBS and then fixed with 0.1% paraformaldehyde/PBS for FACScan analysis, as described in Materials and Methods. A, Fluorescence shift induced by fMLP in control neutrophils is shown. B, LNO_2 inhibits fMLP-induced CD11b expression. C, Percentages of cells in population designated M1 in panels A and B are shown. For all data, values are mean±SD (n=3), and all experiments were repeated at least 3 times on different donors. *$P<0.05$ vs fMLP after ANOVA and Bonferroni post hoc test.

LNO_2 Attenuates Ca^{2+} Mobilization in Response to fMLP

To examine the mechanisms of LNO_2 inhibition of neutrophil activation, Ca^{2+} mobilization in response to fMLP was monitored by fura 2-AM fluorescence. On the addition of 1 μmol/L fMLP to Ca^{2+}-containing samples, a characteristic increase in $[Ca^{2+}_{ic}]$ was observed (Figure 5A). Preincubation of neutrophils with 3 μmol/L LNO_2 significantly attenuated fMLP-induced Ca^{2+} mobilization and accelerated the subsequent rate of Ca^{2+} removal, whereas the addition of LNO_2 to unstimulated cells had no effect (Figure 5). In the absence of external Ca^{2+}, LNO_2 did not alter the kinetics of Ca^{2+} mobilization (Figure 6), indicating that LNO_2 primarily exerted an effect on external Ca^{2+} fluxes in neutrophils.

LNO_2 Elevates cAMP in Neutrophils

LNO_2 inhibits platelet activation via a cAMP-dependent pathway.[11] To determine whether similar mechanisms are operative in neutrophils, cAMP was determined after incubation with LNO_2. After a 1-minute incubation with 3.6 μmol/L LNO_2, a 40% increase in cAMP was observed (Figure 7). However, in the presence of the phosphodiesterase inhibitor IBMX, LNO_2 caused a 3-fold elevation in neutrophil cAMP levels. This indicates that LNO_2 elevates cAMP synthesis through the activation of adenylate cyclase in neutrophils.

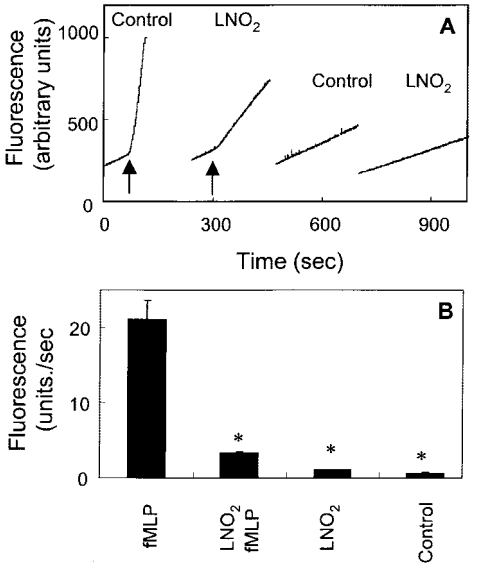

Figure 4. LNO_2 inhibits fMLP-stimulated neutrophil degranulation. Elastase release was measured by hydrolysis of MeO-Suc-Ala-Ala-Pro-Val-MCA as described in Materials and Methods. A, Cells (2×10^6) were incubated with 1 μg/mL cytochalasin B and 40 μmol/L MeO-Suc-Ala-Ala-Pro-Val-MCA at 37°C, with stirring, for 5 minutes before activation using 1 μmol/L fMLP, as indicated by arrow. In some experiments, 3 μmol/L LNO_2 was added 2 minutes before fMLP. B, Rates of fluorescence change after fMLP activation of neutrophils with/without 3 μmol/L LNO_2 were determined. For all data, values are mean±SD (n=3), and all experiments were repeated at least 3 times with neutrophils obtained from different donors. *$P<0.05$ vs fMLP after ANOVA and Bonferroni post hoc test.

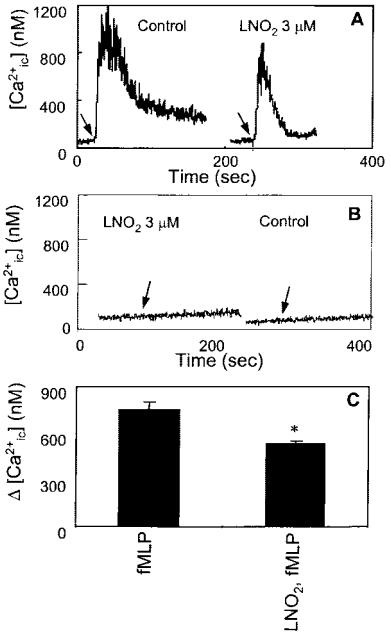

Figure 5. LNO_2 attenuates fMLP-stimulated Ca^{2+} mobilization in neutrophils in the presence of extracellular Ca^{2+}. Neutrophils labeled with fura 2-AM as described in Materials and Methods were stimulated with 1 μmol/L fMLP, as indicated by arrows, in the presence of 1 mmol/L Ca^{2+}, and fluorescence was monitored at 37°C with stirring. A, Ca^{2+} mobilization of neutrophils with/without 2-minute preincubation with 3 μmol/L LNO_2. B, Control experiments showing no effect of 3 μmol/L LNO_2 on control neutrophils. C, Maximum Ca^{2+} elevations recorded on addition of 1 μmol/L fMLP to control or 3 μmol/L LNO_2–treated neutrophils. $[Ca^{2+}_{ic}]$ indicates intracellular Ca^{2+} concentration. For all data, values are mean±SD (n=3), and all experiments were repeated at least 3 times with neutrophils obtained from different donors. *$P<0.01$ vs fMLP alone after independent *t* test.

Discussion

Nitrated lipid has recently been found in human plasma, bovine cardiac tissue, and plasma from a rat model of liver ischemia/reperfusion, with extensive nitration and oxidation of biomolecules also occurring in human atheroma[5,6,31] (D.G. Lim, B.A. Freeman, unpublished data, 2002). Nitrated lipids, including LNO_2, possess vascular protective properties through the inhibition of platelet activation and promotion of smooth muscle relaxation[5,11] (D.G. Lim, B.A. Freeman, unpublished data, 2002). In the present study, the influence of LNO_2 on human neutrophil functional responses was examined to characterize their bioactivity toward proinflammatory leukocytes, which are central to the pathogenesis of atherosclerosis. LNO_2 (0.1 to 1 μmol/L) inhibited neutrophil activation in response to PMA or fMLP, as indicated by the inhibition of $O_2^{\cdot-}$ generation, Ca^{2+} mobilization, azurophilic degranulation, and CD11b expression. This reveals that lipid nitration results in the formation of an antiinflammatory and vascular-protective product that potently attenuates multiple leukocyte functions.

Neutrophil cytotoxicity requires efficient degranulation to release proteases and other degradative enzymes. In the present study, LNO_2 effectively blocked fMLP-induced azurophilic degranulation, indicating that events associated with bacterial killing and cell damage are attenuated by nitrated lipids (Figure 4). The cell surface glycoprotein, CD11b (αM integrin, MAC-1), is expressed as a heterodimer with CD18 (C receptor type 3) on the surface of leukocytes in response to activating agonists such as fMLP and is involved in adhesion to endothelial cells and in transendothelial migration to inflammatory sites.[32,33] Recruitment of leukocytes to the arterial wall is an important event in atherogenesis and plaque rupture.[12,17–19,32,33] This is mediated via leukocyte integrin receptors, including CD11b, and is modulated by a variety of both endothelium- and leukocyte-derived lipid oxidation products and free radicals, including $O_2^{\cdot-}$, NO, prostaglandins, and isoprostanes.[34–42] Inhibition of fMLP-induced CD11b expression by LNO_2 indicates that nitrated lipids also modulate leukocyte responses associated with the development of vascular disease (Figure 3).

Stimulation of neutrophils by fMLP or PMA causes translocation of at least 3 different cytosolic proteins (p67-phox, p47-phox, and p21-rac1) to the membrane, where they interact with 2 membrane-bound proteins (gp91-phox and p22-phox) to form the active $O_2^{\cdot-}$–generating NADPH oxidase complex. To determine whether LNO_2 exerted its inhibitory actions directly on NADPH oxidase or prevented complex assembly, neutrophil membranes were isolated and assayed for $O_2^{\cdot-}$ generation in a reconstitution system after the addition of NADPH. The lack of inhibition by LNO_2 in this assay indicates that LNO_2 exerts its inhibitory effects upstream of NADPH oxidase (Figure 2A).

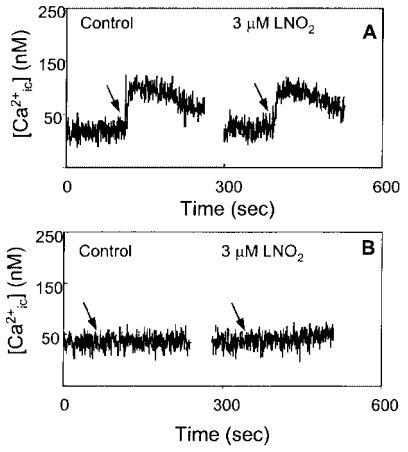

Figure 6. LNO_2 does not alter fMLP-stimulated Ca^{2+} mobilization in neutrophils in the absence of extracellular Ca^{2+}. Neutrophils labeled with fura 2-AM as described in Materials and Methods were stimulated with 1 μmol/L fMLP, as indicated by arrows, in the presence of 100 μmol/L EGTA, and fluorescence was monitored at 37°C with stirring. A, Ca^{2+} mobilization of neutrophils with/without 2-minute preincubation with 3 μmol/L LNO_2. B, Control experiments showing no effect of 3 μmol/L LNO_2 on control neutrophils.

There are at least 2 potential mechanisms by which nitrated lipids may exert cell signaling. The inhibition of platelet activation by LNO_2 requires cAMP synthesis, whereas stimulation of smooth muscle relaxation by LNO_2 or nitrated arachidonate involves cellular metabolism of the nitro functional group to NO[5,11] (D.G. Lim, B.A. Freeman, unpublished data, 2002). An elevation in either cGMP or cAMP can inhibit neutrophil function and is consistent with the observed effects of LNO_2 on agonist-induced Ca^{2+} elevations.[34–39] However, cGMP was ruled out because (1) elevations in response to LNO_2 did not occur and (2) inhibition of sGC did not restore neutrophil activation (Figure 2). In contrast, LNO_2 increased intracellular cAMP, in the presence and absence of IBMX, suggesting an involvement of this metabolite (Figure 7). Previous studies have shown that cAMP inhibits fMLP- but not PMA-stimulated neutrophil $O_2^{\cdot-}$ generation. This indicates that cAMP acts upstream of protein kinase C.[40] Therefore, inhibition of PMA-stimulated $O_2^{\cdot-}$ generation by LNO_2 suggests an additional cAMP-independent mechanism of action. In support, fMLP-activated neutrophils were considerably more susceptible to the inhibitory effects of LNO_2 than when they were stimulated with PMA (Figures 1B and 1D).

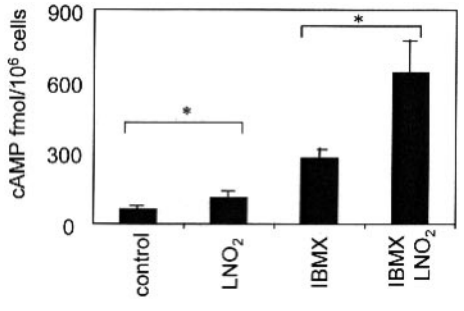

Figure 7. LNO_2 elevates cAMP in neutrophils. Neutrophils (2×10^6/mL) in Krebs buffer were prewarmed to 37°C for 3 minutes. LNO_2 (3.6 μmol/L) with/without 1 mmol/L IBMX was then added, and samples were incubated for 1 minute at 37°C. cAMP was determined as described in Materials and Methods using a commercial radioimmunoassay kit. For all data, values are mean±SD (n=3), and all experiments were repeated at least 3 times on different donors. *$P<0.005$ vs control or IBMX after independent *t* test.

This preparation of LNO_2 is a mixture of 4 positional isomers identified using nuclear magnetic resonance and IR spectroscopy (D.G. Lim, B.A. Freeman, unpublished data, 2002), and this preparation was used because it can be synthesized with a relatively high yield and purity. Levels of nitrated lipid in vivo are too low to allow purification of amounts sufficient for biological studies. Similarly, nitration of linoleate through oxidant pathways (eg, $ONOO^-$ and NO_2) in vivo will also form a mixture of LNO_2 isomers. Metabolism to release NO by smooth muscle cells is unlikely to be isomer specific; however, activation of adenylate cyclase through receptor-dependent pathways may be more effective with ≥1 specific isomer.

Data from the present study and others indicate that lipid nitration results in the formation of compounds that can signal through either NO- or cAMP-dependent pathways, with the biological mechanism of action being highly cell type dependent[5,11] (D.G. Lim, B.A. Freeman, unpublished data, 2002). For example, LNO_2 mediates smooth muscle relaxation through exclusively cGMP-dependent mechanisms, whereas in platelets and neutrophils, there is no role for NO, with its biological effects being at least partly cAMP dependent[11] (D.G. Lim, B.A. Freeman, unpublished data, 2002, and data in the present study). The reasons for this may involve (1) different cellular rates of metabolism of LNO_2 to NO and/or (2) differences in the expression of eicosanoid receptors that activate adenylate cyclase.

In summary, the present study shows that LNO_2 potently blocks multiple neutrophil proinflammatory responses, including degranulation, $O_2^{\cdot-}$ generation, and integrin expression. This indicates that nitrated lipids will attenuate leukocyte migration and subsequent activation in atherosclerotic lesions, demonstrating a further vascular protective property of these compounds in addition to antiplatelet and vasorelaxing effects[5,11] (D.G. Lim, B.A. Freeman, unpublished data, 2002). These data reveal novel mechanisms by which nitrated biomolecules modulate cell activation and show how the presence of the nitro functional group can alter the bioactivity of lipids through the formation of vascular protective and antiinflammatory products.

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References

1. O'Donnell VB, Eiserich JP, Chumley PH, Kirk M, Barnes S, Darley-Usmar VM, Freeman BA. Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem Res Toxicol.* 1999;12:83–92.

2. Gallon AA, Pryor WA. The identification of the allylic nitrite and nitro derivatives of methyl linoleate and methyl linolenate by negative chemical ionization mass spectroscopy. *Lipids*. 1993;28:125–133.
3. Gallon AA, Pryor WA. The reaction of low levels of nitrogen dioxide with methyl linoleate in the presence and absence of oxygen. *Lipids*. 1994;29:171–176.
4. Rubbo H, Radi R, Trujillo M, Telleri R, Kalyanaraman B, Barnes S, Kirk M, Freeman BA. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation: formation of novel nitrogen-containing oxidized lipid derivatives. *J Biol Chem*. 1994;269:26066–26075.
5. Balazy M, Iesaki T, Park JL, Jiang H, Kaminski PM, Wolin MS. Vicinal nitrohydroxyeicosatrienoic acids: vasodilator lipids formed by reaction of nitrogen dioxide with arachidonic acid. *J Pharmacol Exp Ther*. 2001; 299:611–619.
6. Abdalla DSP, Lima ES, DiMascio P, Rubbo H. Nitrated and nitrosylated products from linoleic acid in blood. *Free Radic Biol Med*. 2001;31(suppl 1):S37. Abstract.
7. Podrez EA, Febbraio M, Sheibani N, Schmitt D, Silverstein RL, Hajjar DP, Cohen PA, Frazier WA, Hoff HF, Hazen SL. Macrophage scavenger receptor CD36 is the major receptor for LDL modified by reactive nitrogen species. *J Clin Invest*. 2000;105:1095–1108.
8. Graham A, Hogg N, Kalyanaraman B, O'Leary V, Darley-Usmar VM, Moncada S. Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Lett*. 1993;330: 181–185.
9. Panasenko OM, Briviba K, Klotz LO, Sies H. Oxidative modification and nitration of human low-density lipoprotein by the reaction of hypochlorous acid with nitrite. *Arch Biochem Biophys*. 1997;343:254–259.
10. Podrez EA, Schmitt D, Hoff HF, Hazen SL. Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro. *J Clin Invest*. 1999;103:1547–1560.
11. Coles B, Bloodsworth A, Eiserich JP, Coffey MJ, McLoughlin RM, Giddings JC, Lewis MJ, Haslam RJ, Freeman BA, O'Donnell VB. Nitrolinoleate inhibits platelet activation by attenuating calcium mobilization and inducing phosphorylation of vasodilator-stimulated phosphoprotein through elevation of cAMP. *J Biol Chem*. 2002;277:5832–5840.
12. Aiello RJ, Bourassa PA, Lindsey S, Weng W, Natoli E, Rollins BJ, Milos PM. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 1999; 19:1518–1525.
13. Eriksson EE, Xie X, Werr J, Thoren P, Lindbom L. Direct viewing of atherosclerosis in vivo: plaque invasion by leukocytes is initiated by the endothelial selectins. *FASEB J*. 2001;15:1149–1157.
14. Guo RF, Riedemann NC, Laudes IJ, Sarma VJ, Kunkel RG, Dilley KA, Paulauskis JD, Ward PA. Altered neutrophil trafficking during sepsis. *J Immunol*. 2002;169:307–314.
15. Ibbotson GC, Doig C, Kaur J, Gill V, Ostrovsky L, Fairhead T, Kubes P. Functional alpha4-integrin: a newly identified pathway of neutrophil recruitment in critically ill septic patients. *Nat Med*. 2001;7:465–470.
16. Williams JH, Patel SK, Hatakeyama D, Arian R, Guo K, Hickey TJ, Liao SY, Ulich TR. Activated pulmonary vascular neutrophils as early mediators of endotoxin-induced lung inflammation. *Am J Respir Cell Mol Biol*. 1993;8:134–144.
17. Sligh JE, Ballantyne CM, Rich SS, Hawkins HK, Smith CW, Bradley A, Beaudet AL. Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proc Natl Acad Sci U S A*. 1993;90:8529–8533.
18. Dawson TC, Kuziel WA, Osahar TA, Maeda N. Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis*. 1999;143:205–211.
19. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell*. 1998;2: 275–281.
20. Moilanen E, Vuorinen P, Kankaanranta H, Metsa-Ketela T, Vapaatalo H. Inhibition by nitric oxide donors of human polymorphonuclear leukocyte functions. *Br J Pharmacol*. 1993;109:852–858.
21. Belenky SN, Robbins RA, Rennard RA, Gossman GL, Nelson KJ, Rubinstein I. Inhibitors of nitric oxide synthase attenuate human neutrophil chemotaxis in vitro. *J Lab Clin Med*. 1993;122:388–394.
22. Nicolini FA, Mehta P, Lawson D, Mehta JL. Reduction in human neutrophil chemotaxis by the prostacyclin analog iloprost. *Thromb Res*. 1990;59:669–674.
23. Hayama T, Tomoda S, Takeuchi Y, Nomura Y. Synthesis of conjugated nitroalkenes via nitrosenylation of alkenes. *Tetrahedron Lett*. 1982;23: 4733–4734.
24. Seebach D, Calderari G, Knochel P. Trifluoreoacetyoxyphenylsenenation of nitroolefins regioselective preparation of nitroallylic alcohol derivatives and their use as multiple coupling reagents. *Tetrahedron*. 1985;41: 4861–4872.
25. Segal AR, Jones OTG. The subcellular distribution and some properties of the cytochrome b component of the microbicidal oxidase system of human neutrophils *Biochem J*. 1979;182:181–188.
26. Curnutte JT, Kuver R, Babior BM. Activation of the respiratory burst oxidase in a fully soluble system from human neutrophils. *J Biol Chem*. 1987;262:6450–6452.
27. Babior BM, Kuver R, Curnutte JT. Kinetics of activation of the respiratory burst oxidase in a fully soluble system from human neutrophils. *J Biol Chem*. 1988;263:1713–1718.
28. Cross AR, Parkinson JF, Jones OTG. The superoxide-producing oxidase of leukocytes: NADPH-dependent reduction of flavin and cytochrome b in solubilized preparations. *Biochem J*. 1984;223:337–344.
29. Elbim C, Prevot MH, Bouscarat F, Franzini E, Chollet-Martin S, Hakim J, Gougerot-Pocidalo MA. Polymorphonuclear neutrophils from human immunodeficiency virus-infected patients show enhanced activation, diminished fMLP-induced L-selectin shedding and an impaired oxidative burst after cytokine priming. *Blood*. 1994;84:2759–2766.
30. Sklar LA, McNeil VM, Jesaitis AJ, Painter RG, Cochrane CG. A continuous, spectroscopic analysis of the kinetics of elastase secretion by neutrophils. *J Biol Chem*. 1982;257:5471–5475.
31. Beckman JA, Ye YS, Anderson PG, Chen J, Accavitti MA, Tarpey MM, White CR. Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol Chem Hoppe Seyler*. 1994; 375:81–88.
32. Anderson DC, Springer TA. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1 and p150,95 glycoproteins. *Annu Rev Med*. 1987;38:175–194.
33. Bevilacqua MP, Nelson RM. Selectins. *J Clin Invest*. 1993;91:379–387.
34. Bloemen PGM, van den Tweel MC, Henricks PAJ, Engels F, Kester MHA, van de Loo PGF, Blomjous FJ, Nijkamp FP. Increased cAMP levels in stimulated neutrophils inhibit their adhesion to human bronchial epithelial cells. *Am J Physiol*. 1997;272:L580–L587.
35. Derian CK, Santulli RJ, Rao PE, Solomon HF, Barrett JA. Inhibition of chemotactic peptide-induced neutrophil adhesion to vascular endothelium by cAMP modulators. *J Immunol*. 1995;154:308–317.
36. Bengis-Garber C, Gruener N. Protein kinase A downregulates the phosphorylation of p47phox in human neutrophils: a possible pathway for inhibition of the respiratory burst. *Cell Signal*. 1996;8:291–296.
37. Stokes KY, Clanton EC, Russell JM, Ross CR, Granger DN. NAD(P)H oxidase-derived superoxide mediates hypercholesterolemia-induced leukocyte-endothelial cell adhesion. *Circ Res*. 2001;88:499–505.
38. Lelamali K, Wang W, Gengaro P, Edelstein C, Schrier RW. Effects of nitric oxide and peroxynitrite on endotoxin-induced leukocyte adhesion to endothelium. *J Cell Physiol*. 2001;188:337–342.
39. Tsao PS, Lewis NP, Alpert S, Cooke JP. Exposure to shear stress alters endothelial adhesiveness: role of nitric oxide. *Circulation*. 1995;92: 3513–3519.
40. Sedgwick JB, Berube ML, Zurier RB. Stimulus-dependent inhibition of superoxide generation by prostaglandins. *Clin Immunol Immunopathol*. 1985;34:205–215.
41. Arfors KE, Lundberg C, Lindbom L, Lundberg K, Beatty PG, Harlan JM. A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. *Blood*. 1987;69:338–340.
42. Leitinger N, Huber J, Rizza C, Mechtcheriakova D, Bochkov V, Koshelnick Y, Berliner JA, Binder BR. The isoprostane 8-iso-PGF(2alpha) stimulates endothelial cells to bind monocytes: differences from thromboxane-mediated endothelial activation. *FASEB J*. 2001;15: 1254–1256.