Nitrolinoleate Inhibits Platelet Activation by Attenuating Calcium Mobilization and Inducing Phosphorylation of Vasodilator-stimulated Phosphoprotein through Elevation of cAMP*

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Reactive species formed from nitric oxide (NO) nitrate unsaturated fatty acids such as linoleate (LA) to nitrate derivatives including nitrolinoleate (LNO2). The effect of LNO2 on human platelets was examined to define how nitrated lipids might behave in vivo. LNO2, but not LA or 3-nitrotyrosine, dose dependently (0.5–10 μM) inhibited thrombin-mediated aggregation of washed human platelets, with concomitant expression of P-selectin anticoagulation and selective phosphorylation of VASP at the cAMP-dependent protein kinase selective site, serine 157. LNO2 caused slight mobilization of calcium (Ca2+) from intracellular stores but significantly inhibited subsequent thrombin-stimulated Ca2+ elevations. LNO2 did not elevate platelet cGMP, and its effects were not blocked with inhibitors of NO signaling (oxyhemoglobin, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one. 2-fold elevations in cAMP were found following LNO2 treatment of platelets, and the adenyl cyclase inhibitors 2',5'-dideoxyadenosine and SQ22558 partially restored thrombin-stimulated aggregation. Finally, LNO2 significantly inhibited cAMP hydrolysis to AMP by platelet lysates. These data implicate cAMP in the anti-aggregatory action of LNO2. The platelet inhibitory actions of LNO2 indicate that nitration reactions that occur following NO generation in an oxidizing environment can alter the activity of lipids and lend insight into mechanisms by which NO-derived species may modulate the progression of vascular injury.

Nitric oxide (NO) is an endogenously produced free radical signaling mediator that regulates a variety of biological actions including blood pressure, neurotransmission, and platelet aggregation. The biological chemistry of NO is largely mediated through reaction with transition metals and other radicals, often leading to the formation of potent nitrating and nitrosating species. Aerobically, NO reacts with oxygen (O2) to form nitrogen dioxide (NO2) (k = 2 × 107 M–2 s–1), which further reacts with NO to form dinitrogen trioxide (N2O3) as shown in Equations 1 and 2.

\[
2NO + O_2 \rightarrow 2NO_2 \quad \text{(Eq. 1)}
\]
\[
NO_2 + NO \rightarrow N_2O_3 + H_2O \rightarrow 2NO_3^- + 2H^+ \quad \text{(Eq. 2)}
\]

Under aqueous conditions, N2O3 rapidly hydrolyzes to form nitrite (NO2–), the major decomposition product of NO in aqueous buffers (Equation 2). Although this species is stable at physiological pH, acidification yields nitrous acid (HONO), which in turn generates a complex mixture of oxidizing and nitrating/nitrosating intermediates (e.g. NO3–, NO2–, and N2O5). In vivo, the major stable NO-derived end product of oxidation is nitrate (NO3–); generally assumed to form through the reaction of NO with oxymyoglobin (oxyHb). In vascular diseases such as hypertension and atherosclerosis, the reaction of NO with superoxide (O2–) forming the nitrating/nitrosating peroxynitrite (ONOO–), may also account for NO3– formation and removal of a significant proportion of NO (1, 2). This occurs at diffusion-limited rates (k = \(1.9 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}\)) (3), kinetically outcompeting the reaction of O2– with superoxide dismutase.

Several NO-derived reactive nitrogen species known to be formed in vivo (e.g. ONOO–, N2O3, NO2–, and HONO) react with purified unsaturated fatty acids to yield novel species including nitro, nitrite, and nitroepoxy derivatives (4–7). These reactions generally occur in tandem with oxidation of fatty acids yielding a complex mixture of oxidized and/or nitrated products. Nitration of low density lipoprotein (LDL)1 by ONOO– yields nitro cholyl linoleate (chol-LA-NO2), and human cardiac muscle and hypercholesterolemic plasma contain species with chromatographic and mass spectral properties identical to that of nitrated arachidonate or linoleate (8, 9). This suggests that nitration of unsaturated fatty acid occurs in vivo. Treatment of LDL with nitrating/nitrosating and oxidizing species formed in vivo during myeloperoxidase oxidation of NO2 results in the

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1 The abbreviations used are: LDL, low density lipoprotein; LNO2, nitrolinoleate; DDA, 2',5'-dideoxyadenosine; VASP, vasodilator-stimulated phosphoprotein; AC, adenyl cyclase; LA, linoleate; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; IBMX, 3-isobutyl-1-methyl-xanthine; DEA-NONOate, 2(N-diethylamino)diazenolate-2-oxide; 3-NT, 3-nitrotyrosine; HPLC, high pressure liquid chromatography; ACD, acid citrate dextrose; FITC, fluorescein isothiocyanate; PDE, phosphodiesterase.
formation of an LDL particle recognized by the macrophage scavenger receptor CD36 (10, 11). Macrophage uptake of LDL treated with nitrating/nitrosating species promotes cholesteryl ester synthesis, intracellular cholesteryl and cholesteryl ester accumulation, and foam cell formation (12, 13). These observations raise the possibility that nitrated lipids in LDL may modulate atherogenesis.

In vivo, nitrination, nitrosation, and/or oxidation of biomolecules such as amino acids, lipids, and DNA occur in a variety of inflammatory diseases including atherosclerosis, organ rejection, and chronic inflammation (14). For example, the presence of nitrotyrosine along with protein and lipid oxidation products in these disease processes infers that the formation of nitrating species contributes to the oxidative events associated with diverse aspects of inflammatory disease pathology (15).

Activation of platelets to form thrombi is a major complication of death in atherosclerotic vascular disease. Platelet activation in vivo can be regulated through both NO and eicosanoid-dependent mechanisms, with significant interest in the development of clinical tools that simultaneously modulate both pathways (16). Generation of NO by platelets and endothelial cells in response to agonists such as collagen, ADP, bradykinin, and acetylcholine prevents thrombus formation and adhesion of platelets in vivo. Also, the level of platelet activation is controlled through the action of both pro- (e.g. thromboxanes) and anti- (e.g. prostacyclin) aggregatory eicosanoids derived from the prostaglandin H synthase-1 product prostaglandin H₂ (PGH₂). In this regard, nitrated unsaturated lipids formed by reaction with NO-derived reactive nitrogen species are of potential interest because they may influence platelet activation through either NO or eicosanoid-like reactivity.

In this study, the effects of a chemically synthesized nitro lipid, nitrolinoleate (LNO₂), on thrombin-stimulated platelet activation were determined. This is a model compound that is structurally similar to that generated by reaction of ONOO⁻ with linoleate (4) and was prepared to investigate the potential biological activities of nitrated lipids. Inhibition of thrombin-stimulated platelet aggregation was observed in concert with stimulation of vasodilator-stimulated phosphoprotein (VASP) phosphorylation at serine 157 and attenuated elevations in intracellular Ca²⁺. Nitrolinoleate increased platelet cAMP with two separate adenyl cyclase (AC) inhibitors partially restoring thrombin-induced aggregation of LNO₂-treated platelets. Neither linoleate (LA) nor 3-nitrotyrosine (3-NT) altered effects were specific for LNO₂ and not for all long chain fatty acids or nitrated compounds. These data indicate that LNO₂ inhibits thrombin activation of platelets via cAMP-dependent mechanisms and reveal a novel mechanism by which NO-derived nitrated biomolecules may influence vascular function.

**MATERIALS AND METHODS**

**Synthesis and Purification of Nitrolinoleate**—The synthesis of LNO₂ was modified from previous strategies for the synthesis of conjugated nitrilakines via nitrosylation of alkenes (17, 18). LA [HCl/PhSeBr/AgNO₃ (1.0:3.1:1:0.1:0.1 mol/mol) were combined in tetrahydrofuran:acetone (1:1, v/v; 1 g of linoleate/24 ml of solvent (17)). The mixture was degassed and stirred at 25 °C in the dark for 1.5 h; the supernatant was recovered, and the solvent was removed in vacuo. Lipoic acid was resolvated in 24 ml of tetrahydrofuran, and a 10-fold molar excess of LA (480 mM of H₂O₂) was added to the lipid mixture after cooling to 0 °C. After 20 min, the solution was warmed to −25 °C until gas evolution was noted. Then the solution was cooled to 10 °C followed by stirring at −25 °C for 30 min (18). Lipids were extracted with equal volumes of diethyl ether and saturated NaCl in H₂O. Solvent was removed in vacuo and water contamination was sublimated with acetone. Lipids were then resolved in methanol and chromatographed on a 2.5 × 7.6-cm silica gel (250–400-mesh) column equilibrated with hexane using a hexane:CHCl₃ step gradient (5.0% increments from 0–30% CHCl₃) with LNO₂ predominantly eluting in the 20–30% CHCl₃ fraction as determined by negative ion-mode mass spectrometry analysis. Further separation of the LNO₂-containing fraction was accomplished by HPLC using a 0.1% acetic acid in 50–90% methanol gradient over 46.4 × 250-mm reverse phase 120 Å columns. UV monitoring allowed for the collection of separated peaks which were analyzed further by mass spectrometry. Nitrolinoleate yield was quantified by elemental analysis of nitrogen content using a chemiluminescent nitrogen detector (Antek Instruments, Houston, TX) with caffeine as a standard. After purification and characterization LNO₂ was stored under inert gas at −20 °C.

**Preparation of Platelet-rich Plasma and Washed Platelets—**Whole blood was collected from healthy volunteers who were free from non-steroidal anti-inflammatory drugs for at least 14 days. Platelet-rich plasma was prepared by centrifuging whole blood collected into 3.8% trisodium citrate (blood:citrate, 9:1, v/v) at 2500 g for 10 min and the remaining pellet was resuspended in Ca²⁺-free Tyrode’s buffer (NaCl, 134 mM; NaHCO₃, 12 mM; KCl, 2.9 mM; Na₂HPO₄, 0.34 mM; MgCl₂, 1 mM; HEPES, 10 mM; glucose, 5 mM; pH 7.4) containing ACD (ACD/Tyr, 1:9, v/v). The platelets were washed by centrifuging at 800 × g for 10 min and resuspended in Ca²⁺-free Tyrode’s buffer.

**Platelet Aggregation—**Aggregation of washed platelets (2 × 10⁸ ml⁻¹) was monitored using a PAP4 optical aggregometer (Bio/Data Corp.). To assess platelet function, aggregation in response to 0.04 unit/ml thrombin (bovine, Sigma) was examined. Briefly, 500 µl of platelets (2 × 10⁸ ml⁻¹) were incubated for 3 min at 37 °C in Tyrode’s buffer with 1 mM CaCl₂ before thrombin addition with stirring. Typical aggregation responses were 70–80%, and white blood cell contamination of washed platelet preparations was less than 1%. Where used, 4 µM ODQ was preincubated with platelets for 20 min at room temperature before aggregation. 100 µM 2′,5′-dideoxynucleoside (DDA) was added with platelets for 2 min before assay. LNO₂, LA, or 3-NT were added to washed platelets 2 min before thrombin addition. 20 µM SQ22536 or 13 µM oxyHb were added along with LNO₂.

**Radioimmunoassay of cGMP or cAMP—**For cGMP analysis, platelets (2 × 10⁸ ml⁻¹) were incubated for 3 min at 37 °C in 500 µl of Tyrode’s buffer containing 1 mM CaCl₂, with or without 1 mM 3-isobutyl-1-methylxanthine (IBMX), 100 µM or 1 µM SQ22536, or 1 mM 2′,5′-dideoxynucleoside (DDA) was added with platelets for 2 min before assay. LNO₂, LA, or 3-NT were added to washed platelets 2 min before thrombin addition. 20 µM SQ22536 or 13 µM oxyHb were added along with LNO₂.

**Measurement of Platelet [Ca²⁺]i—**Washed platelets prepared as described were incubated at 4 × 10⁸ ml⁻¹ with 2.5 µM Fura-2/AM (Molecular Probes, Eugene, OR) in Tyrode’s buffer at 37 °C for 45 min (20). Then 12 µl M-ACD was added and platelets were centrifuged at 800 × g for 10 min. Platelets were resuspended at 4 × 10⁸ ml⁻¹ in Tyrode’s buffer, kept at room temperature, and used within 90 min. Fluorescence of platelets (10⁸ ml⁻¹) was measured using a PerkinElmer Life Sciences 50B fluorescence spectrophotometer at 37 °C with stirring. Excitation wavelengths were 340 and 380 nm with emission at 509 nm. Corrections were applied for autofluorescence (unloaded platelets), and calibrations were performed by adding 1 mM CaCl₂ and 1 mM ionomycin (to give Rₘₐₓ and S₀), followed by 5 mM MnCl₂ (to give Rₘₐₓ and S₀ to Fura-2/AM-loaded platelets. [Ca²⁺]i, was calculated using Equation 3,

\[
[Ca^{2+}]_{i} = K_{D} \cdot \frac{S_{0} \cdot (R - R_{min})}{S_{0} - (R - R_{min})} \quad \text{(Eq. 3)}
\]

where \(K_{D}\) is the dissociation constant of Fura2 under intracellular conditions (224 mM), \(S_{0}\) and \(S_{m}\) are the maximum and minimum values of fluorescence at 380 nm, and \(R_{min}\) and \(R_{max}\) are the maximum and minimum values of the ratio (340:380 nm) under Ca²⁺-saturating and Ca²⁺-free conditions, respectively. Fluorescence of platelets was monitored upon addition of 0.04 unit/ml thrombin alone, 10 µM LNO₂, 10 µM
LA, or thrombin following a 2-min preincubation with LNO₂ or LA in the presence of either 1 mM CaCl₂ or 100 μM EGTA.

Phosphorylation of VASP—Platelets (10⁶/ml) in 0.5 ml of Tyrode’s buffer were prewarmed for 1 min to 37 °C before addition of 1 mM CaCl₂ and 100 mM or 1 mM DEA-NOOlate, 10 μM LNO₂, or 10 μM LA. Following a 3-min incubation at 37 °C, 200 μl was removed, diluted with 50 μl of SDS-containing Laemmli buffer, and boiled for 3 min. Samples (corresponding to 4 × 10⁶ platelets) were separated on 8% SDS-polyacrylamide gels for 1.5 h and blotted onto nitrocellulose membranes.

Phosphorylation of VASP at the cGMP-dependent protein kinase–preferred site (serine 239) was detected using a monoclonal antibody specific for that phosphoserine residue (21) (VASP-16C2, Amersham Biosciences, Inc.). VASP phosphorylation at the cAMP-dependent protein kinase–preferred site (serine 157) was detected by a shift of molecular mass from 46 to 50 kDa, using a polyclonal antibody (CD62P, BD PharMingen) or 10 μg/ml IgG1 (Santa Cruz Biotechnology, CA, 0.4 μg/ml) followed by peroxidase-labeled anti-goat IgG and enhanced chemiluminescence detection (Amersham Biosciences, Inc.).

P-selectin Flow Cytometry—Washed platelets (5 × 10⁵ cells total) in Tyrode’s buffer with or without 50 mM LNO₂ or 50 mM LA were added to 10 μl of FITC-conjugated mouse anti-human P-selectin monoclonal antibody (CD62P, BD Pharmingen) or 10 μl of FITC-conjugated mouse IgG₁ monoclonal immunoglobulin isotype control (IgG₁k, BD Pharmingen) containing 4 mM H-Gly-Pro-Arg-Pro-OH (GPRP) and 1 mM CaCl₂. Following a 2-min incubation at 37 °C with or without 50 mM LNO₂, or 10 mM LA, 0.4 unit/ml thrombin was added, and samples were incubated for a further 10 min at 37 °C. Samples were fixed at room temperature with 0.5% paraformaldehyde for 30 min, diluted to 500 μl with Tyrode’s buffer, and stored at 4 °C until FACS analysis. Platelets were analyzed on a FACScan™ flow cytometer (BD Pharmingen) and identified by logarithmic signal amplification for forward and side scatter and for FITC.

Assay of Platelet Phosphodiesterase (PDE) Activity—Platelet homogenate was prepared by incubating washed platelets at 10¹⁵ ml⁻¹ in lysis buffer (10 mM NaH₂PO₄, 2 mM EDTA, 2 mM EGTA, 1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride (AEBSF), 100 units/ml aprotinin, pH 7.6) for 10 min on ice before snap-freezing in liquid N₂. After defrosting on ice, platelet homogenate (equivalent to 1.2 × 10⁶ cells) was incubated with 10 mM MgCl₂, 1 mM [³⁵S]cAMP with or without 12 μM LNO₂ in a final volume of 100 μl of Tris-HCl, pH 7.6, for 30 min at 20 °C. Following this, samples were inactivated by heating to 90 °C for 10 min. Negative controls were generated by heating samples to 90 °C for 10 min before cAMP addition. Nucleotides were separated by HPLC on a Waters Spherisorb 5-micron ODS2 4.6 × 150 mm column, using a gradient of 6.5–50% B over 15 min (A, 10 mM triethylammonium formate; B, acetonitrile) at 0.5 ml/min (22). [³⁵S]cAMP formation by platelet lysates was confirmed by co-elution of radiolabel with high concentrations of unlabeled AMP monitored by absorbance at 254 nm. 0.5-ml fractions were collected into 4 ml of scintillant and analyzed for [³⁵S]cAMP.

RESULTS

LNO₂ Inhibits Thrombin-induced Platelet Aggregation—Addition of 0.5–10 μM LNO₂ to washed platelets 2 min prior to activation with 0.04 unit ml⁻¹ thrombin led to dose-dependent inhibition of aggregation (Fig. 1). Similar concentrations of LA or 3-NT added in the same volumes of ethanol were without effect, indicating that this is specific for the nitrated lipid (Fig. 1B).

LNO₂ Inhibits Thrombin-induced Calcium Mobilization—To determine the anti-aggregatory mechanism of LNO₂, thrombin-induced elevations in intracellular calcium concentrations ([Ca²⁺]i) were measured following a 2-min incubation with LNO₂. Addition of 0.04 unit ml⁻¹ thrombin to wash platelets in the presence or absence of extracellular Ca²⁺ caused characteristic increases in [Ca²⁺]i (Fig. 2, A and B). Addition of 10 μM LNO₂ alone caused a small elevation in [Ca²⁺]i, far lower than that induced by thrombin (Fig. 2, A and B). The magnitude of this response was unaffected by the absence of extracellular Ca²⁺ indicating that it originates from intracellular stores (Fig. 2C). A 2-min preincubation of platelets with LNO₂ suppressed the subsequent thrombin-stimulated platelet [Ca²⁺]i elevation in extracellular Ca²⁺-containing samples by ~50% (Fig. 2, A and C). When extracellular Ca²⁺ was absent, the response to thrombin was also attenuated by LNO₂ treatment (Fig. 2, B and C). However, because LNO₂ also stimulates increases in intracellular Ca²⁺ concentration, the final cytosolic Ca²⁺ concentration was similar in the presence or absence of LNO₂ (Fig. 2B). For all conditions, LNO₂ suppressed the rates of thrombin-induced Ca²⁺ increases up to 85% (Fig. 2D). Finally, in control experiments 10 μM LA did not alter intracellular Ca²⁺ levels and was without effect on thrombin-stimulated elevations (Fig. 2, E and F).

LNO₂ Inhibits Thrombin-induced P-selectin Expression—Exposure of platelets to 0.4 unit ml⁻¹ thrombin caused an increase in the proportion of platelets expressing P-selectin (Fig. 3A). Thrombin-mediated induction of P-selectin expression requires 10-fold higher concentrations of thrombin than for stimulating aggregation or calcium mobilization (observed at low doses of 0.04 unit ml⁻¹). Preincubating platelets with LNO₂ totally abolished thrombin-induced P-selectin exposure, and higher concentrations of LNO₂ (50 μM) were required to offset the actions of higher thrombin concentrations (Fig. 3, B and C). Similar concentrations of LA were without effect (Fig. 3, D–F). These data indicate that in addition to blocking aggregation, nitroilnolate inhibits platelet responses involved in adherence.
Stimulates VASP Phosphorylation—An anti-aggregatory target of NO and eicosanoid signaling in platelets is VASP, a protein involved in platelet cytoskeleton rearrangements, which is phosphorylated at up to three separate sites (serine 157, serine 239, and threonine 278) by cAMP- and cGMP-dependent protein kinases. Although both kinases can phosphorylate all three residues, at early time points serine 157 and serine 239 are preferentially phosphorylated by cAMP- and cGMP-dependent protein kinases, respectively (21). Targeted deletion of VASP results in an enhanced agonist-induced aggregation of platelets, demonstrating its central role in inhibition of platelet activation (23, 24). Following a 3-min incubation of platelets with 10 μM LNO₂, a band at 50 kDa indicating phosphorylation at serine 157 was observed (Fig. 4A); however, no immunoreactivity toward an antibody that detects phosphorylation at serine 239 was found (Fig. 4B). Incubation of platelets with 10 μM LA did not induce VASP phosphorylation at either serine residue (Fig. 4, C and D). For comparison, treatment of platelets with the NO donor DEA-NONOate stimulated phosphorylation at serine 157 but also stimulated extensive phosphorylation at serine 239 (Fig. 4, B and D). In aggregate,
these data implicate VASP phosphorylation at serine 157 in the anti-aggregatory mechanism of LNO₂.

LNO₂ Does Not Alter Platelet cGMP Levels—To examine a role for NO and guanylyl cyclase in platelet inhibition by LNO₂, platelet cGMP content was measured. Platelets treated with LNO₂ either in the absence or presence of the phosphodiesterase inhibitor IBMX did not have elevated cGMP (Fig. 5A). The response of platelets to low concentrations of an NO donor (DEA-NOOate) in comparison showed significant increases in cGMP content. The lack of effect of the NO scavenger oxyHb and guanylyl cyclase inhibitor 1H-(1,2,4)oxadiazole[4,3-a]quinoxalin-1-one (ODQ) (25) on the inhibition of platelet aggregation by LNO₂ further excluded NO involvement in LNO₂-mediated platelet inhibitory actions (Fig. 5B).

**FIG. 3.** LNO₂ but not LA attenuates thrombin-induced P-selectin expression. Washed platelets (5 × 10⁵ cells) in Tyrode's buffer containing 4 mM GPRP, 1 mM CaCl₂, FITC-conjugated mouse anti-human P-selectin monoclonal antibody, or FITC-conjugated mouse IgG₁ monoclonal immunoglobulin isotype control were incubated at 37 °C for 2 min with or without 50 μM LNO₂. Then 0.4 unit/ml thrombin was added and samples incubated for a further 10 min at 37 °C. Samples were fixed and then diluted with Tyrode's buffer and stored at 4 °C until FACScan analysis. A, histogram showing thrombin-induced fluorescence shift resulting from P-selectin exposure. B, inhibition of thrombin-induced fluorescence shift by LNO₂ in the same platelet isolate as A. C, percentage of cells in population designated M1 of A and B (n = 3, mean ± S.D.). Results of a representative experiment are shown, and experiments were repeated on at least three donors. D, histogram showing thrombin-induced fluorescence shift resulting from P-selectin exposure. E, no inhibition of thrombin-induced fluorescence shift by LA in the same platelet isolate as D. F, percentage of cells in population designated M1 of D and E (n = 3, mean ± S.D.). Results of a representative experiment are shown, and experiments were repeated on at least three donors.
LNO₂ in the presence of IBMX indicates that synthesis of cAMP has occurred. Incubation of platelets with LNO₂ in the presence of IBMX led to significant increases in intraplatelet cAMP generation and platelet PDE activity. The anti-aggregatory effect of LNO₂ is attenuated by AC inhibition as detailed under Materials and Methods. A, Western blot probing for serine 157 as determined by a shift in molecular mass from 46 to 50 kDa. B, Western blot probing for immunoreactivity toward an antibody specific for phosphorylation at serine 239. C, Western blot probing for phosphorylation at serine 239. D, Western blot probing for immunoreactivity toward an antibody specific for phosphorylation at serine 239.

The Anti-aggregatory Effect of LNO₂ Is Attenuated by AC Inhibition—To determine whether LNO₂ blocked platelet aggregation via cAMP-dependent mechanisms, the AC inhibitor DDA was included in aggregation assays (26). Because this is a weak inhibitor of AC, aggregation was measured in the presence of several concentrations of LNO₂, which gave partial to weak inhibition of AC. The AC inhibitor SQ22536 that is less effective than DDA also restored aggregation to thrombin (26) (Fig. 6). Inclusion of DDA led to significant recovery of the anti-aggregatory effects of LNO₂. In addition, a second AC isoform hydrolyzing cAMP in intact platelets. To examine PDE3A selectively, our experiments utilized 1 μM LNO₂, which gave partial to weak inhibition of AC, aggregation was measured in the presence of IBMX, 1 μM or 100 μM DEA-NONOate, and 10 μM LA for 3 min at 37 °C and then diluted with Laemmli buffer and boiled. Samples (corresponding to 4 × 10⁶ platelets) were probed for VASP phosphorylation as described under “Materials and Methods”. A, Western blot probing for serine 157 as determined by a shift in molecular mass from 46 to 50 kDa. B, Western blot probing for immunoreactivity toward an antibody specific for phosphorylation at serine 239. C, Western blot probing for phosphorylation at serine 239. D, Western blot probing for immunoreactivity toward an antibody specific for phosphorylation at serine 239.

Although platelets contain several PDE isoforms, PDE3A has a submicromolar $K_m$ for cAMP and is therefore the predominant isoform hydrolyzing cAMP in intact platelets. To examine PDE3A selectively, our experiments utilized 1 μM [³⁵S]cAMP as substrate (27). To ensure linear rates of cAMP hydrolysis, conditions were chosen where less than 10% substrate depletion occurred. Following a 30-min incubation of platelet homogenates corresponding to 1.2 × 10⁶ cells with radiolabeled substrate, significant inhibition (42%) of [³⁵S]cAMP formation was found in the presence of 12 μM LNO₂ (Fig. 7C).
DISCUSSION

Nitrated lipids such as LNO₂ formed via reactions of NO-derived reactive nitrogen species with unsaturated lipid have significant potential to serve as mediators of cell signaling and inflammation. In this study, the influence of synthetic LNO₂ on human platelet functional responses was examined to define how nitrated lipids might behave in vivo. LNO₂ inhibited platelet activation in response to thrombin, as indicated by aggregation and P-selectin exposure, via cAMP-dependent mechanisms including VASP phosphorylation at serine 157 and attenuation of thrombin-induced Ca²⁺ mobilization. In contrast, the parent lipid (LA) and the nitrated amino acid (3-NT) were without effect indicating that this was specific for LNO₂ and not all long chain fatty acids or nitrated compounds.

Nitration/nitrosation of biomolecules through reaction with multiple NO-derived reactive species occurs in diverse inflammatory diseases and has several potential consequences for biological systems. These include (i) loss of structural integrity and cell death, (ii) stimulation/inhibition of cell signaling pathways, and (iii) formation of species with novel bioactivities. This latter effect may occur at low fluxes of reactive nitrogen species in which repair and defense mechanisms effectively

[Fig. 6. AC inhibitors restore aggregation in the presence of LNO₂. A, DDA attenuates the anti-aggregatory effects of LNO₂. Washed platelets (2 x 10⁶ ml⁻¹) in Tyrode’s buffer, 1 mM CaCl₂ were stimulated to aggregate with 0.04 unit ml⁻¹ thrombin with or without a 2-min prior incubation with LNO₂. Where added, 100 μM DDA was added 2 min prior to LNO₂ (n = 3, mean ± S.D.). An asterisk represents p < 0.05 compared with thrombin alone at that concentration of LNO₂, following the unpaired Student’s t test. B, SQ22536 attenuates the anti-aggregatory effects of LNO₂. Washed platelets (2 x 10⁶ ml⁻¹) in Tyrode’s buffer, 1 mM CaCl₂ were stimulated to aggregate with 0.04 unit ml⁻¹ thrombin with or without a 2-min prior incubation with LNO₂. Where added, 20 μM SQ22536 was added along with 5 μM LNO₂ (n = 3, mean ± S.D.). An asterisk represents p < 0.05 following the unpaired Student’s t test. Results of a representative experiment are shown, and experiments were repeated at least three times using different donors.

[Fig. 7. cAMP is elevated by LNO₂ through increased synthesis and inhibition of PDE3A. A, LNO₂ causes elevations in intraplatelet cAMP levels. Washed platelets (2 x 10⁶) in 0.5 ml of Tyrode’s buffer containing 1 mM CaCl₂ were incubated with or without 1 mM IBMX, 10 μM LNO₂ for 1 min at 37 °C. Reactions were stopped by the addition of 6% trichloroacetic acid, and samples were processed as described under “Materials and Methods” for cAMP content (n = 8, mean ± S.D). An asterisk represents p < 0.05 following the unpaired Student’s t test. Results of a representative experiment are shown, and experiments were repeated at least three times using different donors. B, LA does not cause elevations in intraplatelet cAMP levels. Washed platelets (2 x 10⁶) in 0.5 ml of Tyrode’s buffer containing 1 mM CaCl₂ were incubated with or without 1 mM IBMX, 10 μM LA for 1 min at 37 °C. Reactions were stopped by the addition of 6% trichloroacetic acid and samples processed as described under “Materials and Methods” for cAMP content (n = 8, mean ± S.D). C, LNO₂ inhibits platelet cAMP hydrolysis. 10 μl of platelet homogenate was incubated with 1 μM [³²P]cAMP with or without 12 μM LNO₂ for 30 min at 30 °C as described in “Materials and Methods.” Nucleotides were separated and AMP quantified by reverse-phase HPLC as described in “Materials and Methods” (n = 3, mean ± S.D.). An asterisk represents p < 0.005 following the unpaired Student’s t test.
prevent cytotoxicity. For example, ONOO⁻ inhibits platelet activation at least in part due to formation of S-nitrosoglutathione, although other mechanisms, including nitrosylation of proteins such as tissue-type plasminogen activator may also be involved (28–31). Also, ONOO⁻ reacts with glycerol, urate, and glucose to yield organic nitrate derivatives, some of which are metabolized by cells to release NO and cause vasorelaxation (32, 33). Thus, a precedent exists to suggest that NO-derived lipid derivatives may serve as signaling mediators.

Nitric oxide-derived reactive nitrogen species, including ONOO⁻ and NO₂⁻/N₂O₃, react with unsaturated lipid to generate nitrated species. Although the reaction pathways leading to its formation have been characterized (4–7), the biological consequences of lipid nitration remain undefined. Oxidized lipid derivatives, such as eicosanoids and isoprostanes, possess bioactivity through receptor signaling pathways and play central roles in immune responses and inflammatory diseases including asthma, arthritis, and cardiovascular disease. Because of the structure, nitrated lipids may also signal through receptor-dependent mechanisms or directly interfere with eicosanoid signaling. Alternatively, metabolism of the nitrogen-containing group may yield NO in selective cell compartments and cause activation of soluble guanylyl cyclase, similar to nitrated glycerol, glucose, and urate. Human platelets were chosen as the model in which to study the bioactivity of LNO₂ because they respond acutely to both NO and eicosanoids and are centrally involved in regulating vascular function and injury.

Inhibition of platelet aggregation and P-selectin expression by LNO₂ was associated with VASP phosphorylation at serine 157 and decreased Ca²⁺ mobilization in response to thrombin. Both mechanisms are likely to be involved in the inhibitory actions of LNO₂ because (i) elevation of Ca²⁺ alone can stimulate platelet shape change, aggregation, and secretion and (ii) targeted deletion of VASP results in platelet hyperresponsiveness to thrombin and collagen (23, 24). Using VASP knockout mice, inhibition of Ca²⁺ mobilization was found to be independent of VASP phosphorylation in human platelets (24). This suggests that a common stimulus upstream of VASP and Ca²⁺ is separately responsible for these effects of LNO₂. Either cAMP- or cGMP-dependent protein kinases can induce VASP phosphorylation and attenuate Ca²⁺ mobilization; however, serine 157 phosphorylation of VASP is characteristic of cAMP-dependent protein kinase activity. In this study, 2-fold elevations in intraplatelet cAMP were found, and two separate AC inhibitors partially restored aggregation (Figs. 6 and 7). These observations, coupled with increased VASP phosphorylation at serine 157, indicate that these cAMP elevations are sufficient to activate cAMP-dependent protein kinase in LNO₂-treated platelets. Furthermore, 2-fold cAMP increases have been shown to produce maximum activation of cAMP-dependent protein kinases (34, 35) suggesting that this pathway is also responsible for LNO₂-dependent attenuation of calcium mobilization and inhibition of platelet aggregation. In the absence of thrombin addition, LNO₂ alone caused a small release of Ca²⁺ from intracellular stores (Fig. 2). Although we cannot rule out a role for this in the platelet inhibitory actions of LNO₂, the restoration of aggregation by AC inhibition indicates that cAMP-dependent pathways, which attenuate agonist-induced Ca²⁺ elevations, are more likely to be responsible (Fig. 6).

LNO₂ blocked cAMP hydrolysis to AMP by platelet lysates, indicating that cAMP elevation by LNO₂ in intact platelets involves PDE inhibition (primarily PDE3A) (Fig. 7C). However, in the presence of the PDE inhibitor IBMX, LNO₂ also raised intraplatelet cAMP synthesis indicating that activation of AC also occurred (Fig. 7A). These data suggest that dual regulation of AC and PDE activities by LNO₂ may mediate cAMP elevations in platelets.

Unexpectedly, the platelet anti-aggregatory action of LNO₂ did not involve NO. This is distinct from the action of nitrated urate and glucose, which release NO on reduction/metabolism by cells (32, 33). These latter NO donors are nitrates (i.e. R-ONO₂) and are structurally similar to clinically used NO donors such as glycyl trinitrate. In contrast, LNO₂ contains an RNO₂ group (nitro–) and is expected to be considerably more stable and less susceptible to reductive metabolism by cells. Interestingly, LNO₂ and nitrated arachidonate mediate vasorelaxation in an ODQ-inhibitable manner suggesting that unlike platelets, smooth muscle has the capacity to metabolize nitro-lipids to bioactive NO (8).²

Anti-platelet activity of LNO₂ was observed at exogenously added concentrations of 2–5 μM (Fig. 1). Although we do not yet know the in vivo concentrations of nitrated lipids, it is important to note that several parameters can dramatically alter the potency of lipid signaling mediators including partitioning into membranes and lipoproteins and binding to hydrophobic regions of proteins. For example, although endogenous hydroxyeicosatetraenoic acid generation by interleukin-4-stimulated human monocytes in the absence of added arachidonate is generally below the limit of detection, it is sufficient to activate endogenous peroxisome proliferator-activated receptor-γ (PPARγ) (36). In contrast, PPARγ activation by exogenous hydroxyeicosatetraenoic acids requires 10–30 μM (36).

Nitration reactions known to occur in vivo, such as those caused by ONOO⁻ or NO₂ are often associated, but not always causally linked, with deleterious effects such as loss of structural integrity and cell death (37). The data presented here indicate that nitration of lipids can lead to generation of anti-thrombotic mediators, an effect that would be considered vascular-protective. In summary, anti-platelet effects of a nitrated lipid, LNO₂, were found. These observations reveal novel mechanisms by which nitrated biomolecules may modulate cell activation, and they show how the presence of the nitro functional group can alter the bioactivity of lipids.

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Nitrolinoleate Inhibits Platelet Activation by Attenuating Calcium Mobilization and Inducing Phosphorylation of Vasodilator-stimulated Phosphoprotein through Elevation of cAMP

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