15-Lipoxygenase Catalytically Consumes Nitric Oxide and Impairs Activation of Guanylate Cyclase*

(Received for publication, November 25, 1998, and in revised form, March 27, 1999)

Valerie B. O'Donnell‡§, Kenneth B. Taylor¶, Sampath Parthasarathy¶, Hartmut Kühn***, Doris Koesling‡‡, Andreas Friebe‡‡, Allison Bloodsworth‡§, Victor M. Darley-Usmar§ §§, and Bruce A. Freeman§§ ††

From the Departments of ‡‡Anesthesiology, §§Biochemistry and Molecular Genetics, and §§§Pathology and the ††Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, Alabama 35233, the ¶¶Department of Obstetrics and Gynecology, Emory University, Atlanta, Georgia 30322, the **Institute of Biochemistry, Humboldt University, Hessische Strasse 3-4 Berlin, Germany, and the §§Institute of Pharmacology, Freie University, Thielallee 69-73 Berlin, Germany

Analysis of purified soybean and rabbit reticulocyte 15-lipoxygenase (15-LOX) and PA317 cells transfected with human 15-LOX revealed a rapid rate of linoleate-dependent nitric oxide (NO) uptake that coincided with reversible inhibition of product ((13S)-hydroperoxymonoenoic acid, or (13S)-HPODE) formation. No reaction of NO (up to 2 μM) with either native (Ered) or ferric LOXs (0.2 μM) metal centers to form nitrosyl complexes occurred at these NO concentrations. During HPODE-dependent activation of 15-LOX, there was consumption of 2 mol of NO/mol of 15-LOX. Stopped flow fluorescence spectroscopy showed that NO (2.2 μM) did not alter the rate or extent of (13S)-HPODE-induced tryptophan fluorescence quenching associated with 15-LOX activation. Additionally, NO does not inhibit the anaerobic peroxidase activity of 15-LOX, inferring that the inhibitory actions of NO are due to reaction with the enzyme-bound lipid peroxy radical, rather than impairment of (13S)-HPODE-dependent enzyme activation. From this, a mechanism of 15-LOX inhibition by NO is proposed whereby reaction of NO with EredLOO generates EredLOOH, which hydrolyzes to (13S)-HPODE and nitrite (NO2−). Reaction of EredLOOH considerably slower than dioxygenase activity, is then required to complete the catalytic cycle and leads to a net inhibition of rates of (13S)-HPODE formation. This reaction of NO with 15-LOX inhibited NO-dependent activation of soluble guanylate cyclase and consequent cGMP production. Since accelerated NO production, enhanced 15-LOX gene expression, and 15-LOX product formation occurs in diverse inflammatory conditions, these observations indicate that reactions of NO with lipoxygenase peroxy radical intermediates will result in modulation of both NO bioavailability and rates of production of lipid signaling mediators.

Lipoxygenases are a family of ubiquitously expressed non-heme iron-containing enzymes that oxidize the unsaturated fatty acids arachidonate and linoleate to bioactive hydroperoxides and other metabolites (Scheme 1). For example, 5-LOX

\[
\text{E}_{\text{ox}} - \text{H} \rightarrow \text{E}_{\text{ox}} - \text{H}^+ 
\]

generates precursors for leukotrienes, products involved in inflammation and allergic responses (1). 12-Lipoxygenases, present in vascular endothelium, smooth muscle cells, platelets, and leukocytes (2, 3), contribute to vascular cell hypertrophy, proliferation, and hypertensive actions, while 15-LOX is involved in cell development and differentiation, particularly in reticulocytes where 15-LOX oxidation of mitochondrial phospholipids is a trigger for their degradation (2, 4, 5).

A central pathogenic role for 15-LOX in atherosclerosis comes from multiple lines of evidence, in particular the co-localization of 15-LOX mRNA, enzymatic activity, and the relatively specific pattern of isomeric 15-LOX oxygenation products that have been detected in early human and rabbit lesions (6–9). The accumulation and oxidation of low density lipoprotein (LDL) lipids by monocytes and the subsequent accumulation of oxidized lipids and foam cells in the vascular intima is a hallmark of early atherogenesis. In vitro studies have shown

---

* This work was supported by National Institutes of Health Grants P01-HL58418, PO1-HL40456, RO1-HL51245 (to B. A. F. and V. D. U.), and RO1-HL52628 (to S. P.), by a grant from the Parker B. Francis Foundation (to V. B. O.), and by Deutsche Forschungsgemeinschaft Grant Kn 961/2-2 (to H. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom all correspondence should be addressed: Dept. of Anesthesiology, 946 THH, 619 19th St. S., University of Alabama at Birmingham, Birmingham, AL 35233. Fax: 205-934-7437; E-mail: bruce.freeman@uab.edu.

†† To whom all correspondence should be addressed: Dept. of Anesthesiology, 946 THH, 619 19th St. S., University of Alabama at Birmingham, Birmingham, AL 35233. Fax: 205-934-7437; E-mail: bruce.freeman@uab.edu.

The abbreviations used are: LOX, lipoxygenase; LDL, low density lipoprotein; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; HPODE, hydroperoxymonoenoic acid; ETYA, eicosatetraynoic acid; DTPA, diethylenetriamine pentaacetic acid; DEA-NONOate, 2-(N,N-diethylamino)-diazenolate-2-oxide.

---
that macrophage and endothelial cell lipoxygenases readily oxidize externally added LDL and promote metal-dependent lipoprotein oxidation (10–12). In vivo models show that somatic gene transfer of 15-LOX to vessels and transgenic mice co-crossbred with LDL receptor-deficient mice results in increased oxidation of LDL and accumulation of lipid-containing vascular lesions (13, 14). A currently provocative counterpoint to these properties of 15-LOX is the observation that targeted overexpression of rabbit macrophage 15-LOX prevented diet-induced atherosclerosis (15). In contrast, diet-induced atherosclerosis in rabbits is inhibited by administration of a 15-LOX inhibitor having limited direct antioxidant properties (16). In aggregate, these observations encourage better understanding of the interactions of 15-LOX, 15-LOX products, and vascular cells with key mediators of vascular function and atherogenesis, in particular ‘NO.

In vitro, ‘NO can act as a potent antioxidant by scavenging lipid-derived peroxyl and alkoxyl radicals formed in purified or LDL lipids oxidized by Cu^{2+}, azo initiators, peroxynitrite (ONOO\(^-\)), endothelial cells or macrophages (17–24). Inhibition of both plant and mammalian 15-LOX-dependent lipid oxidation by high concentrations of nitric oxide (‘NO) was ascribed to formation of an enzyme-nitrosyl complex (25–27). Nitric oxide can form a nitrosyl complex with the active site of 15-LOX, a single six-coordinate ferrous iron liganded to nitrogen and/or oxygen atoms (28), that is detectable by electron paramagnetic resonance spectroscopy (EPR). The spectrum of the soybean 15-LOX Fe\(^{2+}\)-NO complex contains two species, the first attributed to either high spin ferric iron, formed by transfer of an electron from Fe\(^{3+}\) to ‘NO, or an S = 3/2 system resulting from antiferromagnetic coupling of axial (D > E) high spin ferrous iron to ‘NO (29–31). The dissociation constant (K\(_D\)) for formation of this species is 95 \(\mu\text{M}\) for soybean 15-LOX at pH 7 (31). The second component of the EPR spectra has been suggested to be a high spin Fe\(^{2+}\)-NO complex and requires ‘NO concentrations of at least 400 \(\mu\text{M}\) for detection (30). Three lines of evidence suggested that oxidation of the reduced iron by ‘NO, leading to enzyme activation, might occur following formation of the nitrosyl complex. Addition of ‘NO to anaerobic ferrous 15-LOX resulted in immediate appearance of a pale yellow color, identical to that of the ferric enzyme found on treating native enzyme with HPODE (29). Additionally, EPR and x-ray absorption analysis of rabbit 15-LOX showed that incubation with millimolar concentrations of ‘NO yielded ferric iron species (25). Importantly, the ‘NO concentrations required for formation of the Fe\(^{2+}\)-NO complex significantly exceed those (a) required to inhibit soybean 15-LOX catalytic activity (32) and (b) maximal ‘NO levels typically found in biological systems, typically <1–5 \(\mu\text{M}\) (33–34), suggesting that 15-LOX inhibition does not involve Fe\(^{2+}\)-NO complex formation. Thus, other mechanisms are likely to be operative in the ‘NO-mediated inhibition of lipoxygenase-dependent lipid oxidation.

In addition to the ferrous iron, several species form during 15-LOX catalysis that could potentially react with ‘NO and lead to enzyme inhibition. These include enzyme-bound lipid peroxyl, alkoxyl, and carbon-centered radicals. Termination reactions of ‘NO with non-lipid-derived radicals are fast, occurring at essentially diffusion-limited rates (35, 36). In addition, kinetic studies indicate that ‘NO also reacts extremely rapidly with lipid-derived radicals in aqueous systems (18, 22, 36). Since ‘NO can diffuse into the 15-LOX active site, we hypothesized that enzyme-bound lipid-derived radicals are accessible to ‘NO during turnover.

In biological systems, efficient removal of ‘NO following its synthesis by nitric oxide synthases is critical in maintaining control of vascular tone. While oxyhemoglobin, present in erythrocytes, reacts with and removes ‘NO in the vascular space (37), little is known regarding the processes that remove ‘NO in the subendothelial compartment. The half-life of ‘NO in hemoglobin-free cascade bioassays is only 3–5 s (38), far too short to be accounted for by simple autoxidation, suggesting that cell-dependent ‘NO consumption also occurs. Under pathological conditions ‘NO consumption becomes excessive, with complete loss of the pathways dependent upon activation of soluble guanylate cyclase (39). One component of the inhibition of the ‘NO signaling is the reaction of endothelial-derived relaxation factor with superoxide (O\(_2^\cdot\)) to yield peroxynitrite (ONOO\(^-\)) (39–42). Since reactions of O\(_2^\cdot\) do not account for complete loss of ‘NO signaling to smooth muscle cells (42), other unidentified metabolic pathways that contribute to ‘NO consumption are inferred. Such an alternative are the free radical intermediates populated during the turnover of enzymes mediating electron transfer reactions. During development of diet-induced atherosclerosis in rabbits, impairment of the vascular response to endothelial-derived relaxation factor or ‘NO is a consistent finding (40–42). Since 15-LOX is known to be present in the subendothelial layer in atherosclerotic lesions and ‘NO can concentrate in lipophilic milieu (33), it was of interest to investigate whether reactions of lipid radicals generated by 15-LOX can proceed at a significant enough rate to alter cellular ‘NO levels and impact on ‘NO-dependent signaling.

Herein, the reactions of soybean and mammalian 15-LOX with ‘NO at concentrations encompassing those found under physiological and pathological conditions were examined. Our results indicate that there are two distinct sites for ‘NO reaction during 15-LOX catalysis, and that ‘NO consumption occurs during inhibition of 15-LOX. It was also observed that, during 15-LOX catalysis of lipid oxidation, lipid radical reactions with ‘NO in turn inhibited ‘NO-mediated activation of soluble guanylate cyclase and the subsequent formation of cGMP. In aggregate, these observations reveal that lipoxygenase reactions with ‘NO can inhibit both lipoxygenase catalytic activity and ‘NO-dependent signal transduction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit reticulocyte 15-LOX was purified to electrophoretic homogeneity from the lysate of a reticulocyte-rich blood cell suspension by fractionated ammonium sulfate precipitation and two consecutive steps of fast liquid protein chromatography (43). Soluble guanylate cyclase was purified from bovine lung to homogeneity by immunoaffinity chromatography as previously (29) Linoleic acid was from Nu-Chek Prep (Elysian, MN). Unless stated otherwise, all enzymes and chemicals, including soybean 15-LOX type V was purchased from Sigma.

**Culture of 15-LOX-transfected PA317 Cells**—Murine PA317 fibroblasts stably transfected with either PLLORN or PLLZRN (44) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum supplemented with glutamine and antibiotics. The plasmids PLLORN and PLLZRN are derived from the retroviral vector, pLDRNL, where the LDL receptor cDNA sequence has been replaced with either human 15-LOX cDNA or \(\beta\)-galactosidase cDNA (lacZ), respectively (44, 45). The 15-LOX transfected clones are designated clone 12 and have been derived by clonal selection of the PLLORN-transfected cells, and possess 10–20-fold greater 15-LOX specific activity than the lacZ-infected controls (43).

**Synthesis of (13S)-Hydroperoxycadadienoic acid ((13S)-HPODE)**—(13S)-Hydroperoxycadadienoic acid (13S)-HPODE was synthesized as described (46). Product analysis using both normal and chiral phase HPLC (see “HPLC Analysis of Reaction Products” for details) indicated HPODE products were 97% (13S)-HPODE and 3% (13R)-HPODE.

**15-LOX Assay Systems**—To accurately determine enzyme concentrations, titrations with (13S)-HPODE were monitored fluorimetrically, where quenching of intrinsic tryptophan fluorescence during activation is mediated by 1 mol of HPODE/mol of enzyme (47). 15-LOX activity was assayed spectrophotometrically at 234 for conjugated diene forma-
further 7.6 mHPLC) was carried out on a Spherisorb S5W column (Phase-Sep 250 x 4.6 mm, 5-μm particle size) eluted with n-hexane:2-propanol:acetic acid, 100:2.0:1, v/v, at 1 ml/min. For determination of HPODE enantiomers, a Chiralcel OD column (J.T. Baker, 250 x 4.6 mm, 5-μm particle size) was used with n-hexane:2-propanol:acetic acid, 100:2.0:1, v/v, at 1 ml/min.

Liquid Chromatography-Mass Spectrometry—To examine for nitric oxide consumption by 15-LOX transfectants expressing either human 15-LOX or β-galactosidase were added to 1 ml of 0.1 mM potassium phosphate buffer (pH 7.4), 0.2% sodium cholate, 100 mM DTPA, and monitored for rates of NO consumption at 37 °C. Panel A, nitric oxide consumption by 15-LOX transfectants. Left trace, NO (1.9 μM) was added to 15-LOX transfectants (1.7 x 10⁶ cells) and monitored before and after addition of 500 μM linoleate (LA). Once NO was consumed, two further 1.9 μM NO additions were made. Right trace, decay rate of NO in the absence of cells. Panel B, nitric oxide consumption by β-galactosidase transfectants: NO (1.9 μM) was added to β-galactosidase transfectants (1.7 x 10⁶ cells) and monitored before and after addition of 500 μM linoleate (LA). Once NO was consumed, two further 1.9 μM NO additions were made. Panel C, inhibition of cellular nitric oxide consumption by ETYA. 15-LOX-transfectants (2 x 10⁶ cells) were preincubated in 1 ml of PBS in the NO electrode for 10 min at 37 °C with/without 100 μM ETYA before addition of 1.9 μM NO and 500 μM linoleate (LA). Results shown are of a representative experiment repeated at least three times.

Turnover-Dependent Consumption of NO by Mammalian LOX

Characterization of NO Loss in Reaction Systems—Nitric oxide (1.9 μM) decay in 1 ml of aerobic phosphate buffer followed first order kinetics with a rate constant (kobs) of 4.1 ± 0.6 x 10⁻³ s⁻¹. Aerobic oxidation of NO follows second order kinetics (51), but at the low NO concentrations utilized in this study the rate of NO autoxidation is slow and alternative reactions that follow first order kinetics predominate (e.g. NO-electrode reaction, diffusion into gas phase). Using the calculated kobs, the rate of background NO loss can therefore be calculated at any point during the time course.

Cells Transfected with Human 15-LOX Consume NO during Linoleate Oxidation—Rates of NO decay were higher than in buffer alone when added to murine fibroblast PA317 cells expressing either 15-LOX or β-galactosidase (controls) and no longer followed first order kinetics (Fig. 1A). For example, at 1 μM NO, the rate of decay is 0.25 μM min⁻¹ in buffer alone, or 0.64 ± 0.08 μM min⁻¹ and 0.61 ± 0.07 μM min⁻¹ (mean ± S.D.,

Fig. 1. Nitric oxide is consumed by 15-LOX transfectants. PA317 transfectants expressing either human 15-LOX or β-galactosidase were added to 1 ml of 0.1 mM potassium phosphate (pH 7.4). 0.2% sodium cholate, 100 mM DTPA, and monitored for rates of NO consumption at 37 °C. Panel A, nitric oxide consumption by 15-LOX transfectants. Left trace, NO (1.9 μM) was added to 15-LOX transfectants (1.7 x 10⁶ cells) and monitored before and after addition of 500 μM linoleate (LA). Once NO was consumed, two further 1.9 μM NO additions were made. Right trace, decay rate of NO in the absence of cells. Panel B, nitric oxide consumption by β-galactosidase transfectants: NO (1.9 μM) was added to β-galactosidase transfectants (1.7 x 10⁶ cells) and monitored before and after addition of 500 μM linoleate (LA). Once NO was consumed, two further 1.9 μM NO additions were made. Panel C, inhibition of cellular nitric oxide consumption by ETYA. 15-LOX-transfectants (2 x 10⁶ cells) were preincubated in 1 ml of PBS in the NO electrode for 10 min at 37 °C with/without 100 μM ETYA before addition of 1.9 μM NO and 500 μM linoleate (LA). Results shown are of a representative experiment repeated at least three times.

rapid kinetic stopped-flow measurements of (13S)-HPODE-induced 15-LOX Fluorescence Quenching—As an index of activation, the rate and extent of intrinsic tryptophan fluorescence quenching by (13S)-HPODE was monitored with and without NO. (13S)-HPODE stock (14 μM) was prepared in 2 ml of 0.1 mM potassium phosphate buffer, pH 7.4, with 0.2% cholate and 100 mM DTPA. Soybean 15-LOX was diluted to 0.76 μM in 0.1 mM potassium phosphate buffer, pH 7.4, containing 100 mM DTPA, immediately prior to use. The HPODE and 15-LOX solutions were placed in separate drive syringes for assay, and equal volumes were mixed during each measurement. Rapid kinetic stopped-flow studies were carried out on a Hi-Tech SF-53 stopped-flow spectrophotometer with a dead time of 1.2 ms. Changes in fluorescence emission above 320 nm were monitored using a cut-off filter, with excitation at 280 nm. Nitric oxide was added to HPODE solution with a final concentration of 0.5 mM additions were made. When all linoleate was consumed, NO consumption rates monitored. As NO was consumed, further 7.6 μM additions were made. When all linoleate was consumed, NO uptake slowed and samples were immediately removed and placed on ice until extraction of lipids for HPLC analysis. Controls were prepared by allowing 15-LOX to oxidize 100 μM linoleate in the absence of NO.

Leukomethylene Blue Assay for Hydroperoxides—Sample (50 μl) was added to 100 μl of leukomethylene blue reagent (5 mg of leukomethylene blue, 8 ml of dimethylformamide, 1.4 g of Triton X-100, 5.5 mg of hemoglobin in 100 ml of 0.05 M potassium phosphate buffer, pH 5.0) and absorbance measured at 650 nm using a microplate reader (50).

HPLC Analysis of Reaction Products—Contaminating NO2 was removed by adding equal volumes of 1% sulfanilamide, 3% HCl, and 0.02% N-(1-naphthyl)-ethylenediamine to samples. Following this, lipids were twice-extracted with two volumes of diethyl ether. Extracts were placed in separate drive syringes for assay, and equal volumes were mixed during each measurement. Rapid kinetic stopped-flow studies were carried out on a Hi-Tech SF-53 stopped-flow spectrophotometer with a dead time of 1.2 ms. Changes in fluorescence emission above 320 nm were monitored using a cut-off filter, with excitation at 280 nm. Nitric oxide was added to HPODE solution with a final concentration of 5.4 μM and immediately placed into the drive syringe for assay.

RESULTS

Characterization of NO Loss in Reaction Systems—Nitric oxide (1.9 μM) decay in 1 ml of aerobic phosphate buffer followed first order kinetics with a rate constant (kobs) of 4.1 ± 0.6 x 10⁻³ s⁻¹. Aerobic oxidation of NO follows second order kinetics (51), but at the low NO concentrations utilized in this study the rate of NO autoxidation is slow and alternative reactions that follow first order kinetics predominate (e.g. NO-electrode reaction, diffusion into gas phase). Using the calculated kobs, the rate of background NO loss can therefore be calculated at any point during the time course.

Cells Transfected with Human 15-LOX Consume NO during Linoleate Oxidation—Rates of NO decay were higher than in buffer alone when added to murine fibroblast PA317 cells expressing either 15-LOX or β-galactosidase (controls) and no longer followed first order kinetics (Fig. 1A). For example, at 1 μM NO, the rate of decay is 0.25 μM min⁻¹ in buffer alone, or 0.64 ± 0.08 μM min⁻¹ and 0.61 ± 0.07 μM min⁻¹ (mean ± S.D.,
Turnover-dependent Consumption of NO by Mammalian LOX

Diene conjugation rates were measured in 2 ml of 0.1 M potassium phosphate, 500 μM linoleate, 0.2% cholate, and 100 μM DTPA, pH 7.4 at 37 °C with stirring. Nitric oxide (1.9 μM) was then added during turnover. Rates of NO consumption were measured in 1 ml of phosphate buffer, 0.2% cholate, 500 μM linoleate, 100 μM DTPA, pH 7.4 at 37 °C with stirring, and rates of NO disappearance monitored using an NO electrode. Results shown are mean ± S.D., n = 3.

For example, with 1.9 μM NO, activity was inhibited 80% (Fig. 2A). If NO was added before 15-LOX, inhibition appeared as a prolongation of the lag phase (Fig. 2A). With the soybean 15-LOX, there was less inhibition than with the rabbit 15-LOX (Fig. 2B). For example, when 1.9 μM NO was added during turnover, only 40% inhibition occurred.

For both rabbit and soybean 15-LOX, inhibition was reversible, with time of inhibition directly related to the concentration of added NO. Since soybean 15-LOX does not self-inactivate, full recovery of activity was observed following the inhibition phase. Plotting the time of inhibition versus NO concentration for the rabbit 15-LOX yielded a linear relationship with similar slopes, independent of whether NO was added to samples before 15-LOX (m = 14.7 s μM⁻¹, r = 0.97), or after, during dioxygenase turnover (m = 14.9 s μM⁻¹, r = 0.98) (Fig. 3).

Nitric Oxide Is Consumed during 15-LOX Turnover—Rates of NO consumption by both the rabbit and soybean 15-LOXs were examined in the presence of linoleate (Table I, Fig. 4). NO uptake of NO occurred in the absence of linoleate, or if linoleate was replaced with the 15-LOX product (13S)-HPODE (Fig. 4, A and B). Addition of 750 units/ml CuZn superoxide dismutase to 15-LOX plus linoleate did not affect rates of NO consumption, indicating that superoxide (O₂⁻) was not the species reacting with NO (data not shown). The rates of NO consumption directly paralleled inhibition of 15-LOX activity. The apparent Km for NO consumption was 1.7 ± 0.48 μM for the soybean 15-LOX (Fig. 4C). Since 15-LOX concentrations are 10⁻⁶ to 10⁻⁵ molar lower than NO (13 nM rabbit 15-LOX, 3.5 nM soybean 15-LOX, 1.9 μM NO), it is concluded that NO consumption is a catalytic process requiring dioxygenase turnover.

Effect of NO on Anaerobic Peroxidase Activity—To probe the mechanism of 15-LOX inhibition and NO uptake, effects of NO on anaerobic peroxidase activity were examined (Scheme 2). Since soybean 15-LOX does not self-inactivate, anaerobic peroxidase can be measured by allowing the enzyme to oxidize

### TABLE I

| Lipoygenase   | Conjugated diene formation | Nitric oxide consumption |
|---------------|----------------------------|--------------------------|
|               | –NO                       | +NO                      |
| m mol min⁻¹ μmol⁻¹ 15-LOX |
| Rabbit 15-LOX | 3.6 ± 0.15                | 0.73 ± 0.13              | 0.69 ± 0.03              |
| Soybean 15-LOX| 28.6 ± 1.0                | 18.1 ± 0.119             | 2.9 ± 0.8               |

For example, with 1.9 μM NO, activity was inhibited 80% (Fig. 2A). If NO was added before 15-LOX, inhibition appeared as a prolongation of the lag phase (Fig. 2A). With the soybean 15-LOX, there was less inhibition than with the rabbit 15-LOX (Fig. 2B). For example, when 1.9 μM NO was added during turnover, only 40% inhibition occurred.

For both rabbit and soybean 15-LOX, inhibition was reversible, with time of inhibition directly related to the concentration of added NO. Since soybean 15-LOX does not self-inactivate, full recovery of activity was observed following the inhibition phase. Plotting the time of inhibition versus NO concentration for the rabbit 15-LOX yielded a linear relationship with similar slopes, independent of whether NO was added to samples before 15-LOX (m = 14.7 s μM⁻¹, r = 0.97), or after, during dioxygenase turnover (m = 14.9 s μM⁻¹, r = 0.98) (Fig. 3).

Nitrile Oxide Is Consumed during 15-LOX Turnover—Rates of NO consumption by both the rabbit and soybean 15-LOXs were examined in the presence of linoleate (Table I, Fig. 4). NO uptake of NO occurred in the absence of linoleate, or if linoleate was replaced with the 15-LOX product (13S)-HPODE (Fig. 4, A and B). Addition of 750 units/ml CuZn superoxide dismutase to 15-LOX plus linoleate did not affect rates of NO consumption, indicating that superoxide (O₂⁻) was not the species reacting with NO (data not shown). The rates of NO consumption directly paralleled inhibition of 15-LOX activity. The apparent Km for NO consumption was 1.7 ± 0.48 μM for the soybean 15-LOX (Fig. 4C). Since 15-LOX concentrations are 10⁻⁶ to 10⁻⁵ molar lower than NO (13 nM rabbit 15-LOX, 3.5 nM soybean 15-LOX, 1.9 μM NO), it is concluded that NO consumption is a catalytic process requiring dioxygenase turnover.

Effect of NO on Anaerobic Peroxidase Activity—To probe the mechanism of 15-LOX inhibition and NO uptake, effects of NO on anaerobic peroxidase activity were examined (Scheme 2). Since soybean 15-LOX does not self-inactivate, anaerobic peroxidase can be measured by allowing the enzyme to oxidize

### SCHEME 2

Anaerobic peroxidase activity of 15-LOX. Linoleate (LH) is first oxidized to an alkyl radical by the ferric enzyme (Eox), forming reduced enzyme with bound alkyl radical (Ered·L). In the absence of O₂, the lipid radical dissociates from the active site, leaving reduced enzyme (Ered). To complete the cycle, peroxide product (LOOH) is reduced by a peroxidase activity of the enzyme, forming an alkoxyl radical, LO·, that dissociates to regenerate active enzyme (Eox).

---

### FIG. 2

Nitrile oxide reversibly inhibits 15-LOX. Rabbit (panel A, 6.5 nM enzyme) or soybean (panel B, 0.88 nM enzyme) 15-LOX was added to 2 ml of 0.1 M potassium phosphate (pH 7.4), 0.2% cholate, 100 μM DTPA, 500 μM linoleate, and conjugated diene formation monitored at 235 nm. Nitric oxide (1.9 μM) was added where indicated by arrows. Results shown are of a representative experiment repeated at least three times.

### FIG. 3

Time of inhibition of rabbit 15-LOX by nitrile oxide. Nitrile oxide was added to rabbit 15-LOX oxidation assays, as described in Fig. 2, during turnover (■) or prior to 15-LOX addition (○), and time of inhibition determined.

### TABLE I

| Lipoygenase   | Conjugated diene formation | Nitric oxide consumption |
|---------------|----------------------------|--------------------------|
|               | –NO                       | +NO                      |
| m mol min⁻¹ μmol⁻¹ 15-LOX |
| Rabbit 15-LOX | 3.6 ± 0.15                | 0.73 ± 0.13              | 0.69 ± 0.03              |
| Soybean 15-LOX| 28.6 ± 1.0                | 18.1 ± 0.119             | 2.9 ± 0.8               |
linoleic acid until all O₂ is consumed. At this point, peroxidase activity initiates and can be monitored by measuring oxodiene formation. Sequential additions of 1.9 μM NO had no effect on anaerobic peroxidase activity, with base-line irregularities at the point of NO addition being due to opening/closing the sample chamber (Fig. 5).

**Effect of NO (13S)-HPODE-induced Fluorescence Quenching—**Fluorescence quenching of intrinsic tryptophan fluorescence by (13S)-HPODE is associated with 15-LOX activation and conversion from ferrous to ferric oxidation state (47, 52).

Rapid kinetic stopped flow fluorescence studies were carried out using soybean 15-LOX, since large amounts of enzyme were required. No effect of NO on the rate or extent of (13S)-HPODE fluorescence quenching was observed (Fig. 6).

Nitric Oxide Consumption during 15-Lipoxygenase Activation—High concentrations of native rabbit or soybean LOX did not consume NO in the absence of substrate (Fig. 7, A and B). Addition of equivalent amounts of bovine serum albumin shows that the small decrease in NO concentration on addition of 15-LOX alone was due to dilution or nonspecific effects of adding protein (Fig. 7A). However, addition of (13S)-HPODE to 15-LOX-containing samples resulted in NO consumption (Fig. 7, A and B). Plotting NO uptake versus enzyme concentration demonstrated a linear relationship (m = 0.51 ± 0.03, r = 0.99), with the amount of NO consumed being approximately 2 molar eq/mol of 15-LOX (Fig. 7C). HPLC analysis showed that, during activation of 15-LOX by HPODE, NO did not induce HPODE loss (data not shown).

To examine if NO activates 15-LOX, the characteristic lag phase of 15-LOX dioxygenase activity was examined following preincubation with NO. Soybean 15-LOX (4 nm) was incubated for 15 min at 25 °C with 3.8 μM NO before addition of linoleate. By the end, 95% of the added NO would have been oxidized to NO₂, ensuring that residual NO was insufficient to inhibit dioxygenase activity. This preincubation with NO had no effect on the time of the lag (data not shown), indicating that NO was not activating 15-LOX.

**FIG. 5. Effect of nitric oxide on anaerobic peroxidase activity of soybean 15-lipoxygenase.** Soybean 15-LOX (1.76 μM) was added to 2 ml of 0.1 M potassium phosphate (pH 7.4), 500 μM linoleate, 0.2% cholate, 100 μM DTPA, and formation of oxodiene products of peroxidase activity was monitored at 280 nm. Once the reaction had become fully anaerobic through LOX-mediated oxygen consumption, and peroxidase activity initiated (indicated by the increase in rate of absorbance change), aliquots of 1.9 μM NO were added (as indicated by the arrows) and rates monitored. Results shown are of a representative experiment repeated at least three times.

**FIG. 6. Nitric oxide does not alter rate or extent of (13S)-HPODE-induced fluorescence quenching.** (13S)-HPODE stock (14 μM) was prepared in 2 ml of 0.1 M potassium phosphate buffer, pH 7.4, with 0.2% cholate and 100 μM DTPA. Soybean 15-LOX was diluted to 1.76 μM in 0.1 M potassium phosphate buffer, pH 7.4 containing 100 μM DTPA, immediately prior to use. The HPODE and 15-LOX solutions were placed in separate drive syringes for assay, and equal volumes were mixed during each measurement. Where utilized, NO (5.4 μM) was added to the HPODE solution and immediately mixed with 15-LOX. Changes in fluorescence were monitored above 320 nm using a cut-off filter, with excitation at 280 nm. Traces shown are the average of several independent experiments (n = 3 for controls, n = 7 for NO samples).
Fate of Linoleic Acid Oxidized by 15-LOX in the Presence of NO—To determine the fate of linoleate oxidized by 15-LOX in the presence of NO, lipid products were analyzed by HPLC. Using soybean 15-LOX, a fixed amount of substrate could be completely oxidized (100 μM) in the presence or absence of NO and the yield of products compared. Due to concurrent 15-LOX inhibition by NO, the times for complete linoleate oxidation approximately doubled. For organic solvent extraction of free linoleate and its oxidation products, acidic conditions maximized yield. However, small amounts of NO₂⁻, present as a decomposition product of NO, will nitrate lipid hydroperoxides at low pH, thus depleting LOOH and yielding L(O)NO₂ (53). To avoid this artifact during extraction of 15-LOX products, contaminating NO₂⁻ was first removed by reaction with sulfanilamide/HCl and N-(1-naphthyl)ethylenediamine. Control experiments determined that this completely protects LOOH from nitrination by acidified NO₂⁻ (data not shown). By both reverse phase HPLC and quantitation of total hydroperoxide yields, the predominant product was HPDE (Fig. 8, A and B). No difference in HPDE yield occurred if NO was present during dioxygenase turnover. Analysis by normal phase and chiral phase HPLC showed that the HPDE was predominantly the (13S) isomer (Fig. 8C). Electrospray mass spectrometry revealed no nitrogen-containing oxidized lipid species (data not shown), indicating that the product profile of 15-LOX is unchanged by NO.

The Influence of 15-LOX Catalytic Activity on NO-dependent Activation of Soluble Guanylate Cyclase—Addition of the NO donor DEA-NOONoate, in a concentration that yielded ∼400 nM NO in the absence of 15-LOX-mediated peroxyl radical formation, activated soluble guanylate cyclase formation of cGMP from GTP. Addition of 15-LOX alone had no effect on extents of cGMP formation unless substrate (5 μM arachidonate) was added, whereupon there was an extensive and significant 82% decrease in soluble guanylate cyclase activity and cGMP formation (Fig. 9). Since fatty acids may inhibit soluble guanylate cyclase, control experiments were performed to reveal effects of native and oxidized arachidonate on extents of cGMP formation. 15-Lipoxygenase oxidation of 5 μM arachidonate was allowed to go to completion, prior to addition to reaction systems containing soluble guanylate cyclase, [α-²²P]GTP, and DEA-NOONoate. Soluble guanylate cyclase was not significantly inhibited by either native or oxidized arachidonate (data not shown). This affirmed the NO reaction with and consumption by enzyme-bound peroxyl radical intermediates during catalytic cycling of 15-LOX turnover was responsible for inhibition of guanylate cyclase, rather than direct guanylate cyclase inactivation by oxidized lipid products that are formed during 15-LOX oxidation of arachidonate.

**DISCUSSION**

These results show that the vascular signal transduction actions of both NO and lipoxygenase products can be interdependent, since NO inhibits rates of 15-LOX product formation and, in turn, 15-LOX catalytic activity consumes NO and thus impairs guanylate cyclase activation. These findings may in part explain the anti-atherogenic and anti-cell proliferative actions of NO that have been observed in animal models following l-arginine feeding, administration of NO synthase inhibitors, or transfection with nitric-oxide synthase (54–57). The experiments reported herein were designed to model mechanisms of NO interactions with lipoxygenase-mediated lipid oxidation and to reflect conditions that exist in the vascular compartment. For example, the expression of 15-LOX by cells stably transfected with the human 15-LOX gene is approximately the same as mouse peritoneal macrophages (44); thus, the observed cell 15-LOX-dependent rates of NO consumption are well within those to be expected physiologically (∼0.85 nmol min⁻¹ 10⁶ cells⁻¹; Fig. 1). Maximal rates of NO₂⁻/NO₃⁻ production in activated rat peritoneal macrophages or murine RAW264.7 macrophages are 0.1 and 0.2 nmol min⁻¹ 10⁶ cells⁻¹, respectively (58, 59), much lower than the rates of NO consumption observed here. Therefore, it would be expected that the range of 15-LOX expression in normal and diseased vasculature would have a significant effect on NO available for soluble guanylyl cyclase activation and cell-mediated host defenses. This was confirmed by the observation that NO-activated soluble guanylate cyclase formation of cGMP was profoundly suppressed during the catalytic oxidation of arachidonate by 15-LOX.
Fig. 8. Analysis of lipid product profile following nitric oxide consumption by soybean 15-LOX. Panel A, samples prepared as described under “Experimental Procedures” were analyzed for total LOOH by horseradish peroxidase-catalyzed oxidation of leumymethylene blue (n = 4, ± S.D.) Panel B, following extraction into organic solvent, samples were analyzed for total HPODE content using reverse phase HPLC. Panel C, to examine isomer content of samples prepared in the presence of NO, samples were also analyzed by normal phase (panel C) and chiral phase (inset) HPLC.

To define the mechanism(s) of NO reaction with and consumption by 15-LOX, studies were carried out using purified rabbit reticulocyte and soybean 15-LOX. For both enzymes, reversible inhibition was observed on addition of 1–6 μM NO (Fig. 2). Inhibition coincided with NO consumption, and activity was recovered once NO was depleted. For NO consumption, addition of linoleate but not (13S)-HPODE was required (Fig. 4) and NO consumption was catalytic. These data suggest that an intermediate or product of the dioxygenase cycle react with NO addition but not (13S)-HPODE for reaction with 15-LOX iron. However, using stopped flow fluorescence, no effect of NO on either the rate or extent of 15-LOX intrinsic tryptophan fluorescence quenching was found (Fig. 6) (47, 52). Additionally, native 15-LOX did not consume NO (Fig. 7) and the time of NO inhibition was independent of (13S)-HPODE concentration (Fig. 3). It was recently shown and confirmed herein, that NO prolongs the lag phase of 15-LOX activity (Fig. 4, Ref. 25). Inhibition of 15-LOX did not require addition of NO before substrate, since the length of NO inhibition is the same, whether NO was added either before or during enzyme turnover (Fig. 3). This shows that NO is not preventing 15-LOX activation and infers that enzyme inhibition results from reaction with a dioxygenase intermediate.

15-LOX did not consume NO in the absence of (13S)-HPODE, thus nitrosyl complexes with reduced 15-LOX metal centers did not form. This is not unexpected, since previous studies determined a dissociation constant (Kd = 95 μM at pH 7.0) for the major EPR species formed between NO and soybean 15-LOX, far in excess of NO concentrations used in this study and those found biologically. A second species was also observed by EPR previous study of NO-15-LOX reactions (31). Since formation of this signal required at least 400 μM NO, it is also a biologically unlikely explanation of 15-LOX inhibition by NO.

Two previous reports suggested that high concentrations of NO could oxidize native 15-LOX, leading to either activation or formation of a species more susceptible to peroxide activation (25, 29). In both studies, electron transfer may have occurred following formation of the iron nitrosyl complex, since it was detectable by EPR spectroscopy. Herein, low concentrations of NO did not activate 15-LOX. The high Kd for formation of the EPR-detectable Fe2+NO species infers that under physiological conditions, where NO concentrations will be less than 1 μM, both the formation of ferrous-nitrosyl complexes and activation of 15-LOX by NO is unlikely. During activation by HPODE, consumption of 2 mol of NO/mmol of 15-LOX was observed. Reduction of NO to NO- by the ferrous iron-derived electron, followed by secondary reactions of NO- that can consume NO (e.g., HNO + 2NO → N2O + NO2, k = 109 M−1 s−1) may explain these observations.

Since NO had no effect on 15-LOX tryptophan fluorescence quenching, NO uptake during activation is also unlikely to cause enzyme inhibition (Fig. 6). Therefore, NO must react at an additional site during dioxygenase turnover. The only intermediate that was not excluded experimentally or theoretically is EredNO. Reaction of NO with free LOO- in aqueous solu-
Turnover-dependent Consumption of NO by Mammalian LOX

**Scheme 3.** Potential sites of nitric oxide reaction during 15-LOX oxidation of lipid. Three sites of potential 'NO reaction are shown. (i) During peroxide (LOOH) activation of LOX, 2 mol of NO are consumed, via reaction with an electron (e-) released from the ferrous enzyme (EFe2+) to form nitroxyl anion (NO·-) Secondary reactions of NO· will consume further 'NO molecules, for example, reaction of NO· with O2 or with further 'NO molecules, as shown. (ii) During dioxygenase turnover, 'NO is consumed through reaction with EredLOO to form reduced inactive enzyme (Ered) and an organic peroxynitrite (LOONO). This hydrolyzes to the hydroperoxide (LOOH) and nitrite (NO2·). (iii) at higher 'NO concentrations a ferrous nitrosyl complex can form (E-Fe2+-NO), which slowly decomposes, yielding active enzyme (E*).

**REFERENCES**

1. Yamamoto, S., and Smith, W. L. (1995) J. Lipid Med. Cell Signal 12, 195
2. Hamberg, M., and Samuelsson, B. (1974) Proc. N. Y. Acad. Sci. U. S. A. 81, 3400–3404
3. Yokoyama, C., Shinoz, F., Yoshimoto, T., Yamamoto S., Oates, J. A., and Brash, A. R. (1986) J. Biol. Chem. 261, 16714–16721
4. Kuhn, H. (1996) Prog. Lipid Res. 35, 203–226
5. Rapoport, S. M., Schewe, T., and Thiele, B. J. (1990) in Blood Cell Biochemistry (Harris, J. R., ed) Vol. 1 p. 151, Plenum, New York
6. Kühn, H., Belkner, J., Zais, S., Fahrenkemper, T., and Wohlfel, S. (1994) J. Exp. Med. 179, 1903–1911
7. Yla-Herttuala, S., Rosenfeld, M. E., Parthasarathy, S., Glass, C. K., Sigel, E., Sarkinia, T., Witzum, J. T., and Steinberg, D. (1991) J. Clin. Invest. 87, 1146–1152
8. Folck, V. A., Nivar-Aristy, R. A., Krajewski, L. P., and Catracht, M. K. (1995) J. Clin. Invest. 96, 504–510
9. Belkner, J., Slender, K., and Kuhn, H. (1998) J. Biol. Chem. 273, 32225–32232
10. Parthasarathy, S., Wieland, E., and Steinberg, D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1046–1050
11. Rankin, S. M., Parthasarathy, S., and Steinberg, D. (1991) J. Lipid Res. 32, 449–456
12. O'Leary, V. J., Graham, A., Stone, D., and Darley-Usmar, V. M. (1996) Free Radical Biol. Med. 20, 525–532
13. Yla-Herttuala, S., Luoma, J., Viita, H., Hiltunen, T., and Nikkari, T. (1995) J. Clin. Invest. 95, 2692–2698
14. Harata, D., Kurihara, H., Levkovits, H., Shais, A., and Sigel, E. (1997) Atherosclerosis 134, 275–284
15. Shen, J., Herderick, E., Corhill, J. F., Zegzmid, E., Kim, H. S., Kuhn, H., Guevara, N. V., and Chan, L. (1998) J. Clin. Invest. 98, 2201–2208
16. Sendhory, S. M., Cornizelli, J. A., Welch, K., Tait, B., Trivedi, B., Kalyanaraman, B., Dyer, R. D., Feinmark, S. J., and Daugherty, A. (1995) Br. J. Pharmacol. 120, 1199–1206
17. Mało-Ranta, U., Yla-Herttuala, Metsa-Ketela, T., Jaakkola, O., Moilanes, E., Vourinen, P. and Nikkari, T. (1994) FEBS Lett. 337, 179–183
18. O'Donnell, V. B., Chumley, P. H., Hogg, N., Bloodsworth, A., Darley-Usmar, V. M., and Freeman, B. A. (1994) Blood Cell Biochemistry 36, 15216–15223
19. Hayashi, K., Noguchi, N., and Iki, E. (1990) FEBS Lett. 270, 37–40
20. Goss, S. P. A., Hogg, N. and Kalyanaraman, B. (1995) Chem. Res. Toxicol. 8, 800–806
21. Jessup, W., Mohr, D., Gieseg, S. P., Dean, R. T., and Stocker, R. (1992) Biochim. Biophys. Acta 1100, 73–82
22. Hogg, N., Kalyanaraman, B., Joseph, J., Struck, A., and Parthasarathy, S. (1993) FEBS Lett. 334, 170–174
23. Zayats, M. T., Lambert, L. E., Whitten, J. P., McDonald, I., Mano, M., K., and Mao, S. J. T. (1992) FEBS Lett. 309, 135–138
24. Rubbo, H., Radi, R., Trujillo, M., Tellieri, R., Kalyanaraman, B., Barnes, S., Kirk, M and Freeman, B. A. (1994) J. Biol. Chem. 269, 26066–26075
25. Weisgerber, M., R., Zhang, J., Hidh, H. G., Sier, R., Masler, R., Nalting, H., and Kuhn, H. (1996) FEBS Lett. 388, 229–232
26. Holzhauser, H. G., Wies, R., Rantham, J., Stör, R., and Kuhn, H. (1997) Eur. J. Biochem. 245, 668–618

In summary, our data show that at 'NO and 15-LOX concentrations found in tissues, (a) 15-LOX is inhibited, (b) 15-LOX catalytic activity impairs 'NO-dependent activation of soluble guanylate cyclase, and (c) 15-LOX consumes 'NO through two separate mechanisms. First, during peroxide-mediated activation of 15-LOX, 2 mol of 'NO/mole of 15-LOX are consumed. Second, reaction of 'NO with an intermediate of the dioxygenase cycle, EredLOO, leads to reversible enzyme inhibition by promoting formation of the inactive ferrous enzyme, Ered. While 'NO reaction during 15-LOX catalysis leads to no change in product profile, significant suppression of HPODE generation occurred in concert with consumption of significant quantities of 'NO.

Since modulation of 15-LOX activity by 'NO occurs at biologically relevant 'NO concentrations, suppression of HPODE formation and 'NO consumption is expected in vivo. The Kd for 'NO activation of soluble guanylyl cyclase is approximately 250 nM (64); therefore, varying 'NO levels around these concentrations will have significant impact on cGMP production and resultant smooth muscle relaxation, as revealed in Fig. 9. Thus, consumption of 'NO by the elevated lipoxygenase activities present in a variety of hypertensive vascular diseases would then contribute to their characteristic responses to 'NO (40–42).
