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Inhibition of Lipid Peroxidation in Synaptosomes and Liposomes by Nitrates and Nitrites

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NO is produced endogenously from arginine by the action of NO synthase, and exogenously by nitrovasodilators, including organic nitrates and nitrites. NO has been proposed as a cytotoxic and cytoprotective agent. There is strong evidence that NO acts as an apparent antioxidant in inhibiting lipid peroxidation, via chain termination, and interestingly lipid nitrates and nitrites have been proposed to be products of this chain termination. Both pro- and antioxidant mechanisms may be drawn for nitrates and nitrites; therefore, their effects on lipid peroxidation were measured in two systems, using tocopherol, thiol, and an NO donor for comparison: (1) rat cerebrocortical synaptosomes with Fe(II)-induced lipid peroxidation measured by thiobarbituric acid reactive substances (TBARS), and (2) phospholipid liposomes with an azo-initiator induction system, quantified by a fluorescent probe of peroxide formation. In contrast to the classical nitrate nitroglycerin, novel nitrates which release NO on reaction with thiols and two novel nitrates which spontaneously generate NO in aqueous solution inhibited lipid peroxidation. *i*-Amyl nitrite inhibited lipid peroxidation, and its properties were further studied with ESR spectroscopy. The data show that classical nitrites and novel nitrates are not prooxidants, but inhibit lipid peroxidation.

Introduction

Expansion of research into the pharmacology, biology, and toxicology of nitric oxide (NO) has continued since the important discovery of the biological role and significance of NO. NO is an endogenous chemical messenger molecule, produced from arginine via the action of NO synthase (NOS). Constitutive NOS isoforms generate NO that mediates biological processes, such as tissue relaxation and neurotransmission, whereas inducible NOS isoforms participate in the body's inflammatory response and in pathogen killing (*1*). Despite being an essential biomolecule, NO has long been implicated in the neurotoxic response to cerebral ischemia, and the potential toxicity of NO and its reaction products, in particular peroxynitrite, has been associated with various disease states (*2*).

The primary target of NO, released by constitutive NOS, is the enzyme-soluble guanylyl cyclase (sGC),[1] which, on NO binding, is activated to produce the secondary messenger, cGMP (*3*). However, the chemical reactivity of NO may lead to a separate cascade of biological events. The aberrant reactions of reactive oxygen species (ROS) produced from the diradical, O_2, contribute to oxidative stress, whereas reactions of reactive nitrogen oxygen species (RNOS) produced from reaction of the free radical, NO, are proposed to contribute to nitrosative stress (*2*). These pathways are highly interwoven. The primary RNOS is peroxynitrite ($ONOO^-$), formed by the rapid reaction of NO with the ROS, superoxide ($O_2^{\bullet -}$). Peroxynitrite has been proposed to initiate lipid peroxidation and degradation of other biomolecules, via reactions of its radical decomposition products, primarily hydroxyl radical ($HO^\bullet$) and NO_2 (*4*).

Paradoxically, whereas peroxynitrite, derived from NO, initiates lipid peroxidation, there is substantial evidence, from the use of NO donors, that NO itself inhibits lipid peroxidation, even that induced by peroxynitrite (*5*). Indeed, NO has variously been proposed as a cytotoxic agent, usually via peroxynitrite formation (*6, 7*), and as a cytoprotective agent (*8*−*10*), possessing both prooxidant and antioxidant properties (*11*−*14*). Evidence suggests that the apparent antioxidant activity of NO toward lipid peroxidation results from radical chain termination, rather than via attenuation of the radical initiator. The final lipid radical chain termination products have been

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[1] Abbreviations: AH, ascorbic acid; AH^-, ascorbate; AIBN, 2,2′-azobisisobutyronitrile; ABAP, 2,2′-azobis(2-amidinopropane)dihydrochloride; BODIPY, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-undecanoic acid (BODIPY 581/591-C_{11}); DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-4,5-dihydro-3*H*-pyrrrole-*N*-oxide; DMPO, 5,5-dimethyl-4,5-dihydro-3*H*-pyrrole-*N*-oxide; DPPC, dipalmitoyl phosphatidylcholine; GTN, glyceryl trinitrate (nitroglycerin); sGC, soluble guanylyl cyclase; IAN, *i*-amyl nitrite; LA, lipoic acid; LAH_2, dihydrolipoic acid; LPO, lipid peroxidation; NONOate, diazeniumdiolate salt; NRP, non radical product; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acid; SIN-1, 3-morpholinosydnonimine hydrochloride; Sper/NO, $H_2N(CH_2)_3NH(CH_2)_4-N(NONO)-(CH_2)_3NH_3$, (*Z*)-1-[*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]amino]diazen-1-ium-1,2-diolate; αTH, α-tocopherol; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

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proposed to include nitrite ion (NO_2^-), organic nitrates ($RONO_2$), and organic nitrites (RONO) (eq 1), raising the interesting possibility that lipid nitrates and nitrites are natural membrane components (*4, 15*). The chemical reactivity of nitrates and nitrites has not been exhaustively studied: homolytic decomposition would generate alkoxyl radical, a chain propagator, and either NO or NO_2 (eq 2), whereas α-H atom abstraction would yield aldehyde and NO_x (eq 3) (*16−20*). Thus, it is unclear whether putative lipid nitrates and nitrites would potentiate or inhibit formation of lipid peroxidation products.

$$4NO + 2ROO^{\bullet} + H_2O \rightarrow 2HNO_2 + RONO_2 + RONO \quad (1)$$

$$RONO_x \rightleftharpoons RO^{\bullet} + NO_x \quad (2)$$

where $x = 1, 2$

$$RCH_2ONO_x + R^1O^{\bullet} \rightarrow RCHO + R^1OH + NO_x \quad (3a)$$

$x = 1, 2$; R^1 = H, alkyl

$$RCH_2ONO_x + NO_2 \rightarrow RCHO + HNO_2 + NO_x \quad (3b)$$

$x = 1, 2$; R^1 = H, alkyl.

Nitrates are important therapeutic agents and xenobiotics. Nitroglycerin (GTN) has been in clinical use in the treatment of coronary artery disease for 125 years. In their biological activity, nitrates are NO-mimetics which may act as NO donors, but the mechanism of NO release in vitro and in vivo has not been rigorously defined (*19*). The potential of amyl nitrite (IAN) in treatment of angina was recorded even before that of GTN, although the use of nitrites is now largely recreational. Nitrites may also be drawn as chemically reasonable intermediates in the reductive biotransformation of nitrates (*19, 20*). The effect of nitrites on lipid peroxidation has not been reported. Reports on the influence of GTN on lipid peroxidation include variously, reduction of lipid peroxidation, no effect, and potentiation of lipid peroxidation (*21−23*).

There is a need to explore the effects of nitrates and nitrites on lipid peroxidation. Oxidative damage to lipid and lipoprotein constituents of biological membranes is implicated in many disease states, from atherosclerosis to Alzheimer's Disease (*8, 14, 24*). Of the three most common approaches used to initiate lipid peroxidation in vitro, peroxyl radical precursors [e.g., azo-initiators such as 2,2′-azobis(2-amidinopropane)dihydrochloride (ABAP)], xanthine oxidase, and transition metals, the latter was chosen because of the growing evidence for the role of Fe and Cu cations as causative factors in lipid peroxidation in vivo (*25*). As the lipid reaction medium, rat brain synaptosomes were chosen because of interest in application of novel nitrates in neurodegenerative disorders and the putative link between disruption of Fe homeostasis, Fe-induced oxidative stress, and neurodegeneration (*26, 27*).

The effects on Fe-induced lipid peroxidation, of a classical nitrate (GTN) and a classical nitrite [*i*-isoamyl nitrite (IAN)], were studied, and comparison was made with three novel lipophilic nitrates: nitrate **1**, that may react with simple thiols to give NO as product; and nitrates 2 and **3**, which are the first nitrates discovered to undergo spontaneous reaction in neutral aqueous solution to generate a readily measurable flux of NO. To define the mechanism of action of these nitrates, comparison was made with antioxidants, an NO donor NONOate, thiols, and disulfide, and correlation also was made with rate data for NO release obtained electrochemically and via chemiluminescence assay. The widely used thiobarbituric acid (TBA) assay was employed to measure lipid peroxidation products through TBA reactive substances (TBARS). Although crude from a chemical perspective, this highly reproducible assay provided an effective relative measure of lipid oxidation products that allowed determination of full concentration−response curves. To ensure the generality of these phenomena, comparisons were made with inhibition of ABAP-induced peroxidation of phospholipid liposomes, as assayed by measurement of peroxide formation using a BODIPY fluorescence probe. Further comparisons were also made between α-tocopherol (αTH) and IAN, using ESR trapping and monitoring of radicals generated from azo-initiators. The data reported herein unambiguously demonstrate that nitrites and nitrates are capable of acting as apparent antioxidants in two models of lipid peroxidation.

Experimental Procedures

NONOate (diazeniumdiolate) salts were obtained from RBI (Natick, MA) or Calbiochem (La Jolla, CA). Other chemicals were obtained from Sigma (St. Louis, MO), Aldrich Chemicals (Milwaukee, WI), or BDH (Toronto, Canada), unless otherwise stated. Nitrates **1**, 2, and GTN were synthesized as described in the literature (*28, 29*). Peroxynitrite was synthesized by two routes: first, from reaction of IAN with aqueous H_2O_2 followed by exhaustive washing to remove IAN; second, from reaction of aqueous $NaNO_2$ with an acidic H_2O_2 solution with rapid alkaline quenching. In both cases, excess H_2O_2 was removed with MnO_2, and UV−Vis spectroscopy was used to quantify peroxynitrite and check for contamination.

Synthesis of 3. Synthesis proceeded from the Bunte salt, 2,3-dinitrooxypropane-1-thiosulfonate, which was prepared from 1,2-dinitrooxy-3-bromopropane. 3-Bromopropane-1,2-diol was added dropwise into a cold mixture of HNO_3 (68−70%, 4.0 equiv) and H_2SO_4 (95%, 4.0 equiv) in CH_2Cl_2 (50 mL) at room temperature over 30 min. The organic layer was separated, washed, dried, and concentrated to yield a yellow oil which was purified by flash chromatography on SiO_2 to give 3-bromopropane-1,2-diol dinitrate in 45% yield (*29*). The Bunte salt was prepared by reacting 3-bromopropane-1,2-diol dinitrate with an equimolar portion of $Na_2S_2O_3$ in 3:1 MeOH/H_2O at 50 °C for 10 h and subsequently purifying by flash chromatography on SiO_2 (*29*). The Bunte salt was oxidized with a small molar excess of H_2O_2 (30%) in an EtOH/H_2O mixture (1:1) with a catalytic amount of H_2SO_4 for 2 days. Extraction with CH_2Cl_2, concentration, and purification by flash chromatography on SiO_2 yielded the title compound as a yellow oil (R_f = 0.65; CH_2Cl_2/hexane = 65:35; 5%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.55−5.65 (m, 1H), 4.87−4.94 (dd, 1H, J = 12.94, 2.94), 4.62−4.70 (m, 1H, J = 12.88), 3.13−3.30 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz): 76.74, 69.46/69.42, 36.65/36.63. Mass spectrometry (*m*/*z*, EI$^+$): 380.1 $(M-NO_2)^+$ 290%; 426.1 $(M)^+$ 100%; 427.1 $(M+1)^+$ 10%; 428.1 $(M+2)^+$ 17%; 429.2 $(M+3)^+$ 1.5%; 430.3 $(M+4)^+$ 1.3%; calculated for $C_6H_{10}N_4O_{12}S_3$ 426.0. Elemental analysis: calculated for $C_6H_{10}N_4O_{14}S_2$: C, 16.90; H, 2.36; S, 15.04; calculated for $C_6H_{10}N_4O_{12}S_3$: C, 16.90; H, 2.36; S, 22.56; found: C, 17.27; H, 2.38; S, 21.68.

NO Release. Rate constants determined spectrophotometrically for the hydrolysis or aqueous degradation of the NONOates were measured at 21 or 37 °C as previously described (*30*). NO release from NONOates was detected using a Clark-type NO selective electrode (ISO-NO-II, WPI Inc., Sarasota, FL) as

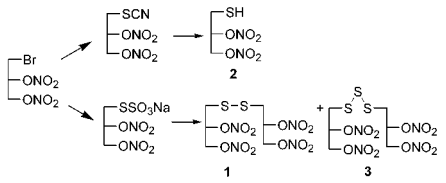
previously described (*30*). NO release was also measured using chemiluminescence detection (Sievers Research Inc., Boulder, CO, model 207B), using standardized NO gas (Scott Specialty Gases, Plumsteadville, PA) injected into the reaction solution to calibrate the system, as previously described (*31*). Volatile organic nitrite did not interfere with the chemiluminescence readings of NO.

Synaptosome Assay. The methodology for measurement of TBARS from synaptosomes was adapted from that of Keller et al. (*32*) Adult Sprague−Dawley rats (250−300 g) were anesthetized with halothane for 20 s and decapitated. The brain was removed and the cerebral cortex separated from white matter. The cerebral cortex was homogenized in a solution containing sucrose 0.32 M, EDTA 2 mM, and TRIS·HCl 10 mM, pH 7.2, using a Teflon pestle. The tissue was 5% w/v in the homogenizing buffer. The homogenate was centrifuged for 10 min at 310*g* at 4 °C. The supernatant was then centrifuged for 10 min at 20000*g* at 4 °C. The pellet was collected, resuspended in Locke's buffer (NaCl 154 mM, KCl 5.6 mM, $CaCl_2 \cdot 2H_2O$ 2.3 mM, $MgCl_2 \cdot 6H_2O$ 1.0 mM, $NaHCO_3$ 3.6 mM, glucose 5 mM, HEPES 5 mM, pH 7.2), and centrifuged for 10 min at 20000*g* at 4 °C. The procedure of washing the pellet was repeated 2−3 times in order to reduce transition-metal ion concentrations. Finally, the pellet was resuspended in Locke's buffer for use in the lipid peroxidation assay. All assays were performed in triplicate and on three separate synaptosome preparations from different animals.

Potential antioxidants and prooxidants were freshly prepared: in Locke's buffer [$FeSO_4$; ascorbic acid (AH); Trolox; cysteine]; in NaOH (10 mM) (NONOates); or in organic solvent—methanol or DMSO [αTH; nitrates; nitrites; PhSH; lipoic acid (LA); dihydrolipoic acid (LAH_2); final dilution ≤2.5% (v/v) organic component]. Pro/antioxidants (or solvent vehicle in control experiments) were added to the synaptosome preparation, followed immediately by freshly prepared aqueous $FeSO_4$ (or buffer in control experiments) and incubated for 30 min at 37 °C (air-equilibrated). After incubation, TBA reagent (TBA 0.375% w/v, Cl_3CCO_2H 15% w/v, HCl 1 M 25% v/v) was added to the homogenate (homogenate:TBA reagent ratio, 1:2 v/v), and the sealed samples were boiled for 15 min at approximately 96−100 °C. The cooled samples were then centrifuged for 10 min at 9000*g* at room temperature. The pink supernatant was transferred into microplates, and the absorbance was measured at 530 nm on a Dynex MRX microplate reader. TBA reagent solutions were freshly made and calibrated using solutions of authentic malondialdehyde.

Liposome Assay. Polyunsaturated fatty acid (PUFA) liposomes were prepared from fresh egg yolk L-α-phospatidylcholine (PC) (Sigma), containing palmitate (ca. 33%), stearate (ca. 14%), oleate (ca. 30%), linoleate (ca. 14%), and arachidonate (ca. 4%). Unsaturated DPPC liposomes were made from dipalmitoyl phosphatidylcholine (Sigma). Small unilamellar liposomes were prepared using a French pressure cell, and checked for uniformity and size using transmission electron microscopy (negative staining technique with 3% uranyl acetate). PCs in chloroform were coated in a thin layer on the walls of a round-bottom flask by blowing purified N_2 to evaporate solvent. Cold phosphate-buffered saline (pH 7.4, 10 mM, NaCl, 50 mM) was added to the dried layer in a volume such that the lipid concentration was 4 mg/mL, and the PCs were suspended by vigorous vortex mixing. The milky suspension was passed 3 times through a 3.5 mL French pressure cell (SLM Instruments Inc., Urbana, IL), and pressure was applied (20 000 psi) to obtain a thin, almost translucent suspension of liposomes. Pressure was applied using a motorized hydraulic press equipped with a pressure regulator. The final phosphatidylcholine concentration for the lipid peroxidation assay was 1 mg/mL, approximately equivalent to the phospholipid concentration in the synaptosome preparations, as estimated by Bradford protein assay of synaptosomes and known tissue protein:phospholipid ratios (*33*). Liposomes were unilamellar and 30−100 nm in diameter, with an average of 50 nm, as assessed by electron transmission microscopy. All assays were performed in triplicate and on three separate liposome preparations.

Scheme 1



Phospholipid peroxidation assays were performed on a Spectramax Gemini XS spectrofluorometer (Molecular Devices) using a quartz-covered 96 well glass micro-plate in triplicate at 37 °C. Potential antioxidants and prooxidants were freshly prepared: in phosphate-buffered saline (pH 7.4, 10 mM, NaCl, 50 mM) (ABAP); in NaOH (10 mM) (NONOate); or in DMSO (Trolox; nitrates; nitrites). Both vehicle (no ABAP and no antioxidant) and ABAP (no antioxidant) controls received the solvent (buffer, DMSO, NaOH aq.) used for the preparation of the antioxidant solutions. The fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-undecanoic acid (BODIPY, Molecular Probes, Eugene, OR) was used to quantify lipid peroxidation using excitation at $\lambda = 540$ nm and emission at $\lambda = 600$ nm, with a 590 nm cutoff filter. The emission intensity in relative fluorescence units (RFU) was measured with time, and normalized relative to 100% RFU immediately prior to addition of antioxidant or vehicle. Liposomes were incubated with BODIPY for 5 min to allow partition into the membrane, followed by incubation with ABAP for a further 10 min. Antioxidants (or vehicle in control experiments) were then added (time zero). The concentration−response curve for 2 and individual data points for IAN and Sper/NO were obtained by measuring percent lipid peroxide formation at 90 min, after sequential addition of BODIPY (1.25 μM), 2 (or IAN or Sper/NO), and then ABAP (1 mM). Assays were performed at 37 °C in triplicate, with controls and normalization performed as described above.

ESR Experiments. All experiments were performed on a Bruker EMX300 spectrometer operating at 9.775 GHz at room temperature (see Figure 10 for settings). Reaction solutions were made up in (1.5−1.8) × 90 mm glass capillaries and incubated in a water bath at 37 ± 1 °C, and cooled prior to reading.

Results

Synthesis. The tetranitrate, **3**, is a product of the catalytic oxidation of 2,3-dinitrooxypropane-1-thiosulfonate, a synthetic precursor of **1** (Scheme 1). The identification of this compound as the symmetric trisulfide over alternative asymmetric dinitrooxypropane products, for example, the asymmetric thiosulfonate ($RS(O)_2SR$), is supported by NMR analysis. This characterization is confirmed by elemental analysis and mass spectroscopy, the latter showing the expected *m*/*z* isotope distribution pattern due to the presence of three sulfur atoms, which is definitive for the trisulfide.

The disulfide dinitrate, **1**, is a member of the SS-nitrate family ($R-SS-CH_2CH(ONO_2)CH_2ONO_2$), that has been the subject of a preliminary communication (*28*). In general, SS-nitrates were shown to be stable in neutral aqueous solution and mixed organic/aqueous solvent systems, but to release NO on reaction with thiol adjuvants at varying rates, that may be controlled by modification of the R-group. In contrast, trisulfide **3** and thiol **2** react spontaneously in aqueous and mixed aqueous/organic solutions to release NO, but are stable on storage for weeks at low temperature in various organic solvents, including DMSO (*28*).

Measurement of NO Release. Electrochemical detection relied on an NO-selective electrode submersed in an unsealed reaction vessel, whereas the more sensitive chemiluminescence method was used to sample the reaction headspace in a sealed vessel (*30*). In the presence of equimolar cysteine, SS-nitrates (e.g., **1**) undergo reaction to yield the symmetrical and mixed denitrated disulfides as major organic products, with concomitant oxidation of cysteine to cystine (*28*). The rate of NO release from **1** and **3** (1 mM) in the presence of cysteine (2 mM) in phosphate buffer (pH 7.4, 100 mM, 37 °C) was measured electrochemically as $d[NO]/dt = 110$ and 330 nM s^{-1} respectively. For comparison, under these conditions, the rate of NO release from the NONOate, Sper/NO (1 mM), was $d[NO]/dt = 560$ nM s^{-1}. Rates of reaction of **1** vary with added thiol; for example, compared to cysteine, the rate of NO release was 4-fold greater with thiophenol (PhSH) and 3-fold less with glutathione (GSH). In the absence of added thiol, and in the presence of thiols of high pK_a, which are poorly ionized at neutral pH, reactions of SS-nitrates such as **1** are slow, and little or no NO release is detected above threshold electrochemical detection limits. In contrast, in aqueous solutions, the thiol **2** and trisulfide **3** released NO in the presence of a large range of thiols, and even in the absence of any adjuvants. The rate of spontaneous NO release estimated using the electrochemical initial rate method for **2** (1 mM) in 60:40 phosphate buffer (pH 7.4, 100 mM)/MeCN was of the same order as that measured for Sper/NO (1 mM) (*28*).

Owing to the volatility of the nitrite IAN, it was chosen to compare NO release from IAN with Sper/NO in the sealed reaction vessel used for headspace chemiluminescence measurements. In phosphate buffer (pH 7.4, 100 mM, containing 1 mM DTPA, 37 °C), rates of NO generation from IAN (0.1 mM) and Sper/NO (0.01 mM) were comparable. The rate constants for NO generation from NONOates also may be estimated from the measured pseudo-first-order rate constants for NONOate decomposition (k_d), assuming the yield of NO: $k_{NO} = (2 \times k_d) = 5.6 \times 10^{-4}$ s^{-1}, in phosphate buffer (pH 7.4, 100 mM, 37 °C) (*31*).

Lipid Peroxidation: Synaptosome Experimental Design. Preliminary lipid peroxidation experiments explored the time course of synaptosome lipid peroxidation, incubating homogenate with $FeSO_4$ (10−150 μM) in Locke's buffer, for time intervals from 15 to 180 min (data not shown). Under these experimental conditions, the level of peroxidation, as measured by TBARS, was seen to be below saturation at 30 min using 50 μM $FeSO_4$. The ability of this system to provide concentration-dependent lipid peroxidation data was demonstrated using the antioxidant α-tocopherol (αTH), and ascorbic acid (AH), which is know to act as a prooxidant in Fe(II)-induced lipid peroxidation systems (Figure 1) (*9*). These conditions thus were chosen for all further synaptosome experiments.

The Fe/synaptosome/TBARS system was designed to provide concentration−response curves for inhibition of lipid peroxidation, which might be quantified by EC_{50} values. Absolute EC_{50} values measured in such systems are highly dependent on experimental conditions, and therefore must be benchmarked against well-studied antioxidants, such as Trolox, a water-soluble chroman carboxylate derivative of αTH. Since the nitrates and nitrites under study might release NO, the NO donor NONOate, Sper/NO, was also studied for comparison.

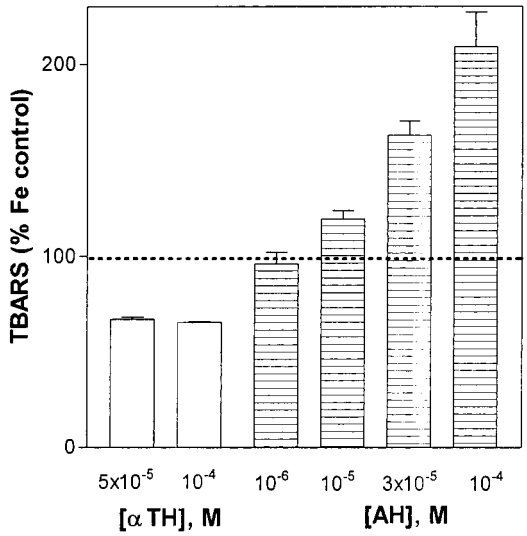


Figure 1. Effect of antioxidant and prooxidant. Inhibition and potentiation of lipid peroxidation induced by $FeSO_4$ (50 μM) as assessed by TBARS determination, on incubation of rat brain synaptosomes with adjuvants: ascorbic acid (AH) or α-tocopherol (αTH). Percentage TBARS detected is given relative to: TBARS in the presence of vehicle and absence of $FeSO_4$ (0%); TBARS in the presence of vehicle and $FeSO_4$ (100%) (shown by dashed line). Error bars show SEM ($n = 3$).

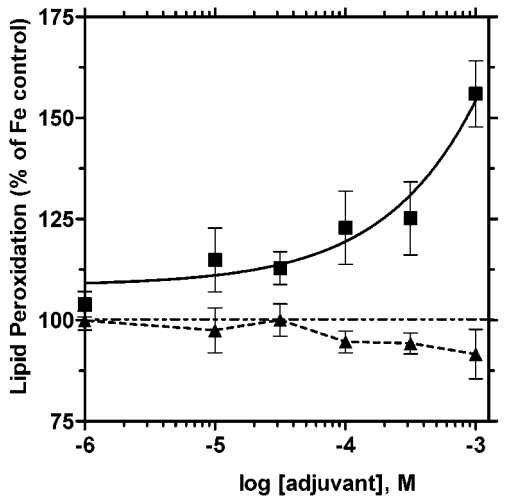


Figure 2. Effect of thiol and disulfide. Lipid peroxidation induced by $FeSO_4$ (50 μM) as assessed by TBARS determination, on incubation of rat brain synaptosomes with lipoic acid (LA, ▲, dashed line) or dihydrolipoic acid (LAH_2, ■, solid line). Percentage lipid peroxidation relative to: control TBARS in the presence of vehicle and absence of $FeSO_4$ (0%); TBARS in the presence of vehicle and $FeSO_4$ (100%). Error bars show SEM ($n = 3$).

Thiols may display mixed pro- and antioxidant activity toward lipid peroxidation. In particular, in the presence of transition metals, either added to lipid preparations or as adventitious metal ions present in tissue homogenates, thiols may act as prooxidants (*34*, *35*). The *vic*-dithiol dihydrolipoic acid (LAH_2) yielded a concentration-dependent prooxidant effect, akin to ascorbic acid, whereas the oxidized disulfide lipoic acid (LA) showed very modest inhibition of lipid peroxidation at the highest concentration applied (Figure 2) (*36*). Cysteine (1 mM) was a prooxidant in the presence of $FeSO_4$, giving 117% of the

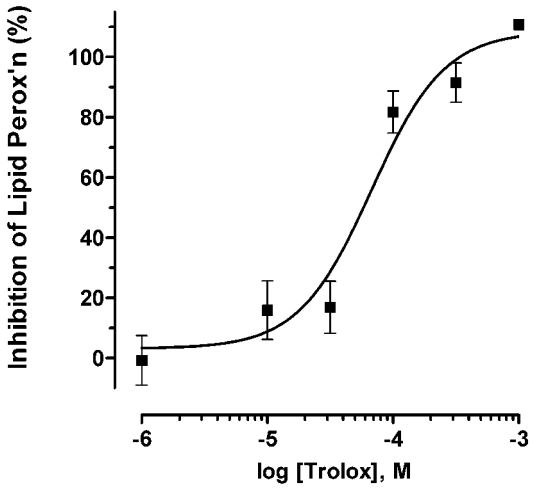


Figure 3. Effect of Trolox antioxidant. Inhibition of lipid peroxidation induced by $FeSO_4$ (50 μM) as assessed by TBARS determination, on incubation of rat brain synaptosomes with Trolox. Percentage inhibition is given relative to: control TBARS in the presence of vehicle and absence of $FeSO_4$ (100%); TBARS in the presence of vehicle and $FeSO_4$ (0%). Error bars show SEM ($n = 3$); data are fitted to a sigmoidal curve ($EC_{50} = 6.8 \times 10^{-5}$ M).

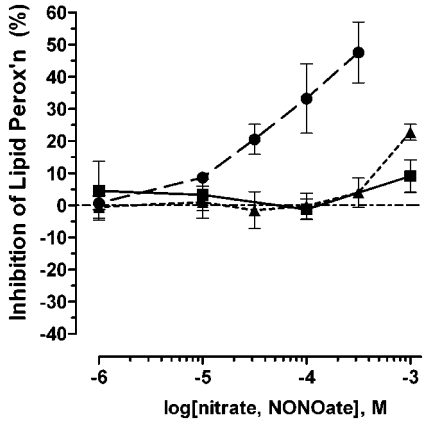


Figure 4. Effect of nitrates. Inhibition of lipid peroxidation induced by $FeSO_4$ (50 μM) as assessed by TBARS determination, on incubation of rat brain synaptosomes with: GTN (varied) + LAH_2 (1 mM) (■, solid line); or **1** (varied) + LAH_2 (1 mM) (▲, short dashed line). For comparison, inhibition data for the NONOate Sper/NO are shown (in the absence of adjuvants; ●, long dashed line). Percentage inhibition relative to: control TBARS in the presence of vehicle and absence of $FeSO_4$ (100%); TBARS in the presence of vehicle, adjuvants, and $FeSO_4$ (0%). In experiments with adjuvant LAH_2, 0% inhibition corresponds to TBARS in the presence of vehicle containing LAH_2. Error bars show SEM ($n = 3$).

lipid peroxidation seen in the presence of $FeSO_4$ alone, whereas PhSH (1 mM) in the presence of $FeSO_4$ gave 110% of the lipid peroxidation seen in the presence of $FeSO_4$ alone (data not shown). Of further consideration in analysis of lipid peroxidation data is the requirement for added thiols in experiments with nitrates. Clearly, any antioxidant effect of nitrates may be masked by the prooxidant effect of the adjuvant thiol. Thus, it was chosen to present the data as "percentage inhibition of lipid peroxidation", by normalizing TBARS measurements to: (i) 100% inhibition of lipid peroxidation (corresponding to TBARS in the presence of vehicle and absence of $FeSO_4$); and (ii) 0% inhibition of lipid peroxidation (corresponding to TBARS in the presence of vehicle, $FeSO_4$, and any added thiol). This methodology and protocol was applied uniformly to all experiments graphed in Figures 3−8.

Lipid Peroxidation: Synaptosomes. The antioxidant Trolox yielded a potent concentration-dependent reduction in TBARS products with $EC_{50} = 6.8 \times 10^{-5}$ M (Figure 3).

GTN alone had no effect on Fe-induced lipid peroxidation (data not shown), nor did varied concentrations of GTN have any significant effect in the presence of added LAH_2 (1 mM) (Figure 4). Indeed, GTN produced no significant effect on lipid peroxidation with any thiol used (e.g., cysteine, PhSH), over the effect of the thiol itself. Nitrate **1** did inhibit lipid peroxidation at higher concentrations in the presence of LAH_2 (Figure 4). Further, in contrast to GTN, nitrate **1** inhibited TBARS formation with the water-soluble thiol cysteine and the more lipophilic thiophenol PhSH (Figure 5). Addition of PhSH (1 mM) yielded a concentration-dependent inhibition curve for lipid peroxidation: $EC_{50} = 1.4 \times 10^{-5}$ M (Figure 5). At high, millimolar concentrations, nitrate **1** showed some prooxidant activity in the presence of cysteine.

Data for inhibition of iron-induced lipid peroxidation, by nitrate **3**, can be fit to a curve leading to 100% efficacy with an EC_{50} of 1.2×10^{-4} M (or fitted without constraint on efficacy to $EC_{50} = 2.7 \times 10^{-4}$ M; efficacy = 78%).

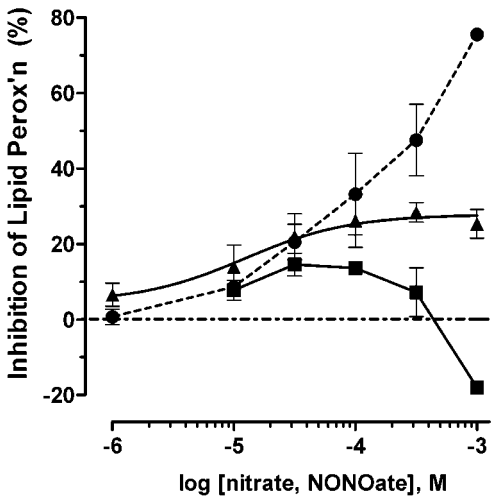


Figure 5. Comparison of NO donor with nitrates. Inhibition of lipid peroxidation induced by $FeSO_4$ (50 μM) as assessed by TBARS determination, on incubation of rat brain synaptosomes with: **1** (varied) + cysteine (1 mM) (■, solid line); or **1** + PhSH (1 mM) (▲, solid line, fitted to sigmoidal curve). For comparison, inhibition data for the NONOate Sper/NO (in the absence of adjuvants; ●, dashed line) are shown. Percentage inhibition relative to controls: TBARS in the presence of vehicle and absence of $FeSO_4$ (100%); TBARS in the presence of vehicle, adjuvant, and $FeSO_4$ (0%). In both experiments with adjuvant thiols, 0% inhibition corresponds to TBARS in the presence of vehicle containing thiol (1 mM). Error bars show SEM ($n = 3$).

Moreover, the SS-nitrate, **1**, in the absence of thiol, showed a modest inhibition of lipid peroxidation, more pronounced at lower concentrations (Figure 6). This antioxidant effect is clearly not an ubiquitous property of disulfides, since lipoic acid (LA) does not show such properties in the identical assay (Figure 2).

Concentration−response curves were derived from TBARS data for the NO donor NONOate, Sper/NO, and for the classical nitrite, IAN (Figure 7). The potency and efficacy of inhibition of lipid peroxidation by Sper/NO and by IAN were both observed to be approximately identical.

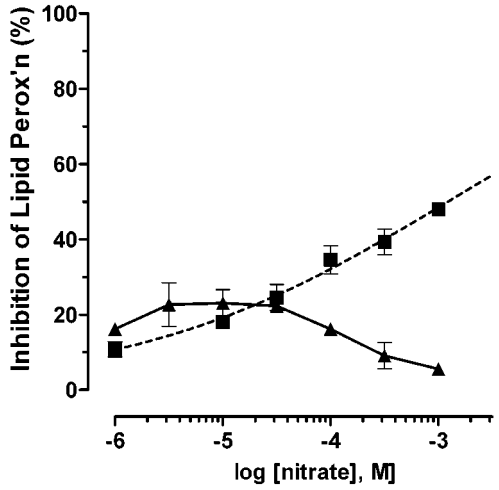


Figure 6. Effect of nitrates without thiol adjuvants. Inhibition of lipid peroxidation induced by $FeSO_4$ (50 μM) as assessed by TBARS determination, on incubation of rat brain synaptosomes with: **3** (varied) (■, dashed line fit to sigmoidal curve); or **1** (▲, solid line). Percentage inhibition relative to: control TBARS in the presence of vehicle and absence of $FeSO_4$ (100%); TBARS in the presence of vehicle and $FeSO_4$ (0%). Error bars show SEM ($n = 3$).

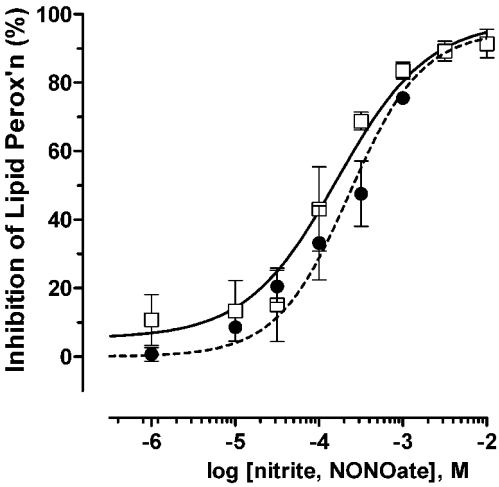


Figure 7. Comparison of NO donor with *i*-amyl nitrite. Inhibition of lipid peroxidation induced by $FeSO_4$ (50 μM) as assessed by TBARS determination, on incubation of rat brain synaptosomes with: (a) IAN (□, solid line); and Sper/NO (●, dashed line). Percentage inhibition relative to: control TBARS in the presence of vehicle and absence of $FeSO_4$ (100%); TBARS in the presence of vehicle, adjuvant, and $FeSO_4$ (0%). Error bars show SEM ($n = 3$). Data are fitted to sigmoidal curves (EC_{50}: IAN = 1.6×10^{-4} M; Sper/NO = 2.0×10^{-4} M).

TBARS measured for the nitrate **2**, in the absence of any adjuvants, revealed similar efficacy for **2** compared to Sper/NO and IAN, but a potency lower by an order of magnitude (Figure 8). The observed behavior of the two nitrates that act as spontaneous NO donors, **2** and **3**, was similar, but measurements on **3** could not be extended to higher concentrations because of solubility. In addition, **2**, which contains both thiol and nitrate functional groups, behaved as a weak prooxidant at lower concentrations (Figure 8).

Lipid Peroxidation: Liposomes. A PUFA liposomal preparation of egg phosphatidylcholine was subjected to peroxidation using a thermal radical source, the azo-initiator ABAP. Formation of lipid peroxides was quantified using the fluorescent probe BODIPY, which relies on trapping of peroxyl radicals. To validate this system, a control reaction of saturated fatty acid DPPC liposomes with BODIPY and ABAP (1 mM) was performed, yielding no peroxidation products over 2 h. The use of peroxynitrite as an alternative lipid peroxidation initiator was explored, but treatment of DPPC liposomes incorporating BODIPY, with peroxynitrite, did result in apparent peroxidation. This was shown to be the result of BODIPY oxidation, because significantly reduced fluorescence was also observed on reaction of peroxynitrite with BODIPY in various solvents in the absence of liposomes.

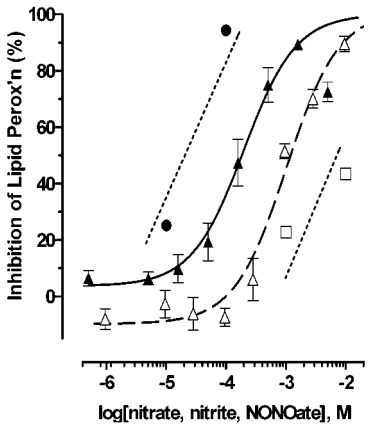


Figure 8. Comparison of liposomes with synaptosomes. Nitrate **2** in: (1) $FeSO_4$-induced lipid peroxidation in rat brain synaptosomes assessed by TBARS determination (△, long dashed line); and (2) ABAP-induced lipid peroxidation in liposomes assessed by loss of BODIPY fluorescence (▲, solid line). ABAP-induced lipid peroxidation in liposomes assessed by loss of BODIPY fluorescence: IAN (□); Sper/NO (●). Percentage inhibition relative to: control response in the presence of vehicle and absence of initiator (100%); response in the presence of vehicle and initiator (0%). Data are fitted to sigmoidal curves: EC_{50}(2, synaptosomes) = 1.0×10^{-3} M, EC_{50}(2, liposomes) = 1.3×10^{-4} M. Error bars show SEM, unless smaller than the size of the symbol ($n = 3$).

For each set of PUFA liposome experiments, response was normalized to 100% (corresponding to fluorescence readings immediately prior to addition of antioxidant or vehicle) and zero (corresponding to quenching of BODIPY fluorescence and completion of reaction with ABAP). The time course of lipid peroxide formation was measured in the absence and presence of ABAP plus vehicle, and in the absence and presence of antioxidant or vehicle. Vehicles were found to have negligible effect on lipid peroxidation. The rate of peroxidation was dependent on the concentration of radical initiator (Figure 9A). Peroxidation initiated by ABAP (1 mM) was completely inhibited by the antioxidant Trolox at 0.01 mM (Figure 9B) and Sper/NO at 0.1 mM (data not shown). Sper/NO and IAN yielded concentration-dependent inhibition of ABAP-induced lipid peroxidation (Figure 9B). Furthermore, the potency of Sper/NO was at least 1 order of magnitude greater than IAN in this assay.

A full concentration−response curve for peroxide formation in PUFA/BODIPY liposomes, induced by ABAP, was obtained for the nitrate, **2**. Transformation of the data to show values for inhibition of lipid peroxidation allowed comparison with the Fe/synaptosome/TBARS data (Figure 8). A sigmoidal concentration−response curve was obtained for **2**, showing deviation at very high nitrate concentration (≥5 mM), but no evidence for the

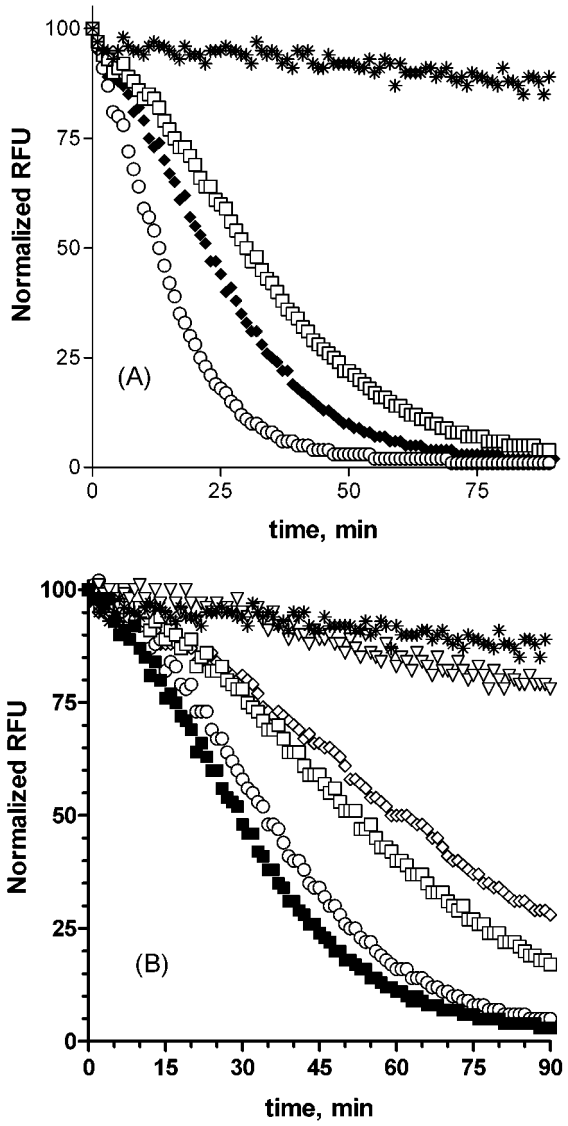


Figure 9. Time course of reduction in BODIPY fluorescence in response to ABAP-induced lipid peroxidation of phospholipid liposomes in phosphate-buffered saline (pH 7.4, 10 mM, NaCl, 50 mM, 37 °C). (Upper panel) Incubation with ABAP (control ∗, 1 mM □, 2.2 mM ◆, 5 mM ○). (Lower panel) Incubation with ABAP (control ∗, 1 mM ■) or with ABAP in the presence of: Trolox (0.01 mM) (▽); IAN (0.1 mM) (◇); IAN (1 mM) (□); or Sper/NO (0.01 mM) (○). Relative fluorescence units (RFU) are normalized to 100% intensity immediately prior to addition of antioxidant or vehicle, with 0% intensity corresponding to complete reaction. All time courses measured in triplicate.

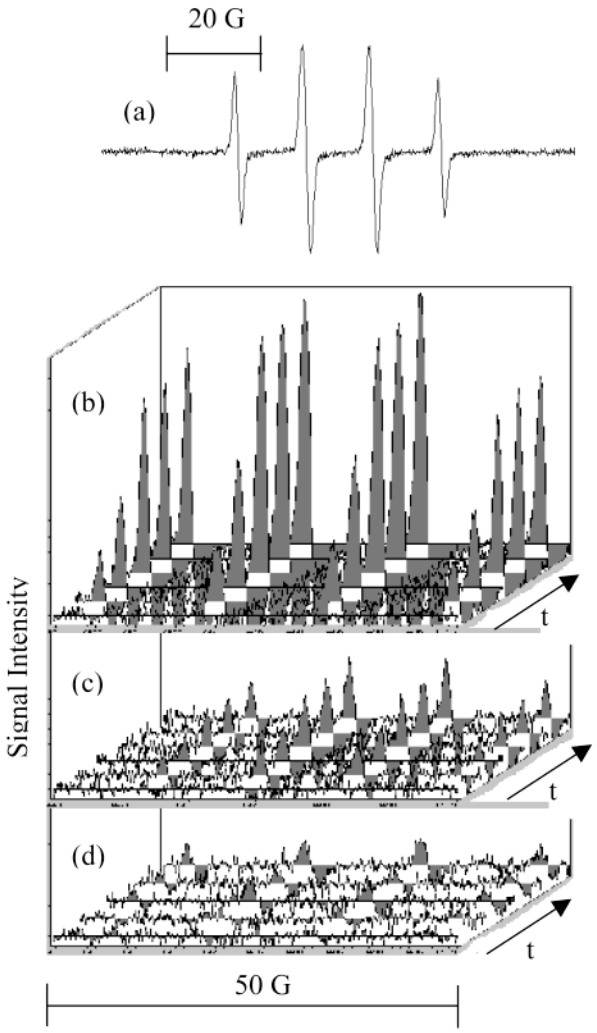


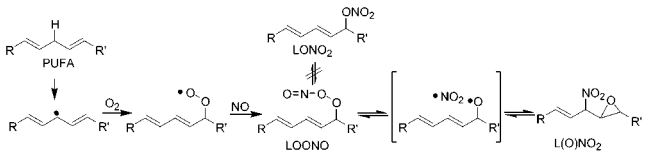
Figure 10. (a) ESR spectrum of DMPO/ABAP adduct (see Scheme 3). (b, c) ESR spectra (intensity >0) of DMPO/adduct superimposed and stacked as a function of time (0, 15, 30, 60, 90 min, *z*-axis) at 37 °C: (b) ABAP (10 mM) in 40% MeCN/phosphate buffer [100 mM, pH 7.4, DTPA (1 mM)]; (c) as (b) with Trolox (0.1 mM); (d) as (b) with IAN (1 mM). *x*-Axis: field 3450−3500 G. *y*-Axis: showing half of full spectrum ($I > 0$); arbitrary intensity units normalized to common scale. DMPO/ABAP adduct: a_H = 14.8 G; a_N = 14.4 G; *g*-factor = 2.0116; $L/G = 0.5$; line width = 1.4; time constant = 10 ms; sweep time = 84 s; RG = 5×10^5; Mod Amp = 1 G; power = 5 mW.

prooxidant effect of 2 seen in synaptosomes (Figure 8). Under identical conditions, lipid peroxides were measured in the presence of IAN and Sper/NO, showing less and greater inhibition potency compared to nitrate 2, respectively (Figure 8).

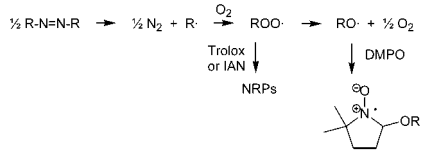
ESR Spin Trapping Measurements. Spin trapping of radicals formed from azo-initiators has previously been followed with respect to time, using radical trapping by 5,5-dimethyl-4,5-dihydro-3*H*-pyrrole-*N*-oxide (DMPO) generating ESR signals attributed to the alkoxy/DMPO radical adducts (*37*). At 37 °C, the ESR spectrum of the radical adduct trapped by DMPO (21 mM) from thermal decomposition of ABAP (10 mM), in oxygen-equilibrated solution, was monitored with time, in the absence and presence of Trolox and of IAN (Figure 10). At higher concentrations of Trolox (1 mM) and IAN (10 mM), no adduct was observed in the ESR spectra, whereas at 10-fold lower concentrations of Trolox and IAN, adduct formation was attenuated (Figure 10). Thus, both Trolox and IAN inhibited formation of the putative alkoxy/DMPO radical adduct. To further examine this antioxidant model system, 5-(diethoxyphosphoryl)-5-methyl-4,5-dihydro-3*H*-pyrrrole-*N*-oxide (DEPMPO) was used in place of DMPO, and the azo-initiators 2,2′-azobisisobutyronitrile (AIBN) and 4,4′-azobis(4-cyanovaleric) acid were used in place of ABAP. Qualitatively identical results were obtained (data not shown). The latter experiments rule out possible nitrosation of the amidine moiety of ABAP by IAN as a contributing factor.

The observed attenuation of ESR signals due to the alkoxy/DMPO radical adduct may result from reaction of the putative antioxidant with intermediate peroxyl and alkoxyl *O*-centered radicals, or reaction with the alkoxy/DMPO radical adduct, which is itself a nitroxyl radical. The competition for trapping of *O*-centered radicals

Scheme 2



Scheme 3



between a putative antioxidant, such as IAN, and a nitrone, such as DMPO, represents a useful test of antioxidant properties (Scheme 3), whereas trapping of the nitroxyl radical adduct does not. To test these possibilities, solutions of ABAP and DMPO were incubated for 15 min before addition of antioxidant. Addition of Trolox (0.1 or 1 mM) led to attenuation and disappearance of alkoxy/DMPO radical adduct signals, whereas addition of IAN (1 or 10 mM) simply led to inhibition of the rate of further adduct formation (data not shown), demonstrating that IAN does not degrade the alkoxy/DMPO adduct directly, but must trap a radical formed from azo-initiator decomposition in the presence of oxygen, possibly the alkoxyl radical (Scheme 3).

Discussion

The major finding of this paper is that antioxidant activity toward lipid peroxidation has been observed and quantified for the first time for novel organic nitrates ($RONO_2$) and for a classical organic nitrite (RONO), in a variety of model systems and in comparison with known antioxidants.

Lipid Peroxidation Systems. Membrane lipid peroxidation is associated with many disease state pathologies, most notably atherosclerosis, but also including traumatic and chronic neurodegenerative disorders (*38*). Although lipid peroxidation may simply be concomitant, a causative role is probable in many cases of cell injury and disease pathogenesis. Lipid peroxidation deleteriously affects the biophysical properties of biological membranes, and releases reactive, potentially cytotoxic products, in particular aldehydes (*32*). Among membrane properties compromised by lipid peroxidation are the selective, active and passive transport of ions and other biomolecules (*39*). A number of initiators have been used to mimic, in vitro, the oxidative stress that leads to lipid peroxidation in vivo, including: (a) azo-initiators (*9*, *12*, *15*, *40*); (b) xanthine oxidase (*4*); (c) lipoxygenase (*41*); (d) peroxynitrite (*7*); (e) cupric and cuprous ions (*11*); and (f) ferric and ferrous ions (*42*). Results from the different methods are often comparable.

Two induction systems for lipid peroxidation were chosen for the present study: ferrous sulfate, because this mode of initiation mimics the iron-induced lipid peroxidation that is likely of biological and pathological significance (eqs 6−9, 11, 12); and azo-initiator (ABAP) induction, which provides a mechanistically more simple system for comparison (eqs 4, 5, 11) (*43*):

$$R{-}N{=}N{-}R \rightarrow 2R^{\bullet} + N_2 \quad (4)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (5)$$

$$Fe^{2+} + O_2 \rightarrow O_2^{\bullet -} + Fe^{3+} \quad (6)$$

$$Fe^{2+} + O_2^{\bullet -} + 2H^{+} \rightarrow Fe^{3+} + H_2O_2 \quad (7)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{-} + HO^{\bullet} \quad (8)$$

$$HO^{\bullet} + H_2O_2 \rightarrow H_2O + HOO^{\bullet} \quad (9)$$

$$ONOO^{-} + H^{+} \rightleftharpoons ONOOH \rightarrow [HO^{\bullet}\cdot NO_2] \rightarrow HO^{\bullet} + NO_2 \text{ or } H^{+} + NO_3^{-} \quad (10)$$

$$LH + ROO^{\bullet} \rightarrow L^{\bullet} + ROOH \quad (11)$$

$$LH + HO^{\bullet} \rightarrow L^{\bullet} + H_2O \quad (12)$$

$$LH + NO_2 \rightarrow L^{\bullet} + HNO_2 \quad (13)$$

H-atom abstraction from homoallylic polyunsaturated fatty acid (PUFA) lipids leads to initial formation of C-centered lipid radicals (eqs 11−13), which undergo chain propagation (or lipid autoxidation) leading to further C- or O-centered peroxyl and alkoxyl lipid radicals (eqs 15−19). Significant differences between Fe-induced and ABAP-induced lipid peroxidation are the ability of the transition metal to catalyze subsequent reactions of lipid peroxidation products (e.g., eq 17) and the susceptibility of metal ion induction to the effects of prooxidants, such as ascorbate (AH^{-}) and thiols (eq 14) (*44*).

$$2Fe^{3+} + (RS^{-} \text{ or } AH^{-}) \rightarrow 2Fe^{2+} + \left(\frac{1}{2}RSSR \text{ or } A + H^{+}\right) \quad (14)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (15)$$

$$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH \quad (16)$$

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + HO^{-} + Fe^{3+} \quad (17)$$

$$LO^{\bullet} \rightarrow L^{\bullet}(O) \quad (18)$$

$$L^{\bullet}(O) + O_2 \rightarrow L(O)OO^{\bullet} \quad (19)$$

Two biologically relevant lipid matrixes were chosen for study: rat brain cerebrocortical synaptosomes chosen for biological significance to neurodegeneration; and phospholipid liposomes selected as a much simpler system for comparison. A number of methods are available for assessing the extent of lipid peroxidation. A commonly used technique to quantify the products of lipid peroxidation, in particular aldehydes, is measurement of TBARS. The drawbacks of this technique have been discussed, and while TBARS may reflect reactive substances in addition to malondialdehyde, the TBARS measure allows highly reproducible determination of lipid

peroxidation products (*45*−*47*). TBARS is an appropriate assay for Fe-induced lipid peroxidation in synaptosomes, which represents a diverse heterogeneous system of vesicles containing protein, lipid, and bound metal ions. The simpler system of ABAP-induced lipid peroxidation in egg phosphatidylcholine liposomes produces only small quantities of TBARS, because subsequent reaction of PUFA hydroperoxides is not accelerated by added or contaminant transition metals; therefore, the fluorescent probe BODIPY was employed to quantify lipid peroxide formation in liposomes (*48*).

The Fe^{II}/synaptosome/TBARS system was benchmarked with the lipid-soluble antioxidant αTH and the water-soluble prooxidant ascorbic acid, showing the expected concentration-dependent prooxidant effect of ascorbic acid, and inhibition of lipid peroxidation by αTH (Figure 1) (*44, 49*). TBARS were quantified after 30 min incubation with $Fe(SO_4)$, which was added immediately after addition of pro/antioxidant solutions. A full concentration−response curve was obtained for inhibition of lipid peroxidation in this system using the more water-soluble antioxidant vitamin E analogue Trolox C (Figure 3). Inhibition curves for the lipid-soluble disulfide LA and *vic*-dithiol LAH_2 showed the prooxidant effect of thiol, again expected in Fe-induced lipid peroxidation systems, in contrast to the negligible effect of the disulfide up to millimolar concentrations (Figure 2) (*50*).

NO and Oxidative Damage. NO has variously been proposed as a cytotoxic agent (*6, 7*) and as a cytoprotective agent (*8, 9, 51*), possessing both prooxidant and antioxidant properties (*11*−*14*). Cytotoxicity attributed to endogenous NO is ascribed to the reactivity of NO reaction products produced under conditions of oxidative stress. Recently species such as NO_2Cl, produced from the main NO metabolite, NO_2^-, have aroused interest, but the primary mediator of oxidative damage is believed to be peroxynitrite (*52, 53*). Over 2000 research papers were published between 1995 and 2000 on aspects of peroxynitrite biology and chemistry. Peroxynitrite is formed by the very rapid reaction of NO with O_2^-. Under physiological conditions, the conjugate acid of peroxynitrite, or the CO_2-adduct, undergoes rapid homolysis to yield the geminate radical pairs $[HO^{\bullet}\cdot NO_2]$ and $[CO_3^{\bullet-}\cdot NO_2]$, respectively. Various products have been identified from the caged pairs: the major reaction pathway is recombination to the rearrangement product, NO_3^-, but cage escape yields the free radicals, NO_2, and hydroxyl or carbonate (*54*). All three free radicals may initiate lipid peroxidation via H-atom abstraction (eqs 12, 13) (*4, 55, 56*). In our hands, peroxynitrite was observed to quench the fluorescence of the BODIPY probe, whether BODIPY was incorporated into PUFA liposomes, into DPPC liposomes, or in simple solution in MeCN/aqueous phosphate buffer mixtures. This direct oxidation reaction of BODIPY by peroxynitrite is under further study.

NO as Antioxidant. Paradoxically, whereas peroxynitrite, derived from NO, initiates lipid peroxidation, albeit at relatively high concentrations, there is substantial evidence, particularly from the use of NO donors, that NO itself inhibits lipid peroxidation (*4, 5*). A number of mechanisms may be drawn for inhibition of lipid peroxidation by NO. Although initial focus was on the interaction of NO with Fe-centers (*57*), evidence now suggests that the apparent antioxidant activity of NO toward lipid peroxidation results from radical chain termination, rather than via attenuation of the radical initiator (*12, 14, 58*).

To reconcile the sometimes paradoxical data on prooxidant and antioxidant activities of NO, contemporary theories have variously proposed the importance of: the flux of NO; the timing of NO release; and the formation of "NO_x" chemical species other than NO, including RNOS (*2*). Whereas all of these theories may have merit, it is likely that some of the paradoxical behavior of NO derives from the fact that the experimental use of NO donors is preferred over the use of purified NO gas. Analysis of data from NO donors (*59, 60*) is complicated by the release of products in addition to NO, reactions of the reactive intermediates involved in release of NO, and uncertainty as to the exact chemical mechanisms of NO release. NONOates are arguably the simplest class of genuine NO donors in their mechanism of NO release, yielding up to 2 equiv of NO in an acid-catalyzed hydrolytic degradation, which can be monitored spectrophotometrically (*61*).

The NO donor, Sper/NO, was shown to inhibit lipid peroxidation in synaptosomes in a concentration-dependent manner, leading toward complete inhibition of lipid peroxidation at supermillimolar concentrations (Figure 7). Goss et al. studied the time course of low-density lipoprotein (LDL) oxidation induced by Cu^{II}, ABAP, and SIN-1 (a putative peroxynitrite source) in the presence of NONOates, with reactivity spanning that of Sper/NO (*11*). Quite reasonably, the effectiveness of the NONOates in inhibiting lipid peroxidation, as assessed by TBARS and other methods, was related to the rate of NO release, the rate of oxidation, and the rate of loss of NO, such that the most reactive NONOate was ineffective as an antioxidant over the long period of oxidation studied, 1−10 h. Our synaptosome system, was subjected to a shorter 30 min oxidation with $FeSO_4$, and with a half-life of approximately 45 min for Sper/NO degradation; at pH 7.4 in phosphate buffer, Sper/NO, as expected, acted as an inhibitor. In this assay, Sper/NO was 2-fold fold less potent than Trolox (Figure 7). In PUFA liposomes, we also observed Sper/NO to show concentration-dependent inhibition of ABAP-induced peroxide formation, with Sper/NO (100 μM) and Trolox (10 μM) both giving complete protection (Figure 9).

Lipid Nitrates and Lipid Nitrites. Chain termination in lipid peroxidation leads to production of a variety of non radical products (NRPs), including aldehydes (eq 20). The classical chain-breaking antioxidant α-TH (the major constituent of vitamin E) terminates two radical chains per molecule, again leading to a variety of NRPs (eqs 21, 22) (*62*). Chain breaking by NO can hypothetically yield a variety of N,O-containing NRPs, including lipid nitrites, nitrates, and pernitrites (eqs 23, 24, 26−28). Simple alkyl pernitrites (ROONO) would be expected to show reactivity similar to peroxynitrous acid (HOONO), that is, to undergo rapid rearrangement to the nitrate (eq 25). The formation of nitro (LNO_2), nitrosooxy (nitrite, LONO), and nitrooxy (nitrate, $LONO_2$) lipid products has been supported by MS analysis of lipid peroxidation in the presence of dissolved NO solution, NO donors, and even peroxynitrite, although product isolation and full characterization is awaited (*15, 41, 63, 64*). The interesting observation that the stoichiometry of NO chain termination requires 2−4 molecules of NO per chain has led to proposal of the overall reaction stoichiometry shown in eq 1.

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow NRPs \quad (20)$$

$$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet} \quad (21)$$

$$LOO^{\bullet} + TO^{\bullet} \rightarrow NRPs \quad (22)$$

$$LOO^{\bullet} + NO \rightarrow LOONO\ (k = 2 \times 10^{9}\ M^{-1}\ s^{-1}) \quad (23)$$

$$LO^{\bullet} + NO \rightarrow LONO\ (k = 3 \times 10^{9}\ M^{-1}\ s^{-1}) \quad (24)$$

$$LOONO \rightarrow [LO^{\bullet}\cdot NO_2] \quad (25a)$$

$$[LO^{\bullet}\cdot NO_2] \rightarrow LO^{\bullet} + NO_2 \text{ or } LONO_2 \text{ or } L(O)NO_2 \quad (25b)$$

$$LO^{\bullet} + NO_2 \rightarrow LONO_2 \quad (26)$$

$$L^{\bullet}(O) + NO_2 \rightarrow L(O)NO_2 \quad (27)$$

$$L(O)OO^{\bullet} + NO \rightarrow L(O)OONO \quad (28)$$

Hydroxyl radical photochemically generated from alcoholic inorganic nitrite solutions yields peroxyl radicals which react with NO very rapidly, 10^3 more rapidly than with ascorbate (*65*). The primary products from reaction of ethylperoxyl radical with NO are reported to be (1) ethyl nitrate (<2%) and (2) NO_2 + ethoxy radical (98%), with secondary products including ethyl nitrite, ethyl nitrate, ethyl pernitrate, acetaldehyde, and nitroxyl (*66*). Hydroxyl radical produced from methyl nitrite photolysis will react with a saturated alkane, yielding a peroxyl radical in the presence of O_2, which on reaction with NO yields alkyl nitrate as product (*67*). Further support for nitrate formation in reaction of *O*-centered radicals with NO and NO_2 comes from the reaction of NO_2/N_2O_4 with *tert*-butyl peroxide, which yields approximately 80% *tert*-butyl nitrate and 10% *tert*-butyl nitrite (*68*). However, in the autoxidation of the PUFAs methyl linoleate and linolenate with NO_2, N,O-containing lipid products were too labile to be isolated (*69*). Furthermore, although allylic nitrite and nitro products were inferred from spectroscopic data, allylic nitrates were not, possibly owing to intramolecular trapping of the allylic pernitrite intermediate (Scheme 2; see eq 25). *Thus, there is support from experimental data for the formation of nitrolipids, lipid nitrites, and lipid nitrates from both peroxynitrite-induced lipid peroxidation and inhibition of lipid peroxidation by NO. The probability then exists that such compounds represent natural components of lipid membranes.*

So-called non reactive products (NRPs) from radical chain termination are species which often possess cytoxic potential themselves, often through chemical reactivity leading to formation of covalent adducts with biomolecules (*38*). However, lipid nitrate and nitrite NRPs have the potential not only to undergo further reaction but also either to propagate or to inhibit the chain reaction. The putative pernitrite ester produced from chain termination by reaction of lipid peroxyl radical with NO is likely to decompose to yield a significant flux of chain-propagating free radicals, including alkoxyl radical and NO_2 (eqs 2, 3) (*65, 66*). But what of the reactivity of lipid nitrate and nitrite NRPs?

Nitrates and Nitrites. It is a mistake to consider these chemical species collectively, since the chemical reactivity of nitrites and nitrates is very different. The organic nitrite functional group is relatively labile toward hydrolysis, which is susceptible to acid catalysis, and provides an effective nitrosating agent, reacting with a variety of nucleophiles and readily undergoing transnitrosation (*20, 70, 71*). Conversely, the organic nitrate functional group is hydrolytically stable, undergoing denitration only in strong alkali, and represents a poor nitrating agent (*19*).

Study of the photochemistry of nitrites has yielded a variety of interesting reactions, some of synthetic utility, leading to nitroxides, oximes, alcohols, nitrates, and nitrosoalkanes as isolable products. Both nitrites and nitrates will undergo photochemical homolysis reportedly to yield alkoxyl radical, NO or NO_2, and secondary products, but for nitrates, this process has a higher energy barrier (eqs 2, 3) (*16−18, 72, 73*). Thermal homolysis and reactions of nitrates and nitrites with free radicals are less well studied, although thermal radical reactions of synthetic utility have been reported. After thermal or photochemical homolysis, H-atom abstraction by the initial radical products, NO_2, $RO^{\bullet}$, or NO, is well precedented, to yield aldehyde (eq 3) and other products (*74*).

The reduction of a nitrate to NO is a $3e^-$ reduction. Classical nitrates, such as GTN, require biotransformation, which is probably enzyme-mediated, to release significant amounts of NO. GTN itself reacts only slowly with thiols at physiological pH, and only a small fraction of the reaction flux leads to NO as product (*19*, *30, 31*). Under aerobic conditions, negligible amounts of NO (2.5 nM/min) are produced in the presence of cysteine (2 mM), from GTN (1 mM), in a reaction which is probably catalyzed by trace transition metals, and inhibited by O_2 (*31*). It is misleading to regard classical nitrates as simple NO donors. However, there is ample evidence that NO is released on administration of GTN to whole cell and tissue preparations, but that these biotransformation mechanisms are compromised in broken cell preparations (*19*, *75*). Thus, classical nitrates, including xenobiotics and lipid nitrates, may yield NO on biotransformation, in vivo.

The reduction of an organic nitrite to NO is a more facile $1e^-$ reduction, and much larger fluxes of NO can be detected from nitrites in neutral, aqueous solution, even in the absence of added thiols (*30*). There is a body of work on the heterolytic reactions of nitrites, but there exist little data on NO release from homolytic reactions (*71*). Comparison of nitrites (RONO) with nitrosothiols (RSNO) is useful. Nitrosothiols are NO donors, which undergo homolysis to NO and disulfide, usually requiring trace metal ion catalysis, or photochemical initiation (*76*). Spin-trapping in the reactions of phenol with either a nitrosothiol or *i*-pentyl nitrite gave evidence for a thiyl radical intermediate from the nitrosothiol, but no alkoxyl radical intermediate from the nitrite, an observation echoed in study of mixed solutions of nitrosothiols and nitrites (*71, 77*). Nitrosothiols, argued to be acting via NO release, have been shown to act as antioxidants in a variety of systems (*40*).

From consideration of chemical data, there is potential for nitrates and nitrites to undergo degradation to yield prooxidant species, including alkoxyl radical and NO_2, and to yield the antioxidant NO. In the case of nitrites, a further possibility is a nonradical pathway, such as hydrolysis, or transnitrosation with a suitable nucleophile, since nitrites are good nitrosylating agents. Ob-

servations on the effect of nitrites on lipid peroxidation have not been reported. Published data on the influence of the classical nitrate GTN on lipid peroxidation include, variously, reduction of lipid peroxidation, no effect, and potentiation of lipid peroxidation (*21*−*23*).

Nitrates, Nitrites, and Lipid Peroxidation. We observed no perturbation of Fe-induced lipid peroxidation in synaptosomes by the classical nitrate GTN (data not shown). Classical nitrates are poor NO donors in vitro, even in the presence of thiols (*31*), whereas SS-nitrates, such as **1**, may react with thiol to generate a significant flux of NO (*28*). Furthermore, novel nitrates 2 and 3 spontaneously release NO in aqueous and mixed aqueous/organic solvent systems (*28*). The novel nitrates, **1**−**3**, are compounds with interesting biological activity, differentiated from that of classical nitrates (*26*).

Measurements of lipid peroxidation in the presence of nitrates and thiol adjuvants are complicated by the prooxidant capacity of thiols in Fe-induced lipid peroxidation (Figure 3). Therefore, to discern the effects of the nitrates themselves, the data must be normalized for the effect of prooxidant, adjuvant thiol. Thus, the negligible effect (ca. 0% inhibition of lipid peroxidation) reported for GTN + LAH_2 indicates that the amount of lipid peroxidation was unchanged from that seen with LAH_2 (i.e., GTN did not inhibit the prooxidant effect of LAH_2 on Fe-induced lipid peroxidation). Similar results were seen for GTN with water-soluble thiol (cysteine) and lipid-soluble thiol adjuvants (LAH_2, PhSH). The SS-nitrates react with thiols to yield NO, and the SS-nitrate, **1**, did show a concentration-dependent inhibition (≤30%) of lipid peroxidation in the presence of LAH_2 (Figure 4). Furthermore, the reaction of the lipophilic nitrate, **1**, with lipophilic PhSH produced significantly greater inhibition of lipid peroxidation than seen with hydrophilic cysteine (Figure 5). Although these results are compatible with inhibition of lipid peroxidation by NO produced from the SS-nitrate, the use of nitrates that spontaneously generate NO overcomes complications from the background thiol prooxidant effect.

The nitrate **3** spontaneously releases a flux of NO in aqueous solution, detectable electrochemically, whereas **1** releases very low concentrations of NO, detectable only by much more sensitive chemiluminescence methods. Nitrate **3** showed significant, concentration-dependent inhibition of lipid peroxidation, but an accurate EC_{50} could not be determined within solubility limits (Figure 6). Surprisingly, nitrate **1** inhibited lipid peroxidation equivalently at low concentrations (Figure 6). It is not unreasonable that **1** may react with components of the complex synaptosome lipid peroxidation medium to release concentrations of NO sufficient to give 20−30% inhibition of lipid peroxidation. In contrast, there is no ambiguity with nitrate 2, which is a spontaneous NO donor in aqueous solution, and which efficiently inhibited Fe-induced lipid peroxidation in synaptosomes with an EC_{50} of 1×10^{-3} M (Figure 8). Nitrate 2, which contains a thiol group, might be expected to show some prooxidant capacity, and although some evidence for this may be seen at low concentrations, at higher concentrations, 2 is able to almost completely inhibit lipid peroxidation. The classical nitrite IAN also efficiently inhibited Fe-induced lipid peroxidation in synaptosomes with an EC_{50} of 1.6×10^{-4} M^{-1}, an order of magnitude higher potency than nitrate 2 (Figure 7).

Synaptosomes vs Liposomes. Mechanistic information may be gleaned from comparison of observations on lipid peroxidation in the two systems: (i) synaptosome/Fe^{II}/TBARS; and (ii) liposomes/ABAP/BODIPY. Synaptosome preparations are useful for studies relevant to neuronal oxidative damage, and neurodegeneration, but represent a complex matrix containing a large variety of biomolecules and their fragments. Phosphatidylcholine liposomes represent a much simpler matrix for study of lipid peroxidation, especially with use of the thermal azo-initiator ABAP. The study of the combination of both simple and biologically relevant lipid systems is essential for understanding of antioxidants as is exemplified by studies on αTH, wherein antioxidant activity is seen in simple model systems, but prooxidant activity is observed in LDL itself (*62*). The use of an azo-initiator in liposomes, importantly, serves to determine whether antioxidant activity observed in synaptosomes is purely the result of interactions with Fe ions: (a) by complexation and/or oxidation of Fe^{II}; (b) by inhibition of Fenton chemistry; or (c) by an Fe^{II}-catalyzed reduction of nitrate or nitrite to yield NO. TBARS measurements are not satisfactory for the ABAP/liposome system, because the secondary formation of TBARS products, from initial peroxidation, is slow in the absence of transition metals. Therefore, BODIPY, a fluorescent probe, was employed for quantification of lipid peroxidation. BODIPY has been used in one previous lipid peroxidation study; therefore, control experiments were required to validate the methodology (*48*). TBARS are secondary oxidation products, including in particular MDA, formation of which might be inhibited by nucleophilic trapping, or inhibition of peroxide degradation, rather than by chain-breaking antioxidant activity. Observations on the BODIPY measurement of lipid peroxides thus again provide a useful comparison.

In liposome time course experiments, 0.01 mM Sper/NO was seen to be approximately equivalent to 1 mM IAN in inhibiting lipid peroxidation, in dramatic contrast to the observations in synaptosomes (Figures 7 and 9). As was observed in synaptosomes, the NO donor, Sper/NO, was considerably less effective than Trolox at equimolar concentrations, due to fact that the concentration of NO at any time-point during the experiment was obviously much lower than the initial concentration of the NO donor. Using a slightly modified methodology, a full concentration−response curve was obtained for inhibition of lipid peroxidation in liposomes by nitrate 2, showing a leftward shift relative to synaptosomes, corresponding to a 10-fold increase in potency in the less complex matrix (Figure 8). The order of potency observed in synaptosomes was: IAN ≥ Sper/NO > 2, in contrast to liposomes: Sper/NO > 2 > IAN. The flux of NO released, in phosphate-buffered solution over 20 min, from IAN (0.1 mM), Sper/NO (0.01 mM), and 2 (0.2 mM), measured using chemiluminescence detection, was similar. The potency of an NO donor as an inhibitor of lipid peroxidation is dependent on the time of incubation. Labile NO donors would be rapidly exhausted, whereas relatively stable NO donors would release too low a flux of NO to significantly inhibit oxidation. The half-life for decomposition of Sper/NO to NO is approximately 45 min at pH 7.4; thus, over the time course of peroxidation in liposomes (90 min) and synaptosomes (30 min), neither IAN, Sper/NO, nor 2 would be expected to be exhausted.

Inhibition of Peroxidation Monitored by ESR. Spin traps, such as the nitrone DMPO, will scavenge the free radicals formed in lipid peroxidation to form radical adducts which are quantifiable by ESR spectroscopy. In the presence of oxygen, but absence of lipid, thermal decay of an azo-initiator will form peroxy radicals (eqs 4, 5). The ESR spectrum observed in the thermal decomposition of ABAP in the presence of DMPO has been assigned to the DMPO/ABAP radical adduct formed from alkoxyl radical trapping (Scheme 3) (*37*). A competition experiment, in which the nitrone competes with an alternate radical scavenger, such as Trolox or IAN, illustrates the radical scavenging efficiency relative to the nitrone (Scheme 3). Both Trolox and IAN quenched DMPO/ABAP radical adduct formation in the presence of excess DMPO (Figure 10). Furthermore, control experiments demonstrated that IAN did not simply quench formation of the DMPO/ABAP radical adduct by accelerating the decomposition of the adduct itself. These ESR spin trap competition experiments provide further verification of the chain-breaking antioxidant potential of nitrites.

Mechanism of Inhibition of Lipid Peroxidation. Despite the widespread use of NONOates as NO donors, a kinetic study of hydrolytic degradation has only recently appeared (*78*). This study raised complexities associated with aggregation, complexation, and metal ion effects on reactivity, which add to the question of why some NONOates release far less than the theoretical yield of 2 mol equiv of NO. There is a reasonable body of research on mechanisms of reaction of nitrites with nucleophilies, but not on reactions leading to NO release in solution (*71*). We are only beginning to explore the reactivity of novel nitrates, such as 2 and the SS-nitrates (*28*). It is chemically quite reasonable that the rate of NO generation from Sper/NO, IAN, and nitrate 2 would be sensitive to acceleration or inhibition by components of the incubation medium, in particular in the heterogeneous synaptosome lipid peroxidation medium.

Comparison between the synaptosome and liposome systems shows that neither reactions with Fe ions nor inhibition of secondary TBARS product formation is responsible for the observed lipid peroxidation inhibition data, although such reactions may contribute to the observed inhibitor potency. The NO donor rationale for the observed inhibitory action toward lipid peroxidation by NONOates, nitrites and nitrates, rests on breakdown to NO, which has chain-breaking antioxidant properties. Differences in relative inhibitor potency can be explained by perturbations of NO flux by components of the lipid peroxidation incubation medium. In addition to the flux of NO, the lipophilicity of the NO donor must be relevant, since both organic nitrates and nitrites are lipophilic and present in the synaptosome and liposome lipid domains in high local concentrations, in contrast to the hydrophilic NONOate. The lipophilicity of initiator and antioxidant and, indeed, the location within lipid domains are known to be important factors in inhibitor efficiency (*79*).

The NO donor rationale raises questions. While Sper/NO reacts to release 2 mol equiv of NO and amine byproduct, NO release from nitrites via homolysis theoretically will generate chain-propagating alkoxyl radical (eq 2). An alternative to the NO donor argument is direct reaction of nitrites and nitrates with lipid peroxyl radicals, which again would be facilitated by the lipophilicity of nitrates and nitrites enhancing reaction rate and consequent chain termination. Although a balanced equation can be drawn for nitrate 2 (eq 29), the underlying chemistry remains to be defined. Furthermore, direct reaction of nitrites does not remove the problem that radical trapping produces further propagating radical products (eq 30). Other possible mechanisms may be drawn (e.g., eqs 3, 31). This chemistry is under examination.

$$2HSCH_2CHRONO_2 + 2LOO^{\bullet} \rightarrow (HOCHRCH_2S)_2 + O_2 + 2\,LOONO \quad (29)$$

$$RONO + LOO^{\bullet} \rightarrow RO^{\bullet} + LOONO \quad (30)$$

$$RCH_2ONO + LOO^{\bullet} \rightarrow RCHO + LOOH + NO \quad (31)$$

Summary. The primary objective of this work was to determine, unambiguously and for the first time, the capacity of organic nitrites and nitrates to inhibit or potentiate lipid peroxidation in biologically relevant model systems. Novel nitrates and classical nitrites were shown to be inhibitors of Fe-induced lipid peroxidation in rat cerebrocortical synaptosomes, a system chosen for its relevance to putative mechanisms of neurodegeneration. The generality of these phenomena was verified by measurements of inhibition of lipid peroxidation in phosphatidylcholine liposomes using an azo-initiator, and quantification of lipid peroxidation using a fluoresecent probe, and further supported by spin trap ESR studies. The capacity of nitrates and nitrites to inhibit lipid peroxidation is significant: first, because of the roles of nitrates as important therapeutic agents and nitrites as xenobiotics and potential intermediates in nitrate biotransformation; but second, because lipid nitrates and nitrites have been proposed to be natural products from the antioxidant reactions of NO with lipid alkoxyl and peroxy radicals. It is reasonable to speculate that a protective role against lipid peroxidation may be played by both exogenous and endogenous nitrates and nitrites.

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