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tert-Butyl Hydroperoxide/Hemoglobin-Induced Oxidative Stress and Damage to Vascular Smooth Muscle Cells

Different Effects of Nitric Oxide and Nitrosothiols

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ABSTRACT. The goal of the present work was to determine whether nitric oxide (NO) released from different donors (NONOates and nitrosothiols) can act as a protective antioxidant against oxidative stress and cytotoxicity induced by extracellular hemoglobin/*tert*-butyl hydroperoxide (Hb/*tert*-BuOOH) in vascular smooth muscle cells (VSMCs). No changes in phospholipid composition were found in VSMCs incubated with oxyhemoglobin (oxyHb)/*tert*-BuOOH. Using our newly developed HPLC-fluorescence technique for measurement of site-specific oxidative stress in membrane phospholipids, we produced VSMCs in which endogenous phospholipids were metabolically labeled with an oxidation-sensitive fluorescent fatty acid, *cis*-parinaric acid. In these cells, we were able to reliably quantitate oxidative stress in major phospholipid classes—phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol—induced by *tert*-BuOOH in the presence of oxyHb or methemoglobin (metHb). The oxidative stress was accompanied by cytotoxic effects of oxyHb/*tert*-BuOOH and metHb/*tert*-BuOOH on VSMCs. We further found that an NO donor, (Z)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen 1-ium-1,2-diolate (PAPANONO), but not nitrosothiols, protected VSMCs against oxidative stress and cytotoxicity induced by Hb/*tert*-BuOOH. The protective effect of PAPANONO was most likely due to its ability to form NO-heme Hb (detectable by low temperature EPR spectroscopy and visible spectrophotometry). These findings are important for further understanding the physiological antioxidant role of NO against oxidative stress induced by hemoproteins as well as for pathological hypertensive events induced by extracellular Hb via NO depletion. BIOCHEM PHARMACOL **57**;9:989–1001, 1999.

KEY WORDS. nitric oxide; nitrosothiols; oxidative stress; *tert*-butyl hydroperoxide; smooth vascular muscle cells; hemoglobin

Antioxidant deficiency and oxidative stress have been associated with a plethora of pathological conditions and diseases such as cancer, Alzheimer's disease, diabetes, coronary artery disease, and hypertension [1–5]. In particular, disturbances of intracellular Ca^{2+} homeostasis caused by oxidative stress and membrane lipid peroxidation may be accountable, at least in part, for the dysregulation of contraction/relaxation cycles in smooth muscle cells and alteration of vascular tone [6–8]. Specific mechanisms involved in the generation of reactive oxygen species and organic free radicals causative of oxidative stress in smooth muscle cells are still to be elucidated.

Extracellular Hb†† is increased in a number of pathological conditions, such as preeclampsia, trauma, and hemorrhagic injury [9, 10] and can catalytically enhance oxidative

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†† *Abbreviations*: Hb, hemoglobin; NO, nitric oxide; VSMCs, vascular smooth muscle cells; *tert*-BuOOH, *tert*-butyl hydroperoxide; oxyHb, oxyhemoglobin; metHb, methemoglobin; hSA, human serum albumin; NAC, *N*-acetylcysteine; FBS, fetal bovine serum; PC, phosphatidycholine; PEA, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SPH, sphingomyelin; DPG, diphosphatidylglycerol; LPC, lysophosphatidylcholine; PnA, *cis*-parinaric acid; BHT, butylated hydroxytoluene; AMVN, 2,2′-azobis(2,4-dimethyl-valeronitrile); SNAP, nitrosylated penicillin; PAPANONO, (Z)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen 1-ium-1,2-diolate; GS-NO, nitrosylated glutathione; and NAC-S-NO, nitrosylated *N*-acetylcysteine.

damage in vascular smooth muscle, thus affecting contractility [11]. This catalytic role of Hb becomes particularly prominent when it forms an extremely potent oxidant, oxoferryl-Hb, upon interaction with hydrogen peroxide or organic peroxides [12, 13].

Hypertensive events are often seen during the administration of extracellular Hb-based O_2 carriers; they have been attributed to depletion of NO and hence disruption of the NO-signaling pathways [14, 15]. The intervention by endothelial NO in both the physiological vascular effects of lipid peroxidation and the biochemical mechanism(s) of oxidative stress was suggested to affect and complicate overall contractility outcomes [16]. At the biochemical level, antioxidant effects of NO may be important for the regulation of oxidative stress through: (i) interaction with catalytic transition metal centers to prevent formation of potent oxidants, and/or (ii) direct free radical scavenging, i.e. termination of lipid peroxidation [17–20]. We demonstrated recently that interaction of NO with heme- and non-heme-iron catalytic sites prevents production of oxoferryl-hemoproteins and protects erythroleukemia K562 cells [21] and cardiac myocytes against oxidative stress and the cytotoxic effects of alkyl hydroperoxides [21, 22].

The goal of the present work was to determine whether the protective effects of NO against oxidative stress and cytotoxicity induced by extracellular Hb/*tert*-BuOOH can be observed in VSMCs. Since nitrosothiols have been implicated recently in a number of physiological and biochemical functions ascribed to NO [23, 24], we compared the effectiveness of two different types of NO donors—NONOates and nitrosothiols—as antioxidant protectors against Hb/*tert*-BuOOH-induced oxidative stress.

MATERIALS AND METHODS

Reagents

PnA (Z-9, E-11, E-13, Z-15-octadecatetraenoic acid) was purchased from Molecular Probes, Inc. The purity of each lot of PnA purchased was determined by UV spectrophotometry (Shimadzu UV 160U spectrophotometer) using the molar extinction $\epsilon_{304\ nm}$ in ethanol, $80 \times 10^3\ M^{-1}\ cm^{-1}$. FBS, fatty acid-free hSA, phospholipids, *tert*-BuOOH, metHb, polyoxyethylenesorbitan monolaurate (Tween 20), sodium molybdate, malachite green base, BHT, Hanks' balanced salt solution, and glutathione were purchased from the Sigma Chemical Co. HCl, acetone, and ammonium hydroxide were purchased from Fisher Scientific. Methanol, chloroform, hexane (HPLC grade), 2-propanol (HPLC grade), water (HPLC grade), and sodium nitrite were purchased from the Aldrich Chemical Co. Silica G plates (5 × 5 cm) were purchased from Whatman. α-Minimum essential medium (MEM-20) was purchased from GibcoBRL/Life Technologies. AMVN was obtained from Polysciences, Inc. Medium for cell culture contained 10% FBS (Sigma), gentamycin, penicillin, streptomycin, fungizone, and L-glutamine (GibcoBRL). Elastase, collagenase, and soybean trypsin inhibitor were obtained from the Sigma Chemical Co. PAPANONO was purchased from the Alexis Corp. Phospholipid standards were purchased from Avanti Polar Lipids.

VSMC Culture

The superior mesenteric artery was removed from Sprague–Dawley rats under sterile conditions and placed in Hanks' balanced salt solution at 37°. VSMCs were isolated from the superior mesenteric artery of the rat by the technique of McGuire *et al.* [25]. Excess adventitial fat and connective tissues were removed. Endothelial cells were removed by passing a 0.011-inch radius wire through the lumen of the artery. The artery was cut into small rings (approximately 0.5 mm) and treated with collagenase (1 mg/mL), elastase (0.5 mg/mL), and soybean trypsin inhibitor (0.5 mg/mL) in Hanks' balanced salt solution in an incubator at 37° for 20 min. Tissues were rinsed twice with 1 mL of MEM-20 with L-glutamine (2 mM) containing 20% FBS, gentamycin (50 μg/mL), fungizone (0.5 μg/mL), penicillin (100 IU), and streptomycin (10 μg/mL). The rings were transferred to 6-well culture plates coated with gelatin and incubated in a drop of medium for 4 hr at 37° in a tissue culture incubator (5% CO_2, 95% O_2) after which 1 mL of MEM-20 medium was gently added for incubation overnight. This incubation technique is necessary for attachment of the smooth muscle cells from explanted tissue to the surface of the dish. Explants were removed after migration of VSMCs and visualized (approximately 7 days). Purity of smooth muscle cell cultures was verified by immunohistochemical staining with anti-α smooth muscle actin (mouse monoclonal clone 1A4, Sigma). Cells between passages 4 and 7 were plated at a density of 2×10^5 cells/75 mL tissue culture flask (Greiner Laboratories, GmbH) and grown in MEM-20 (containing 10% FBS, fungicides, and anti-microbial agents as described above).

Extraction of Cell Lipids

Total lipids were extracted from VSMCs (3×10^6 cells) using the procedure of Folch *et al.* [26]. The lipid extract was dried under nitrogen and dissolved in 0.2 mL of 2-propanol:hexane:water (4:3:0.16, by vol.).

HPTLC Assay of Phospholipid Composition of VSMCs

The phospholipid classes in the extracts were separated by two-dimensional HPTLC on silica G plates. The plates were first developed with a solvent system consisting of chloroform:methanol:28% ammonium hydroxide (65:25:5, by vol.) After drying the plates with a forced air blower to remove the solvent, the plates were developed in the second dimension with a solvent system consisting of chloroform:acetone:methanol:glacial acetic acid:water (50:20:10:10:5, by vol.) The phospholipids were visualized by exposure to iodine vapor and identified by comparison with

migration of authentic phospholipid standards. The spots identified by iodine staining were scraped, and the silicic acid was transferred to tubes. Lipid phosphorus was determined by the sub-micro method as described by Bottcher *et al.* [27]. The identity of each phospholipid was established by comparison with the R_f values measured for authentic standards.

PnA-Based Assay of Oxidative Stress in Phospholipids of VSMCs

Incorporation of PnA into membrane phospholipids of VSMCs was performed as follows. PnA was incorporated into VSMCs by addition of its hSA complex (PnA–hSA) to minimal essential medium without additives. The complex was prepared as described by Ritov *et al.* [28]. PnA–hSA complex was added to the cells during their log phase of growth (3×10^6 cells) to give a final concentration of 5 μg PnA/10^6 cells, and the cells were incubated in minimal essential medium with 10% FBS for 2 hr at 37° in a CO_2 incubator to allow incorporation of PnA into phospholipids. At the end of a specified incubation period, the cells were washed twice with serum-free minimum essential medium with and without hSA (0.5 mg/mL) to remove the excess of unbound PnA.

N-*Acetyl-nitrosocysteine and NO-Glutathione Solutions*

NAC-S-NO and GS-NO were synthesized from glutathione and NAC by incubating them in 0.5 N HCl with an equimolar (200 mM) concentration of $NaNO_2$ as described in Ref. 29. The completeness of the reaction and the purity of the products were confirmed by UV spectra (335 nm) [30].

Treatment of VSMCs with tert-*BuOOH, oxyHb, or metHb and Different NO Donors*

VSMCs preloaded with PnA were incubated for 1 hr in the presence of oxyHb or met/Hb (5 μM) and *tert*-BuOOH (150 μM) at 37° in a CO_2 incubator. The effects of PAPANONO, NO-S-NAC, and GS-NO were studied by adding the above reagents to cells, first prior to oxyHb and *tert*-BuOOH exposures, and again 30 min following application of NO donors. Incubated cells were scraped, collected, and tested for viability by trypan blue exclusion and subjected to phospholipid analysis.

HPLC Analysis of Cell Lipids

Lipid extracts were separated by HPLC using an ammonium acetate gradient essentially as described by Geurts van Kessel *et al.* [31]. The lipid extracts were applied to a 5-mm Supelcosil LC-Si column (4.6 × 250 mm) equilibrated with a mixture of 1 part solvent A:2-propanol:hexane:water (57:43:1, by vol.) and 9 parts solvent B:2-propanol:hexane:40 mM aqueous ammonium acetate (57:43:10, by vol.), pH 6.7. The column was eluted during the first 3 min with a linear gradient from 10% solvent B to 37% solvent B, then for 3–15 min with an isocratic gradient at 37% solvent B, 15–23 min with a linear gradient to 100% solvent B, and 23–45 min with an isocratic gradient at 100% solvent B; the solvent flow rate was maintained at 1 mL/min. The separations were performed using a high performance liquid chromatograph (Shimadzu model LC-600) equipped with an in-line configuration of fluorescence (model RF-551) and UV-VIS (model SPD-10A V) detectors. The effluent was monitored by absorbance at 205 nm to detect lipids, and the fluorescence of PnA was measured by emission at 420 nm after excitation at 324 nm. UV and fluorescence data were processed and stored in digital form with Shimadzu EZChrom software. The identity of phospholipids in the chromatogram was established by collecting each of the peak fractions and subjecting them to HPTLC analysis as described above. Lipid phosphorus was determined using a modification of the method for microdetection described by Chalvardjian and Rubnicki [32].

EPR and Spectrophotometric Assays of Interaction of NO and Nitrosothiols with oxyHb in Model Systems

OxyHb (2.5 μM) was incubated with either 100 μM PAPANONO or 100 μM NAC-S-NO for 60 min at 37°. Transformation of oxyHb in the presence of PAPANONO and NAC-S-NO was monitored spectrophotometrically and by using EPR spectrometry. Visible (VIS) spectra were obtained in the 350 to 700 nm range [17]. EPR spectra were recorded in the range of 320 ± 25 mT as described previously [21]. In low temperature EPR measurements, the concentrations of oxyHb and NO donors were 50 μM (200 μM on per heme basis) and 2.0 mM, respectively. We had to use higher concentrations of oxyHb and NO donors in EPR measurements (as compared with optical spectroscopic assays and experiments with cells) to obtain resolved EPR spectra. In all experiments, we maintained the same 40:1 ratio of NO-donor/oxyHb.

Hemoglobin Solution

Commercial Hb was mainly in the met (ferric) form. We reduced metHb (1 mM solution in 50 mM phosphate buffer, pH 7.4) to its ferrous (oxyHb) form using a fourfold excess of sodium dithionite. Pure oxyHb was obtained by separation on a Sephadex G-25 column preequilibrated with 50 mM phosphate buffer, pH 7.4. The concentration of oxyHb/metHb was calculated as described previously by Winterbourn [33] using the oxyHb extinction coefficient at 577 nm of 15.0 mM^{-1} cm^{-1}.

Statistical Evaluation

Data are expressed as means ± SEM of at least three experiments. The statistical significance of differences between determinations was calculated by ANOVA, and was set at $P < 0.05$.

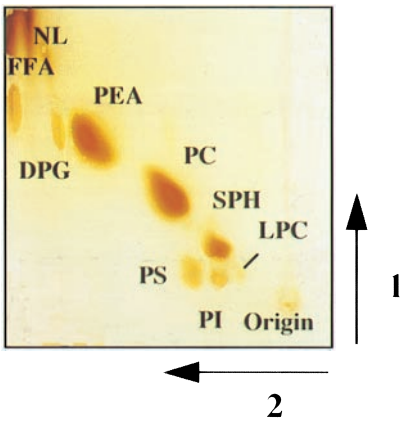

FIG. 1. Typical HPTLC two-dimensional chromatogram of a total lipid extract from logarithmically growing VSMCs. Abbreviations: FFA, free fatty acid; NL, neutral lipids; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; DPG, diphosphatidylglycerol; and LPC, lysophosphatidylcholine.

RESULTS

Assessment of Hb/tert-BuOOH-Induced Oxidative Changes in Phospholipids of VSMCs

In our initial experiments, we attempted to determine whether changes in phospholipid composition occur in VSMCs exposed to Hb/*tert*-BuOOH. To this end, we used a relatively sensitive HPTLC assay for different classes of membrane phospholipids. VSMCs were incubated for 1 hr at 37° in the presence of 5 μM oxyHB and 150 μM *tert*-BuOOH. After incubation, total lipid extracts were prepared, and lipids were separated by HPTLC. Figure 1 shows a typical chromatogram. Seven different phospholipid spots were reliably detected by HPTLC of lipids extracted from control VSMCs (Fig. 1). PC represented about half of the total phospholipids, with PEA the next most prominent phospholipid (about 25%). Additionally, the phospholipids in the order of their abundance—SPH > PS > PI ≫ DPG ≫ LPC—were detectable on the HPTLC plates. No significant difference in phospholipid distribution was detected in VSMCs following oxidative stress induced by oxyHb/*tert*-BuOOH (Table 1). The slight increase in content of LPC, a relatively minor phospholipid, in oxyHb/*tert*-BuOOH-treated cells may be due to PC hydrolysis, which is known to be exacerbated by oxidative stress [34, 35].

Oxidatively modified phospholipids are known to undergo rapid remodeling that involves phospholipase A_2- and acyl transferase-catalyzed mechanisms activated by oxidative stress [36, 37]. Hence, lack of HPTLC-detectable changes in the phospholipid composition of VSMCs exposed to oxyHb/*tert*-BuOOH might be due to effective repair of phospholipids via reacylation pathways [38, 39]. Therefore, we applied our newly developed PnA-based assay that permits detection of site-specific oxidative stress in membrane phospholipids of cells despite repair mechanisms.

VSMCs were incubated at 37° in the presence of PnA–hSA for 2 hr to incorporate the fluorescent fatty acid into cellular phospholipids. Total lipid extracts were prepared, and the constituent phospholipids were separated by HPLC. A typical fluorescence emission profile of the column eluate is shown in Fig. 2. Major fluorescence peaks were identified using authentic phospholipid standards and included PI, PEA, PS, and PC. The identity of the fluorescence peaks was also confirmed by HPTLC of the collected HPLC fractions. Values for specific incorporation of PnA into membrane phospholipids of VSMCs are presented in Table 2. This shows that the incorporation of PnA in the various phospholipids was differential, and the amount of PnA incorporated was in the following order: PC > PEA > PI > PS. Control incubations of cells with hSA alone showed no fluorescent HPLC components under the excitation and emission limits used.

To determine the effect of Hb/*tert*-BuOOH and Hb on any change in fluorescence of PnA-labeled phospholipids of VSMCs, cells were washed three times with PBS and incubated for 1 hr at 37° in the absence/presence of 150 μM *tert*-BuOOH and 5 μM oxyHb aerobically in the dark. Changes in emission intensity of fluorescence of individual phospholipids were determined by HPLC at the end of the incubation. These results (Fig. 3) show that *tert*-BuOOH or

TABLE 1. Effect of *tert*-BuOOH and oxyHb on the phospholipid composition of VSMCs

	Phospholipid content (% of total phospholipids)	
Phospholipids	**Control**	***tert*-BuOOH (150 μM) + oxyHb (5 μM)**
Phosphatidylcholine	49.2 ± 2.6	49.8 ± 2.8
Phosphatidylethanolamine	25.9 ± 1.6	24.7 ± 1.7
Phosphatidylinositol	6.1 ± 0.7	6.0 ± 0.7
Phosphatidylserine	7.7 ± 0.6	7.2 ± 0.6
Sphingomyelin	9.5 ± 0.5	9.9 ± 0.8
Diphosphatidylglycerol	1.6 ± 0.7	1.8 ± 0.1
Phosphatidylglycerol	Tr	Tr
Lysophosphatidylcholine	Tr	0.6 ± 0.2

Amounts of total phospholipids applied at the origin of chromatograms usually correspond to 2.5 μg of phospholipid phosphorus. Tr: trace, less than 0.5%. VSMCs were incubated in the absence and presence of *tert*-BuOOH (150 μM) and oxyHb (5 μM) for 1 hr at 37° in minimum essential medium. Lipids were extracted and resolved by HPTLS as described in Materials and Methods. Each value is the mean percent of total phospholipids ± SEM ($N = 3$).

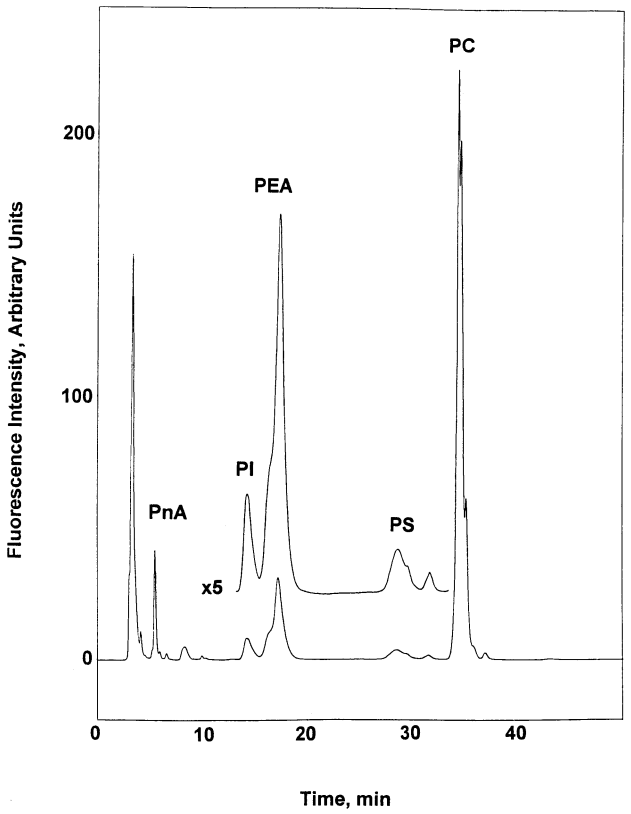

FIG. 2. Normal phase HPLC chromatograms of total lipids extracted from 1 × 10^6 VSMCs. Fluorescence emission intensity: excitation at 324 nm, emission at 420 nm. Cells were incubated with hSA–PnA complex (5 μg of PnA/0.5 mg hSA/10^6 cells) in minimum essential medium with 10% FBS for 2 hr at 37° and then were washed with serum-free minimum essential medium with or without hSA (0.5 mg/mL). Lipids were extracted and resolved by HPLC as described in Materials and Methods. PnA = free *cis*-parinaric acid. See legend of Fig.1 for the definitions of the other abbreviations.

oxyHb alone caused some oxidation of PnA-labeled phospholipids. An additive increase in oxidation of PnA-labeled phospholipids was produced by the *tert*-BuOOH/oxyHb combination.

In the presence of oxyHb alone or metHb alone, peroxidation of phospholipids did not exceed 20% of the initial amount in the individual classes of PnA-labeled phospholipids (Fig. 3). While PAPANONO caused some protection against Hb-induced oxidation, the effects did not reach the level of statistical significance. *tert*-BuOOH alone, however, caused 30–40% oxidation of PnA-labeled phospholipids (Fig. 3). In this case, PAPANONO exerted significant protective effects against both *tert*-BuOOH-induced phospholipid oxidation and *tert*-BuOOH-induced cytotoxicity (data not shown). When VSMCs were incubated with metHb/*tert*-BuOOH, similar phospholipid peroxidative changes were observed in PC and PEA contents. However, metHb/*tert*-BuOOH caused a less pronounced peroxidation of PI and PS than oxyHb/*tert*-BuOOH (Fig. 3). VSMCs contain endogenous myoglobin [40] as well as other heme- and non-heme-iron proteins that are able to catalytically decompose *tert*-BuOOH to produce different radicals (e.g. *tert*-butoxyl radicals, protein-centered peroxyl radicals) [18]. Not surprisingly, hydrogen peroxide and organic peroxides have been demonstrated to induce oxidative stress in smooth muscle cells [41–43]. The conditions of our experiments (concentrations of *tert*-BuOOH, incubation time) were, most likely, non-saturating for the amounts of endogenous (iron)-catalytic sites at which the hydroperoxide was decomposed into radicals. As a result of this, the effect of exogenously added oxyHb (or metHb) and *tert*-BuOOH on oxidation of PnA-labeled phospholipids in the cells was additive.

TABLE 2. Specific incorporation of PnA into membrane phospholipids of vascular smooth muscle cells

Phospholipids	Specific incorporation (mol PnA/mol phospholipid)
Phosphatidylcholine	1:18
Phosphatidylethanolamine	1:34
Phosphatidylinositol	1:40
Phosphatidylserine	1:55

Cells were incubated with PnA–hS complex for 2 hr at 37° in minimum essential medium with 10% FBS. Data are values of three experiments.

Reacylation of phospholipids during exposure to oxidants might interfere with the loss of PnA from individual phospholipids caused by oxidative stress. To exclude the potential interference of a reacylation reaction (that might cause a decrease of actual oxidation of PnA-labeled phospholipids), excess free PnA was thoroughly removed after completion of phospholipid labeling by washing cells with fatty acid-free hSA. As shown in Fig. 2, the peak of free PnA was remarkably smaller than the peaks of the major PnA-labeled phospholipids (such as PC and PEA). On a quantitative basis, the total amount of free PnA was 7.2 ± 1.3 and 7.0 ± 0.8 ng PnA/μg of total lipid phosphorus in control cells and cells treated with oxidants, respectively. Hence, the amount of free PnA was not changed significantly by oxidative stress. Moreover, this amount of free PnA in the cells represented only about 2.2 to 3.5% of total PnA metabolically integrated into cell lipids (328.2 and 200.7 ng PnA/μg of total lipid phosphorus in control or metHb/*tert*-BuOOH-treated cells, respectively). Thus, we conclude that the observed decreased fluorescence of PnA-labeled phospholipids was a consequence of increased peroxidation of the fatty acid residues and not of decreased incorporation of free PnA in the steady-state pool of phospholipids.

Effects of PAPANONO and Nitrosothiols on OxyHb/tert-BuOOH-Induced Peroxidation of Phospholipids in VSMCs

To further study the effects of different NO donors on oxyHb/*tert*-BuOOH-induced peroxidation of membrane phospholipids, PAPANONO, GS-NO, and NAC-S-NO were incubated with VSMCs. We found that PAPANONO

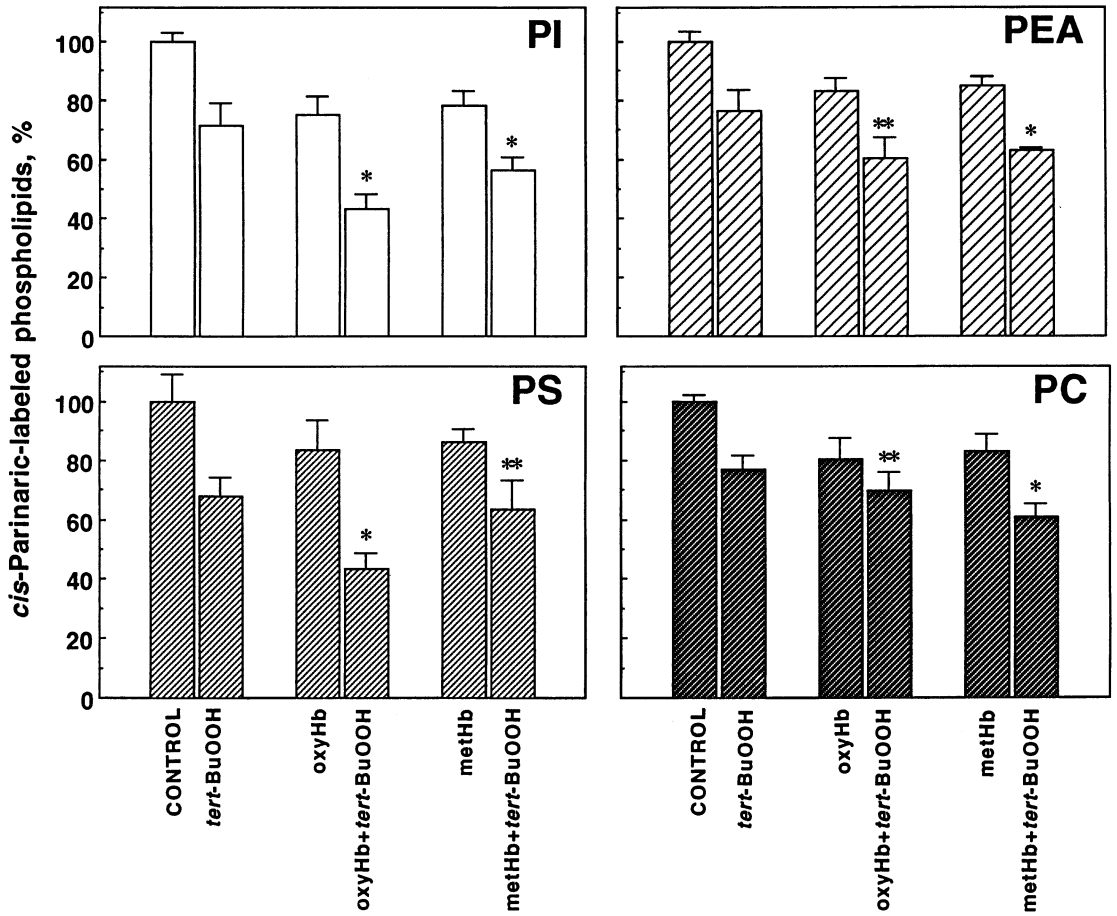

FIG. 3. Effects of *tert*-BuOOH, oxyHb, and metHb on the oxidation of PnA-labeled phospholipids in VSMCs. PnA-loaded VSMCs were incubated for 1 hr at 37° in the presence of oxyHb (5 μM) or metHb (5 μM) and *tert*-BuOOH (150 μM) in minimum essential medium in a CO_2 incubator. After incubation, the cells were scraped, and aliquots of the cell suspension were taken for phospholipid analysis. The absolute values for different classes of PnA-labeled phospholipids in the control samples (100%) were (ng PnA/μg of total lipid phosphorus): 13.4 ± 0.4 for PI, 66.1 ± 2.4 for PEA, 11.6 ± 1.1 for PS, and 237.0 ± 4.8 for PC. All values are means ± SEM. For control, *tert*-BuOOH, and oxyHb/*tert*-BuOOH, N = 7; for oxyHb, metHb, and metHb + *tert*-BuOOH, N = 5. Key: (*) $P < 0.003$, and (**) $P < 0.02$ vs control.

acted as a potent antioxidant protector against both oxyHb/*tert*-BuOOH- (Fig. 4) and metHb/*tert*-BuOOH-induced phospholipid peroxidation (Fig. 5). In the absence of oxyHb/*tert*-BuOOH, no effect of PAPANONO was observed. In contrast to PAPANONO, neither GS-NO nor NAC-S-NO exerted any protective effect against phospholipid peroxidation induced by oxyHb/*tert*-BuOOH in VSMCs (Fig. 4, Table 3). No protective effect was achieved at the NAC-S-NO concentration of 200 μM, i.e. a concentration 4-fold greater than that of PAPANONO, which significantly inhibited the oxyHb/*tert*-BuOOH-induced peroxidation of phospholipid (Fig. 4). Both nitrosothiols had no effect on the phospholipid pattern of VSMCs in the absence of oxyHb/*tert*-BuOOH.

Effects of Hb/tert-BuOOH and NO Donors on Cell Viability

Viability of VSMCs decreased more than 2.5- and 1.5-fold after incubation with oxyHb/*tert*-BuOOH or metHb/*tert*-BuOOH, respectively (but not with oxyHb alone, metHb alone, or *tert*-BuOOH alone, data not shown). PAPANONO completely protected VSMCs against the cytotoxic effect of oxyHb/*tert*-BuOOH or metHb/*tert*-BuOOH. No protection was afforded by either GS-NO or NAC-S-NO against oxyHb/*tert*-BuOOH-induced cytotoxicity (Table 4).

Effects of PAPANONO, GS-NO, or NAC-S-NO on OxyHb in a Model System

To understand the mechanism(s) responsible for the different actions of PAPANONO and nitrosothiols on Hb/*tert*-BuOOH-induced oxidative stress and cytotoxicity, we studied the effects of the NO donors on the formation of NO-Hb and metHb from oxyHb in model systems.

Incubation of oxyHb with PAPANONO, a donor of NO, caused a rapid oxidation of ferrous Hb to ferric Hb (data not shown) with subsequent nitrosylation of heme iron by an excess of NO to yield heme iron nitrosyl complex. Indeed,

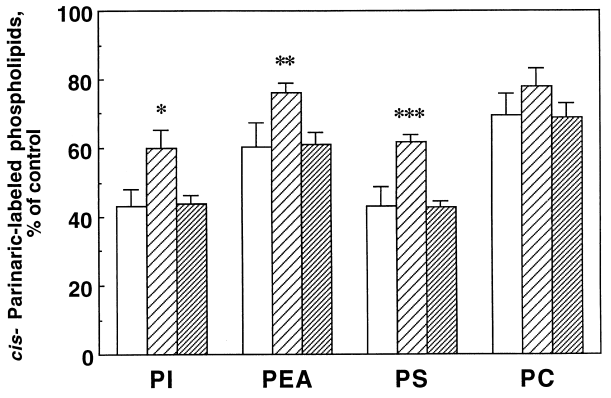

FIG. 4. Effects of PAPANONO and NAC-S-NO on the oxidation of PnA-labeled phospholipids induced by oxyHb/*tert*-BuOOH in VSMCs. Open columns: oxyHb/*tert*-BuOOH; widely hatched columns: 50 μM PAPANONO + oxyHb/*tert*-BuOOH; and narrowly hatched columns: 50 μM NAC-S-NO + oxyHb/*tert*-BuOOH. PnA-loaded VSMCs were incubated for 1 hr at 37° in minimum essential medium in the presence of oxyHb (5 μM) and *tert*-BuOOH (150 μM) in a CO_2 incubator. PAPANONO (50 μM) or NAC-S-NO (50 μM) was added to the cells before the addition of oxyHb and *tert*-BuOOH. After incubation, cells were scraped, and phospholipids were resolved by HPLC. The absolute values for different clones of PnA-labeled phospholipids in the control samples (100%) were (ng PnA/μg of total lipid phosphorus): 13.4 ± 0.4 for PI, 66.1 ± 2.4 for PEA, 11.6 ± 1.1 for PS, and 237.0 ± 4.8 for PC. All values are means ± SEM ($N = 3$). Key: (*) $P < 0.013$, (**) $P < 0.006$, and (***) $P < 0.001$ vs oxyHb/*tert*-BuOOH.

both visible and EPR spectra of oxyHb were changed dramatically after a 60-min incubation at ambient temperature in the presence of PAPANONO (Figs. 6 and 7). The characteristic peaks of oxyHb at 541 and 576 nm were shifted; heme iron nitrosyl complexes of Hb absorbing at 545 and 569 nm, respectively, were observable (Fig. 6) [17]. The Soret band of oxyHb (maximum at 412 nm) shifted to 420 nm (not shown). The time course of the reaction between PAPANONO and oxyHb (Fig. 6, inset A) demonstrates a sharp decline of oxyHb and subsequent monotonous accumulation of heme-nitrosylated Hb. Simultaneously, a pronounced EPR signal of the heme iron nitrosyl complexes manifested in the low-temperature EPR spectrum of the composition of oxyHb/PAPANONO (Fig. 7, spectrum 2). A large unresolved symmetrical EPR signal with the major features $g = 2.04$ (maximum), and $g = 1.98$ (trough), and a shoulder at $g = 2.07$ was assigned to the hexacoordinated heme iron nitrosyl complexes of Hb [44].

Incubation of oxyHb with NAC-S-NO was accompanied by redox transformation of ferrous oxyHb to its ferric form, metHb. After a 60-min incubation at ambient temperature, the spectrum of oxyHb was replaced completely with the spectrum of met/Hb (major peaks at $\lambda_{max} = 630$, and $\lambda_{max} = 405$ nm Soret band, not shown) (Fig. 6). The time course of this interaction shows a continuous decline of oxyHb and accumulation of metHb (Fig. 6, inset B), which was not accompanied by any formation of heme-nitrosylated Hb. The formation of the heme iron nitrosyl complexes was not revealed in the visible spectra. Similarly, no characteristic signals of heme iron nitrosyl complexes were detected in low temperature EPR spectra (Fig. 7).

Effects of PAPANONO on Oxoferryl-Hb in a Model System

To obtain direct evidence for the interaction of NO with oxoferryl-Hb, we conducted low-temperature EPR measurements of the system oxyHb/*tert*-BuOOH plus PAPANONO. Exposure of oxyHb (50 μM) to *tert*-BuOOH (1.5 mM) gave rise to a broad two-line anisotropic EPR signal (Fig. 8). The *g* values at the zero crossing point and a low-field maximum observed in the EPR spectra were 2.010 and 2.036, respectively. This EPR signal had the profile and characteristic features of the oxoferryl-Hb free radical species (the protein-centered peroxyl radicals, most likely tryptophan-centered peroxyl radical) [45]. Neither *tert*-BuOOH alone (data not shown) nor oxyHb alone (Fig. 8) gave any detectable signals in the low-temperature EPR spectra. We next studied the effects of PAPANONO on oxoferryl-radicals produced by *tert*-BuOOH/oxyHb. The generation of oxoferryl-derived radicals was prevented completely when PAPANONO was added to the incubation mixture containing *tert*-BuOOH and oxyHb (Fig. 8). These results suggest that interaction of NO with oxyHb/*tert*-BuOOH resulted in the reduction of oxoferryl-derived radicals similarly to that previously described for the effects

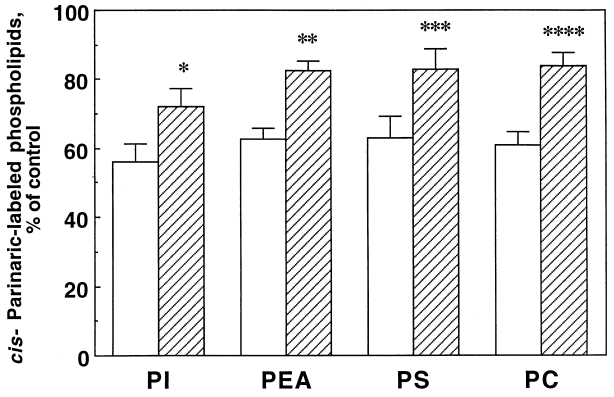

FIG. 5. Effect of PAPANONO on the oxidation of PnA-labeled phospholipids induced by metHb/*tert*-BuOOH in VSMCs. Open columns: metHb/*tert*-BuOOH; and widely hatched columns: 200 μM PAPANONO + metHb/*tert*-BuOOH. PnA-loaded VSMCs were incubated for 1 hr at 37° in minimum essential medium in the presence of metHb (5 μM) and *tert*-BuOOH (150 μM) in a CO_2 incubator. PAPANONO (200 μM) was added to the cells at two times (100 μM × 2), before addition of metHb and *tert*-BuOOH and after a 30-min incubation in the presence of both metHb and *tert*-BuOOH. After incubation, cells were scraped, and phospholipids were resolved by HPLC. The absolute values for different classes of PnA-labeled phospholipids in the control samples (100%) were (ng PnA/μg of total lipid phosphorus): 13.4 ± 0.4 for PI, 66.1 ± 2.4 for PEA, 11.6 ± 1.1 for PS, and 237.0 ± 4.8 for PC. All values are means ± SEM ($N = 5$). Key: (*) $P < 0.014$, (**) $P < 0.001$, (***) $P < 0.04$, and (****) $P < 0.003$ vs metHb/*tert*-BuOOH.

TABLE 3. Effects of PAPANONO, GS-NO, and NAC-S-NO on the oxidation of PnA-labeled phospholipids in VSMCs induced by *tert*-BuOOH and oxyHb

Additions	N	PI	PEA	PS	PC
			PnA (ng/μg of total lipid phosphorus)		
Control	7	13.4 ± 0.4	66.2 ± 2.4	11.6 ± 1.1	237.0 ± 4.8
tert-BuOOH + oxyHb	7	5.8 ± 0.7*	39.9 ± 4.6*	5.0 ± 0.6*	164.7 ± 14.8*
tert-BuOOH + oxyHb + PAPANONO	7	9.4 ± 1.2†	55.3 ± 3.2†	9.4 ± 1.7†	199.8 ± 20.6†
tert-BuOOH + oxyHb + GS-NO	3	5.4 ± 0.5*	40.6 ± 2.9*	4.5 ± 0.7*	162.6 ± 18.0*
tert-BuOOH + oxyHb + NAC-S-NO	3	6.4 ± 0.6*	36.9 ± 2.4*	4.0 ± 0.3*	158.5 ± 13.0*
			PnA (ng/μg of lipid phosphorus in phospholipid fraction)		
Control	7	219.7 ± 6.6	255.6 ± 9.3	161.1 ± 15.3	481.7 ± 7.11
tert-BuOOH + oxyHb	7	94.6 ± 11.1*	154.1 ± 17.7*	69.6 ± 8.7*	344.8 ± 30.7*
tert-BuOOH + oxyHb + PAPANONO	7	154.7 ± 19.6†	213.4 ± 12.4†	130.8 ± 23.5†	402.2 ± 41.9†
tert-BuOOH + oxyHb + GS-NO	3	88.3 ± 8.2*	156.9 ± 11.2*	62.2 ± 9.5*	330.4 ± 36.6*
tert-BuOOH + oxyHb + NAC-S-NO	3	105.2 ± 9.4*	142.4 ± 9.2*	56.2 ± 4.2*	322.3 ± 26.5*

PnA-loaded VSMCs were incubated with *tert*-BuOOH (150 μM) and oxyHb (5 μm) in the presence or absence of PAPANONO (200 μM), GS-NO (200 μM), and NAC-S-NO (200 μM) for 1 hr at 37° in minimum essential medium. Lipids were extracted and resolved by HPLC as described in Materials and Methods. All values are means ± SEM; N = number of experiments.

*$P < 0.05$ vs control cells.

†$P < 0.05$ vs cells treated with oxyHb-*tert*-BuOOH.

of *tert*-BuOOH on Hb-containing erythroleukemia cells [21].

DISCUSSION

Catalytically active non-heme and heme iron in cells and biologic fluids (which is not bound to redox-inactive complexes by ferritin and transferrin, respectively) may be involved in the generation of reactive oxygen species and the initiation of different cytotoxic (and genotoxic) processes [46–48]. In particular, iron-catalyzed decomposition of hydrogen peroxide and organic hydroperoxides (e.g. lipid hydroperoxides) yielding free radicals (e.g. hydroxyl, alkoxyl, peroxyl radicals) as well as formation of oxoferryl-related radical species is believed to be responsible for oxidative damage to critical biomolecules [48, 49]. Not surprisingly, hemolysis and release of hemoglobin from red blood cells accompanying a number of pathological conditions [50] is associated with massive oxidative stress and cell injury [51]. Our results indicate that both oxyHb and metHb were able to cause oxidative stress in phospholipids of VSMCs in the presence of *tert*-BuOOH. This suggests that *tert*-BuOOH-induced formation of oxoferryl-Hb was, most likely, involved in the initiation of oxidative stress in VSMC phospholipids:

$$HbFe^{2+}{-}O_2 + tert\text{–}BuOOH \rightarrow HbFe^{3+} + OH^- + tert\text{–}BuO^{\cdot} + O_2$$

$$HbFe^{3+} + tert\text{–}BuOOH \rightarrow HbFe^{4+}{-}OH + tert\text{–}BuO^{\cdot}$$

$$HbFe^{4+} - OH + tert\text{–}BuO^{\cdot} + O_2 + H_2O \rightarrow {}^{\cdot}O_2HbFe^{4+} - OH + tert\text{–}BuOH + OH^-$$

A direct chemical interaction of NO with hydroperoxides at non-heme- and heme-iron catalytic sites was demonstrated to prevent the generation of different free radical species, thus precluding free radical-induced damage of critical biomolecules [17, 20, 52, 53]. Hence, protective effects of NO donors were reported in short-term incubations of hydroperoxides with cells in which oxidative damage was dependent on the non-heme-iron [20, 52, 53] or heme-iron catalyzed reactions [17, 18]. We have reported

TABLE 4. Effect of PAPANONO, GS-NO, and NAC-S-NO on the viability of VSMCs during oxidative stress induced by *tert*-BuOOH and oxyHb or metHb

Additions	Viability (%)
Control	81.2 ± 9.0
tert-BuOOH + oxyHb	31.5 ± 9.2*
tert-BuOOH + metHb	52.0 ± 9.0*
tert-BuOOH + oxyHb + PAPANONO	80.1 ± 13.1†
tert-BuOOH + metHb + PAPANONO	79.0 ± 12.0†
tert-BuOOH + oxyHb + GS-NO	29.9 ± 14.4*
tert-BuOOH + oxyHb + NAC-S-NO	28.5 ± 18.2*

VSMCs (3×10^6 cells) were incubated with *tert*-BuOOH (150 μM) and oxyHb (5 μM) or metHb (5 μM) in the presence or absence of PAPANONO (200 μM), GS-NO (200 μM) and NAC-S-NO (200 μM) for 1 hr at 37° in minimum essential medium. All values are means ± SEM (N = 3).

*$P < 0.05$ vs control cells.

†$P < 0.05$ vs cells treated with Hb-*tert*-BuOOH.

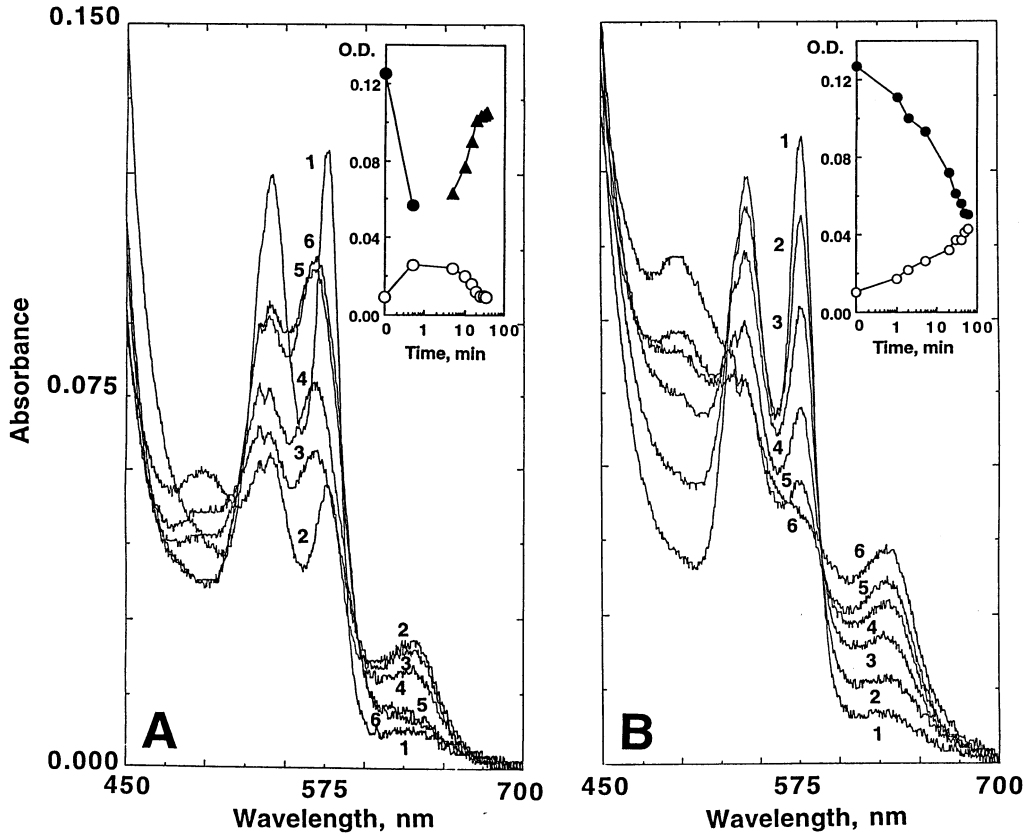

FIG. 6. Visible absorbance spectra of the reaction products of oxyHb (2.5 μM) with (A) PAPANONO (100 μM) and (B) NAC-S-NO (100 μM). (A) 1, control (no PAPANONO); 2–6, in the presence of PAPANONO; 2, 0.5 min; 3, 5.0 min; 4, 10 min; 5, 20 min; 6, 30 min. Inset: Time course of oxyHb (●), heme-nitrosylated Hb (▲), and metHb (○). (B) 1, control (no NAC-S-NO); 2–6, in the presence of NAC-S-NO; 2, 1 min; 3, 5 min; 4, 20 min; 5, 40 min; 6, 60 min. Inset: Time course of oxyHb (●) and metHb (○).

recently that oxidative stress induced by *tert*-BuOOH in hemoglobin-rich cells (human erythroleukemia K562 cells) or myoglobin-rich cells (rat neonatal cardiomyocytes) could be eliminated or diminished by NO [21, 22]. This protective effect of NO may represent a new, physiologically important antioxidant function of NO against oxidative stress catalyzed by hemoproteins. The mechanism, however, functions at the expense of NO oxidation at heme-catalytic sites, hence depleting NO as discussed below.

The results of this work demonstrated that NO indeed protected VSMCs against oxyHb/*tert*-BuOOH- or metHb/*tert*-BuOOH-induced phospholipid peroxidation and cytotoxicity when PAPANONO was used as an NO donor. The protective effect was due, most likely, to a chemical reduction of *tert*-BuOOH by NO at the catalytic site that prevented formation of oxoferryl-Hb. In line with this, formation of heme-nitrosylated Hb was detectable in low temperature EPR spectra as well as in visible spectra of oxyHb incubated for 1 hr in the presence of PAPANONO. Extensive evidence in favor of the above mechanism was presented in our earlier report on *tert*-BuOOH-dependent oxidation in Hb-containing erythroleukemia cells [21]. Since both oxyHb [54] and metHb [14] can be effectively nitrosylated by NO, we found, as expected, that PAPANONO protected phospholipids against *tert*-BuOOH-dependent oxidation catalyzed by both the reduced and oxidized forms of Hb. The protective effect of NO against hydroperoxide-induced toxicity may have pharmacological significance in lieu of occupational and environmental exposures to organic peroxides and hydrogen peroxide [55].

In addition to the interaction of NO with catalytic transition metal centers to prevent the formation of potent oxidants, the antioxidant effect of NO may be realized through direct free radical scavenging of alkoxyl and peroxyl radicals [17–20]. The latter mechanism has been investigated recently in detail [56]. Basically, both of these mechanisms can contribute to the inhibition of lipid peroxidation. However, in the presence of relatively high concentrations of hemoproteins to which NO binds with high affinity, the role of direct radical scavenging may become quantitatively negligible as compared with that caused by interactions with hemoproteins. We have observed that lipid peroxidation induced in HL-60 cells by a lipophilic azo-initiator of peroxyl radicals, AMVN, was blocked completely by NO mainly through a direct radical-scavenging pathway.* In contrast, NO did not protect

* Fabisiak JP, Tyurina YY, Tyurin VA, Laso JS and Kagan VE, Manuscript in preparation.

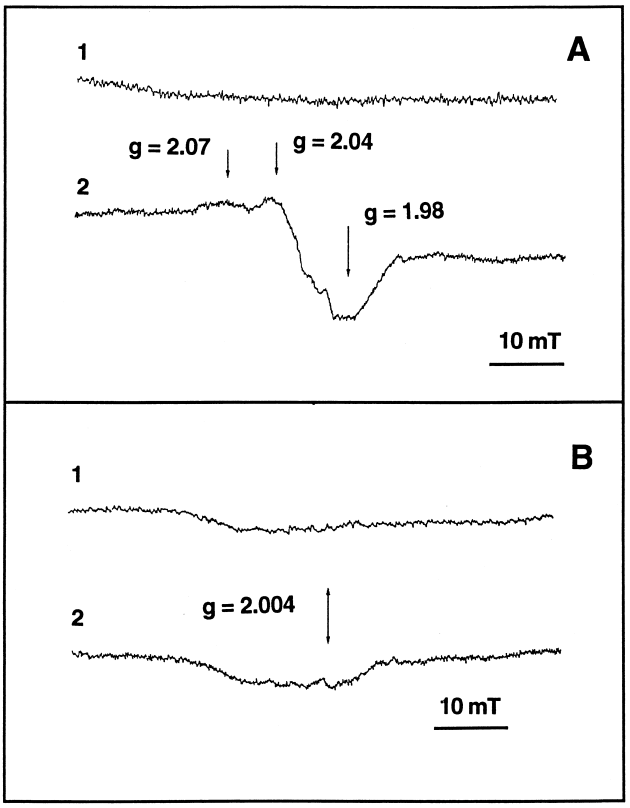

FIG. 7. Low-temperature EPR spectra of the reaction products of oxyHb with (A) PAPANONO, and (B) NAC-S-NO. (1) 50 μM oxyHb in 100 mM sodium phosphate buffer (pH 7.4); (2) same as (1) but in the presence of either 2.0 mM PAPANONO or 2.0 mM NAC-S-NO.

human erythroleukemia K.VP.5 cells overexpressing Hb against AMVN-induced phospholipid peroxidation, suggesting that NO-binding with Hb suppressed its participation in scavenging alkoxyl and peroxyl radicals [21]. We have previously performed experiments with human erythroleukemia cells that overexpress Hb and hence are able to effectively decompose *tert*-BuOOH [21]. We conducted a comparative study of the effect of PAPANONO on oxidative stress in phospholipids of these cells with that in K.VP.5 cells transfected with inducible NO synthase (iNOS). We found that in both cases NO caused a remarkable protection against *tert*-BuOOH-induced oxidative stress in phospholipids. In iNOS-transfected K.VP.5 cells, however, the effect was caused by two major factors: (i) direct interaction of NO with *tert*-BuOOH-derived radicals at heme-catalytic sites, and (ii) post-transcriptionally decreased levels of both heme- and non-heme-iron proteins in the transfected cells as compared with mock-transfected K.VP.5 cells [57]. The latter effect was, most likely, due to the effects of NO on iron-regulatory proteins [58].

In contrast, nitrosothiols (GS-NO and NAC-S-NO) exerted no protection against either phospholipid peroxidation or cytotoxicity. Using low temperature EPR spectroscopy or conventional visible spectrophotometry, we were not able to detect heme-nitrosylated Hb after a 1-hr incubation of oxyHb with NAC-S-NO. The time-course of the reactions between PAPANONO and oxyHb confirmed the accumulation of heme-nitrosylated Hb after the initial formation of metHb. On the other hand, only monotonous accumulation of metHb (that was not accompanied by any detectable formation of heme-nitrosylated Hb) was observed in the presence of NAC-S-NO. Nitrosothiols have been reported to *S*-transnitrosylate Hb [23]. Moreover, allosteric transition associated with oxygenation of Hb increases the rate of its *S*-nitrosylation, such that in oxy conformation (R state), *S*-nitrosylation is faster than in the deoxy conformation (T state) [23].

It is likely that *S*-nitrosylated Hb was not able to catalyze reduction of *tert*-BuOOH at the heme-site and hence did not protect against *tert*-BuOOH-dependent oxidative stress. Another possibility is that nitrosothiols are much less effective as NO donors than PAPANONO [59]. In a recent study, the protective effects of different NO donors against cytotoxicity induced by hydrogen peroxide correlated with their ability to release NO [60]. In particular, both NONOates and nitrosothiols (e.g. GS-NO and SNAP) were shown to have similar effectiveness as NO donors (under the same conditions) and protectors against oxidative stress. While nitrosothiols are relatively stable in simple model systems

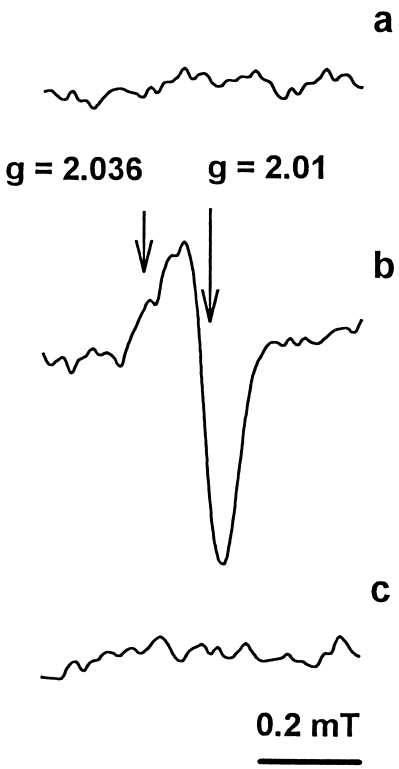

FIG. 8. Low-temperature EPR spectra of the reaction products of oxyHb with *tert*-BuOOH in the absence and in the presence of PAPANONO. (a) 50 μM oxyHb in 100 mM sodium phosphate buffer (pH 7.4); (b) same as (a) but in the presence of 1.5 mM *tert*-BuOOH; and (c) same as (b) but in the presence of 2.0 mM PAPANONO.

(e.g. in phosphate buffer at pH 7.4), it is important that spontaneous decomposition of nitrosothiols may be substantially enhanced by reduced low molecular weight thiols [61], as well as by reactive sulfhydryls present on cell surfaces [61, 62].

It has been suggested that the reaction of NO with Hb that has been taken up by endothelial cells or located in the space between endothelial cells and smooth muscle cells is accountable for most of the NO depletion responsible for vasoconstriction and the hypertensive events associated with extracellular Hb [14]. Even if the concentration of such extracellular Hb (e.g. released by hemolysis) is not high enough, under conditions of oxidative stress, it may still be sufficient to scavenge the levels of NO produced by arterial endothelial cells (by constitutive NO synthase). While in red blood cells NO-induced conversion of oxyHb to metHb is enzymatically reversed by NAD(P)H-metHb reductase activities [63], extracellular metHb cannot be recycled effectively back to oxyHb. Accumulation of peroxidation products (e.g. lipid hydroperoxides) during oxidative stress may enhance the consumption of NO in a continuous oxoferryl-Hb-catalyzed redox process [21]. Thus, hemolysis in combination with oxidative stress, which happens in a number of diseases (e.g. preeclampsia) [64], may stimulate the reactions of NO with Hb and cause depletion of NO, resulting in vasoconstriction.

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