Nitric Oxide Prevents Oxidative Damage Produced by tert-Butyl Hydroperoxide in Erythroleukemia Cells via Nitrosylation of Heme and Non-heme Iron

ELECTRON PARAMAGNETIC RESONANCE EVIDENCE*

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We studied protective effects of NO against tert-butyl-hydroperoxide (t-BuOOH)-induced oxidations in a subline of human erythroleukemia K562 cells with different intracellular hemoglobin (Hb) concentrations. t-BuOOH-induced formation of oxoferryl-Hb-derived free radical species in cells was demonstrated by low temperature EPR spectroscopy. Intensity of the signals was proportional to Hb concentrations and was correlated with cell viability. Peroxidation of phosphatidylcholine, phosphatidylyethanolamine, phosphatidylserine, phosphatidylinositol, and cardiolipin metabolically labeled with oxidation-sensitive cis-parinaric acid was induced by t-BuOOH. An NO donor, (Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)amino]-diazen-1-ium-1,2-diolate, produced non-heme iron dinitrosyl complexes and hexa- and pentacoordinated Hb-nitrosyl complexes in the cells. Nitrosylation of non-heme iron centers and Hb-heme protected against tert-BuOOH-induced: (a) formation of oxoferryl-Hb-derived free radical species, (b) peroxidation of cis-parinaric acid-labeled phospholipids, and (c) cytotoxicity. Since NO did not inhibit peroxidation induced by an azo-initiator of peroxyl radicals, 2,2′-azobis(2,4-dimethylvaleronitrile), protective effects of NO were due to formation of iron-nitrosyl complexes whose redox interactions with tert-BuOOH prevented generation of oxoferryl-Hb-derived free radical species.

Nitric oxide (NO)1 is an important physiological regulator of biological responses such as vasodilation, blood coagulation, neurotransmission, renal function, inflammation, and antitumor immune surveillance (1–6). Paradoxically, it can simultaneously exert adverse effects on cells. Cytotoxic effects of NO are believed to be produced through three major pathways as follows: (i) direct modification of proteins by NO via nitrosylation of sulfhydryl groups, heme and non-heme sites, and possibly tyrosyl residues (e.g., modification of poly(ADP-ribose) synthetase, ribonucleotide reductase, and enzymes of mitochondrial electron transport) (4–10); (ii) NO-induced activation of enzymes involved in posttranscriptional regulation of protein expression (e.g., transferrin/ferritin pathway for iron mobilization) (11, 12); and (iii) oxidative damage to critical biomolecules such as nucleic acids, proteins, and lipids. The latter is mainly associated with the production of peroxynitrite (13–17).

It has been demonstrated recently that NO can also act as an antioxidant, thus protecting cells against oxidative damage (17–19). In Chinese hamster V79 lung fibroblasts and human umbilical vein endothelial cells, this antioxidant effect of NO was associated with its ability to scavenge lipid alkoxyl and peroxyl radicals (18–20). The balance between intracellular antioxidant and pro-oxidant effects of NO in vivo remains to be elucidated.

It has been suggested that the interaction of NO with hemoglobin and myoglobin may prevent hydroperoxide-induced formation of oxoferryl hemoproteins, thus blocking subsequent generation of oxygen-derived reactive species and oxidative damage (18, 19, 21–25). In line with this, our previous studies demonstrated that NO was capable of inhibiting oxoferryl-induced oxidation in simple model systems such as tert-butyl-hydroperoxide (t-BuOOH)/hemoglobin or t-BuOOH/myoglobin (24). The proposed antioxidant mechanism of NO involves reduction of oxoferryl-derived radicals (24). Whether this mechanism operates in cells remained unclear.

In the present work, we attempted to elucidate the antioxidant role of NO against intracellular oxoferryl hemoglobin-induced oxidative stress. We studied the effects of NO on t-BuOOH-induced, heme and non-heme iron-dependent oxidation of membrane phospholipids and cytotoxicity in a sub-paramagnetic resonance; HIN, the hexacoordinated heme iron nitrosyl complexes; PIN, the pentacoordinated heme iron nitrosyl complexes; NOC-15 or PAPA NONOate, (Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)-amino]-diazen-1-ium-1,2-diolate; DPFC, L-α-phosphatidylcholine, dipalmitoyl (C18:1 cis-9; PC, phosphatidylcholine; PS, phosphatidylserine; PEA, phosphatidylethanolamine; PI, phosphatidylinositol; CL, cardiolipin; mT, millitese; HPLC, high performance liquid chromatography.

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line of human erythroleukemic K562 cells in which concentrations of endogenous hemoglobin can be easily manipulated.

**MATERIALS AND METHODS**

Human hemoglobin (Hb), tert-butylhydroperoxide, sodium hydrosulfite (dithionite), hemin, and L-α-phosphatidylethanolamine, dipalmitylo-(C18:1 (cis)-9) (DPPC) were obtained from Sigma. Potassium phosphate (monobasic) was purchased from Fisher. 9-cis,11-trans-13-trans-15-cis-octadecatetraenoic acid (cis-parinaric acid, PnA) was purchased from Molecular Probes, Inc. (Eugene, OR). NOC-15, z-V-[N-(3-aminosulfonyl)-propyl]-zyxinino-(4-azonyloninfolin) dianzen-1-ium chloride (PAPA NONOate) was from Cayman Chemical Co. (Ann Arbor, MI). AMVN, 2,2′-azo-bis(2-methyl)-iminonitril was obtained from Polysciences, Inc. (Warrington, PA); Sephadex G-25 columns were obtained from Pharmacia LKB (Uppsala, Sweden).

**Cells and Media**—KVP-5 cells used for most of the experiments described are a subline of human erythroleukemia K562 cells selected for resistance to the anticancer agent etoposide (26). Cells were grown in continuous culture in Dulbecco’s modified Eagle’s medium in the presence of 7.5% iron-supplemented calf serum. KVP-5 cells were chosen for study because it was found that intracellular Hb was 25 pmol/10⁶ cell compared with 5 pmol/10⁶ cell in parental K562 cells. This permitted us to discriminate a Hb-dependent t-BuOOH toxicity to the cells from the total iron-catalyzed toxicity. To additionally increase the intracellular amount of Hb in KVP-5 cells, the growth medium was supplemented with hemin (25 μmol/10⁶ cell/ml) to ensure that the concentration of Hb exceeded the concentration of membrane receptors for Hb (27).

Thus a part of hemin from the growth medium was integrated into intracellular hemoglobin, and the rest of it (bound to serum proteins in the medium) was removed by centrifugation; no “loose” membrane-bound hemin was present in the cells used in the experiments performed.

**Incorporation of PnA into Cell Phospholipids**—PnA was incorporated into cells by addition of its HSA complex (PnA-HSA) to cell suspensions (31). Cells in log phase growth were rinsed twice with L1210 buffer, diluted to a density of 1.0 × 10⁶ cells/ml, and then incubated with PnA-HSA complex (final concentration of PnA 4 μg/ml) in L1210 buffer at 37 °C for 2 h. After incubation, cells were pelleted by centrifugation and then washed twice with isotonic buffer with HSA. The total amount of PnA metabolically incorporated into membrane phospholipids was less than 1% of fatty acid residues. PnA-treated cells were incubated in the presence of t-BuOOH (100 nmol/10⁶ cells) and/or NOC-15 (20 and 80 nmol/10⁶ cells) as described above. Total lipid extracts from the cells were obtained using a Folch procedure (32). The lipid extract was dissolved in 3:4:0.16 (v/v) hexane/isopropl alcohol/water (0.15 ml).

**HPLC Analysis of Cell Lipids**—Lipid extracts were separated by a normal phase HPLC as described previously (31). A 5-μm Supelcosil LC-Si column (4.6 × 250 mm) was employed with the following mobile phase flowing at 1 ml/min: solvent A (57:43:1 isopropl alcohol/hexane/H₂O), solvent B (57:43:1 isopropl alcohol/hexane, 40 μmol aqueous ammonium acetate, pH 7.0), 0–3 min linear gradient from 10% B to 37% B, 3–15 min isocratic at 37% B, 15–23 min linear gradient to 100% B, 23–45 min isocratic at 100% B. A Shimadzu HPLC system (model LC-600) fitted with a fluorescence detector (model RF-551) was used. Fluorescence of PnA in eluates was monitored by emission at 420 nm after excitation at 324 nm. Fluorescence data were processed and stored in digital form with Shimadzu EZchrom software.

**Hemoglobin Solutions**—Commercial Hb was mainly in the fer- ric form. We reduced met-Hb (1 mM solution in 100 mM phosphate buffer, pH 7.4) to its ferrous (oxy-Hb) form using 4-fold excess of sodium dithionite. Pure oxy-Hb was obtained by separation on a Sephadex G-25 column equilibrated with 100 mM phosphate buffer, pH 7.4. The concentration of oxy-Hb/met-Hb was calculated as described previ- ously by Winterbourn (33) using oxy-Hb extinction coefficient at 577 nm 15.0 μm⁻¹ cm⁻³⁻¹. Deoxygenation of oxy-Hb was performed by incubating it in a nitrogen atmosphere.

**Preparation of Hemin-containing Liposomes**—Liposomes were prepared from a stock solution of 2 mM DPPC in CHCl₃/CH₃OH (1:1) containing 0.2 mM met-Hb. The solvent was evaporated under a stream of N₂. Phosphate buffer (100 mM, pH 7.4) was added, and the resulting suspension was sonicated (three 30-s bursts at 65 watts) with aCole-Palmer Instrument Co. 4713-18 sonic dissi- poler Ultrasonicator (Chicago, IL). To reduce the oxidized form of Hb to its ferrous form we used sodium dithio- nite (0.8 μM). Removal of excess dithionite was achieved by gel filtration through a Sephadex G-25 column equilibrated with 100 mM phosphate buffer. Thus prepared hemin-containing liposomes were used for EPR and spectrophotometric measurements.

**EPR Measurements**—Log phase cells were separated from the growth media as mentioned above. Two different sets of EPR measurements were performed. Cells adjusted to either 1 × 10⁶ cell/ml or 80 × 10⁶ cell/ml were incubated for 10 min in the absence or presence of NOC-15 (20 and 80 nmol/10⁶ cells, releasing NO with a half-life of 76 min), following which 100 nmol of t-BuOOH/10⁶ cells was added to an aliquot for an assigned concentration of N₂O. Phosphate buffer (100 mM, pH 7.4) was added, and the resulting suspension was sonicated (three 30-s bursts at 65 watts) with a Cole-Palmer Instrument Co. 4713-18 sonicator Ultrasoundicator (Chicago, IL). To reduce the oxidized form of Hb to its ferrous form we used sodium dithio- nite (0.8 μM). Removal of excess dithionite was achieved by gel filtration through a Sephadex-G-25 column equilibrated with 100 mM phosphate buffer. Thus prepared hemin-containing liposomes were used for EPR and spectrophotometric measurements.

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The growth medium was supplemented with hemin (25 nM) for 24 h and subsequently treated with alamethicin; 3, K/VP.5 cells treated with alamethicin, 4, cell growth medium obtained after sedimentation of K/VP.5 cells incubated for 24 h in the presence of hemin (thus obtained medium was diluted 5-fold by addition of 100 mM phosphate buffer, pH 7.4, containing 1% of Triton X-100. Liposomes were prepared from a stock solution of 2 mM DPPC in CHCl3/CH3OH (1:1) containing 0.2 mM hemin. The solvent was evaporated under a stream of N2. Phosphate buffer (100 mM, pH 7.4) was added, and the resulting suspension was sonicated (three 30-s bursts at 65 watts) with a Cole-Parmer Instrument Co. 4710 Series Ultrasonicator. To reduce ferric form of hemin to its ferrous form, we used sodium dithionite (0.8 mM). Removal of excess dithionite was achieved by gel filtration of liposomes through a Sephadex G-25 column preequilibrated with 100 mM phosphate buffer.

**RESULTS**

EPR Evidence for NO-dependent Protection Against t-BuOOH-induced Cytotoxicity

The EPR spectra of both control K/VP.5 cells (25 pmol Hb/10⁶ cells) and K/VP.5 cells enriched with Hb (90 pmol Hb/10⁶ cells) displayed only nonspecific free radical signals at g = 2.004 (Fig. 2, A1 and B1), probably resulting from components of the mitochondrial respiratory chain (35, 36).

EPR Spectra of K/VP.5 Cells Incubated with NOC-15

Control K/VP.5 Cells—Incubation of control (nontreated with hemin) K/VP.5 cells with two different concentrations of NOC-15 (20 and 80 nmol/10⁶ cells) resulted in EPR signals with identical profiles (Fig. 2, A2 and A3). We registered four-line anisotropic spectra with (i) principal features at g∥ = 2.04 and g⊥ = 2.015; (ii) additional features at g = 2.07 (a maximum), and g = 1.989 (a trough); (iii) a free radical signal at g = 2.004 (Fig. 2, A2 and A3). Similar spectra, with an axial anisotropic feature at g∥ = 2.04 and g⊥ = 2.015, have been previously observed upon exposure of various types of cells to NO (5, 8, 35–37), and were assigned to the characteristic EPR signals of protein- and nonprotein nonheme iron-dinitrosyl complexes (35–39). We and others (7, 12, 40) have detected EPR signals with similar features at g∥ = 2.04 and g⊥ = 2.015 in control parental K562 cells (with a very low level of endogenous Hb, 5 pmol/10⁶ cells) treated with NO (spectra not shown).

Hb-enriched K/VP.5 Cells—In Hb-enriched K/VP.5 cells, treatment with the NO donor (20 and 80 nmol/10⁶ cells) caused EPR spectra with profiles different from those of control K/VP.5 cells. A five-line anisotropic signal with incompletely resolved superfine structure was detected (Fig. 2, B1 and B3). The features g∥ = 2.04 and g⊥ = 2.015 were assigned to non-heme iron dinitrosyl complexes (35, 36, 38, 39, 41) similar to that in control K/VP.5 cells. The signals in Hb-enriched cells, however, also exhibited additional multiplet signals at g = 2.07, g = 2.025, g = 2.004 and g = 1.989.

The features at g = 2.07 and g = 1.989 in both control- and Hb-enriched K/VP.5 cells can be assigned to nitrosyl complexes of heme iron, apparently resulting from nitrosylation of intracellular Hb. Indeed, these spectral components in the Hb-enriched K/VP.5 cells were much more intense than in control cells, although partly obscured by signals from non-heme iron dinitrosyl complexes at g∥ = 2.04 and g⊥ = 2.015 (Fig. 2, B2 and B3). Similar signals of nitrosyl complexes of hemoglobin have been shown previously in model systems (42–44). Moreover, essentially the same spectra were observed in erythrocytes upon exposure to NO (37, 45). To confirm this assignment of the features g = 2.07 and g = 1.989 to the signal of nitrosylated Hb in K/VP.5 cells, and to identify the additional features at g = 2.025 and g = 2.004 in Hb-enriched cells, we compared EPR signals from the cells with those obtained from pure Hb or hemin (incorporated in DPPC liposomes) upon addition of NO (see below).

EPR Spectra of Heme Iron Nitrosyl Complexes of Hb and Hemin

Nitrosylated Hb has previously been demonstrated to produce EPR signals with two different profiles that have been assigned to nitrosyl species of pentacoordinated ferrrous complexes with the proximal histidine-Fe⁴⁺ bond significantly stretched and nitrosyl species of hexacoordinated ferrous complexes (42–46).

![Comparison of visible spectra of hemoglobin released from KVP.5 treated with alamethicin with the spectra of hemin-containing cell growth medium and hemin/DPPC liposomes.](http://www.jbc.org/)

**FIG. 1.** 
Comparison of visible spectra of hemoglobin released from KVP.5 treated with alamethicin with the spectra of hemin-containing cell growth medium and hemin/DPPC liposomes. A, 1–3, supernatants obtained after centrifugation of KVP.5 cells treated with alamethicin. 1, KVP.5 cells preincubated with hemin for 24 h and subsequently treated with alamethicin; 2, treated with alamethicin; 3, KVP.5 cells treated with alamethicin. 4, cell growth medium obtained after sedimentation of KVP.5 cells incubated for 24 h in the presence of hemin (thus obtained medium was diluted 5-fold by fresh Dulbecco’s modified Eagle’s medium). B, 1–3, cell debris after treatment with Triton X-100, same as (A, 1–3). 4, hemin/DPPC after treatment with Triton X-100. Conditions for the cell incubations are as follows. Cells were grown in continuous culture in Dulbecco’s modified Eagle’s medium in the presence of 7.5% iron-supplemented calf serum. To additionally increase the intracellular amount of Hb in KVP.5 cells, the growth medium was supplemented with hemin (25 μM) for 1 and 24 h. Log phase cells were separated from growth medium containing hemin by centrifugation (1,500 × g for 5 min). Cell pellet was rinsed twice with Li212 buffer containing 115 mM NaCl, 5 mM KCl, 5 mM NaH₂PO₄, 1 mM MgCl₂, 10 mM glucose, and 25 mM Heps, pH 7.4. Ten million cells resuspended in Li212 buffer were treated with an amphotillic channel-forming peptide, alamethicin (50 μM), to release intracellular Hb. After centrifugation (100,000 × g for 20 min), the supernatant was collected, and pelleted cell debris were solubilized in 100 mM phosphate buffer, pH 7.4, containing 1% of Triton X-100. Liposomes (final concentration of hemin was 25 μM) were prepared from a stock solution of 2 mM DPPC in CHCl₃/CH₃OH (1:1) containing 0.2 mM hemin. The solvent was evaporated under a stream of N₂. Phosphate buffer (100 mM, pH 7.4) was added, and the resulting suspension was sonicated (three 30-s bursts at 65 watts) with a Cole-Parmer Instrument Co. 4710 Series Ultrasonicator. To reduce ferric form of hemin to its ferrous form, we used sodium dithionite (0.8 mM). Removal of excess dithionite was achieved by gel filtration of liposomes through a Sephadex G-25 column preequilibrated with 100 mM phosphate buffer.
Since α subunits of Hb have a higher affinity for NO than β subunits (42, 43, 45), nitrosylation of Hb at nonsaturating concentrations of NO can lead to the formation of the pentacoordinated heme iron nitrosyl (PIN) complexes, where the NO ligand ends up bound primarily to α subunits of Hb (ααNO or ββoNαNO) (42–45). The PIN complexes produce an EPR signal with a three-line splitting characterized by hyperfine coupling $A_z = 1.61 \text{ mT}$ in the high magnetic field region centered at $g_s = 2.010$ and a broad shoulder in the low magnetic field region (44). We observed a spectrum with these features after incubation of 20 μM Hb with 20 μM NOC-15 (in 100 mM phosphate buffer, pH 7.4) for 10 min. b, Hb-enriched K/V5.5 cells incubated with 20 nmol of NOC-15/10^6 cells (obtained by a subtraction of the reconstructed EPR spectrum (A4) from the spectrum (B2)). c, 25 μM hemin incorporated into DPPC liposomes (see “Materials and Methods”) incubated with 20 μM NOC-15. d, 25 μM hemin incorporated into DPPC liposomes (see “Materials and Methods”) incubated with 80 μM NOC-15. e, Hb-enriched K/V5.5 cells incubated with 80 nmol of NOC-15/10^6 cells obtained by a subtraction of the reconstructed EPR spectrum (A4) from the spectrum (B3). Incubation, spectrometer conditions, and computer manipulations were the same as in Fig. 2. Spectra a, b, and e are magnified versions of the same spectra as 5a, 5b, and 4a, respectively, shown on Fig. 2.
Hexacoordinated heme iron nitrosyl (HIN) complexes are characterized by a large unresolved symmetrical spectrum centered at $g = 2.026$ and a shoulder at $g = 2.07$ (42–45). The HIN complexes can be observed in completely saturated Hb, when NO ligands to the hexacoordinated heme iron of both $\alpha$ and $\beta$ subunits ($\beta$NO$_{2}$NOaNObNO). In our experiments, this type of spectrum was observed after 1 min of exposure of 20 $\mu$M Hb (100 mM phosphate buffer, pH 7.4) to NO gas in anaerobic conditions (Fig. 2, B4b). A shoulder at $g = 2.07$ was clearly discernible.

**EPR Determinations of Nitrosyl Heme Iron Complexes in K/VP.5 Cells**

To identify and characterize signals of nitrosyl-Hb complexes in the ESR spectra of KVP.5 cells also containing signals of nitrosyl non-heme iron complexes, we used the spectra of pure nitrosylated Hb (obtained in model experiments) to dissect “pure” EPR signals of non-heme iron dinitrosyl complexes. We then used these spectra to identify EPR signals of HIN and/or PIN complexes of nitrosylated Hb in the control and Hb-enriched KVP.5 cells exposed to two different concentrations of NOC-15.

**Non-heme Iron Dinitrosyl Complexes in K/VP.5 Cells**—If the EPR spectra from control and heme-treated cells represent a superposition of the signals from nitrosylated non-heme iron complexes and nitrosylated Hb, subtraction of the spectrum of nitrosylated Hb (normalized appropriately, see below) from the spectrum of the cells should yield the EPR spectrum of non-heme iron dinitrosyl complexes. Indeed, subtraction of the EPR spectrum of the HIN complex of $\beta$NO$_{2}$NOaNObNO obtained from the reaction mixture of 20 $\mu$M Hb with NO (the EPR spectrum of nitrosylated Hb shown in Fig. 2, B4b), from the spectrum of the control KVP.5 cells (incubated with either 20 or 80 nmol of NOC-15/10$^6$ cells (Fig. 2, A2 and A3)), resulted in a new spectrum (Fig. 2, A4) with two features characteristic of non-heme iron dinitrosyl complexes and free radicals, respectively (34, 39, 44–48). The best fit of these spectra was obtained when 10% of the EPR spectrum of nitrosylated Hb (Fig. 2, B4b) was subtracted from the spectrum of the control cells (Fig. 2, A2 and A3). Since the control cells incubated with 20 and 80 nmol of NOC-15/10$^6$ cells produced similar EPR spectra (Fig. 2, A2 and A3), not surprisingly, the contribution of the HIN complexes of nitrosylated Hb to the spectra was identical.

**Nitrosoyl Hb Complexes in K/VP.5 Cells**—We subtracted the EPR signals of non-heme iron dinitrosyl complexes from the spectra of Hb-enriched KVP.5 cells exposed to NOC-15, reasoning that this procedure would reveal signals of different forms of heme-nitrosylated HIN and/or PIN Hb complexes formed intracellularly. By subtracting spectrum A4 of Fig. 2 (superposition of the signals from non-heme iron dinitrosyl complexes and free radicals) from spectrum B2 of Fig. 2 (the spectrum of Hb-enriched KVP.5 cells exposed to 20 nmol of NOC-15/10$^6$ cells), we recovered an EPR spectrum with a three-line splitting characterized by hyperfine coupling $A_2 = 1.62$ mT in the high magnetic field region centered at $g_b = 2.010$ (Fig. 3a). The profile of the reconstructed spectrum was similar to that of PIN complexes of nitrosylated pure Hb (Fig. B5). The PIN spectrum could not be assigned to membrane-bound hemin-NO complexes or complexes of hemin-NO with intracellular proteins other than apohemoglobin. The characteristic spectral band (395 nm) of hemin was not detectable in visible spectra of either cell membranes (membrane pellet solubilized in 1% Triton X-100) or intracellular content (supernatant obtained after treatment of cells with alamethicin) (Fig. 1). Low temperature EPR spectra for PIN complexes of hemin-NO in lipid membranes and toluene glass (49, 50) differ from the spectrum of PIN complexes of hemoglobin-NO in both profiles of the spectra and hyperfine splitting constants (Fig. 3, a versus c and d).

When the subtraction procedure was applied to the spectrum from Hb-enriched KVP.5 cells exposed to 80 nmol of NOC-15/10$^6$ cells (Fig. 2, B3), a large unresolved symmetrical spectrum centered at $g = 2.026$, and a shoulder at $g = 2.07$ (Fig. 2, B4a) was obtained. This spectrum apparently resulted from nitrosylation of both $\alpha$ and $\beta$ subunits due to saturation of Hb by higher concentrations of NO. The profile of the reconstructed spectrum was very similar to the EPR spectrum of the HIN Hb complex (Fig. 2, B4b). The appearance of additional spectral excursions at $g = 2.025$, $g = 2.010$, and $g = 2.004$ in the reconstructed spectrum apparently resulted from traces of the PIN complex (37).

Two different concentrations of NOC-15 (20 and 80 nmol/10$^6$ cells) produced identical profiles in EPR spectra of control KVP.5 cells (Figs. 2, A2 and 1, A3). Subtraction of spectrum A4 (Fig. 2) from either spectrum A2 or A3 gave identical EPR spectra characteristic of HIN Hb complexes (g = 2.026 and g = 1.989) (not shown). The magnitudes of the signals at $g = 1.989$ (trough) in these calculated spectra were approximately 3.5-fold less than that obtained from Hb-enriched KVP.5 cells exposed to a higher concentration of NOC-15 (Fig. 2, B4a). This suggests that the ratio of HIN Hb complexes in Hb enriched to that in control cells was almost the same as the ratio of intracellular Hb in the respective cells.

In summary, exposure of KVP.5 cells to NO released from NOC-15 leads to the formation of nitrosyl complexes of heme- and non-heme iron that gave rise to intensive EPR signals with a partially resolved structure (Fig. 2, A2, A3, B2, and B3). These signals were superpositions of the signals resulting from several distinct intracellular paramagnetic complexes of NO. Therefore, the profiles of the EPR spectra were dependent on both the level of intracellular Hb (Fig. 2, A2 and B2) and the concentration of NO (Fig. 2, B2 and B3). The presence of PIN or HIN Hb complexes suggests that partial or complete saturation of Hb heme was achieved by exposure of Hb-enriched KVP.5 cells to two different concentrations of NOC-15. Complete saturation of both heme-Hb and non-heme iron by NO was found in the control KVP.5 cells at both NOC-15 concentrations. These results set the stage for subsequent experiments aimed at elucidation of NO’s protective mechanism(s) against t-BuOOH-induced oxidative damage.

**EPR Evidence for a Protective Effect of NO Against t-BuOOH-induced Oxoferryl-Hb Free Radical Species in K/VP.5 Cells**

**Formation of Oxoferryl-Hb Species in K/VP.5 Cells Exposed to t-BuOOH—Exposure of KVP.5 cells to t-BuOOH (100 nmol/10$^6$ cells) gave rise to broad EPR signals with partly resolved hyperfine structure (a splitting constant 1.23 mT) (Figs. 4 and 5, A3 and B3). The g values at zero crossing point and a trough minimum observed in the EPR spectra were 2.010 and 2.0027, respectively. The amplitude of these EPR signals was correlated with the levels of intracellular Hb, and the profile was identical to those previously identified by Shiga and Imaiuzumi (51) and similar to the EPR signal characterized as protein centered tyrosyl radicals (Hoganson and Babcock (52)). Therefore, these EPR signals observed in KVP.5 cells were tentatively assigned to oxoferryl-Hb-derived free radical species produced in redox reaction of t-BuOOH and heme iron. The amplitude of t-BuOOH-induced EPR signals in Hb-enriched KVP.5 cells was 3-fold higher than in control KVP.5 cells (Fig. 5, B3 and A3, respectively), the ratio which is very close to the ratio of Hb and the ratio of HIN NO-Hb complexes in the respective cells. The EPR signal obtained from the K/VP.5 cells...
Exposed to t-BuOOH was different from the EPR signal of hemin (in DPPC liposomes) incubated with t-BuOOH (Fig. 4). Indeed, reaction of 20 μM hemin with 0.1 mM t-BuOOH resulted in the appearance of an alkoxyl radical-like EPR signal centered at g = 2.012 (53, 54) (Fig. 4).

Protective Effects of NOC-15—Preincubation of both control and Hb-enriched K/VP.5 cells with 80 nmol of NOC-15/10⁶ cells for 10 min prevented the appearance of the EPR signal of the oxoferryl-Hb free radical species upon exposure to t-BuOOH (Fig. 5, A4 and B4). This effect was accompanied by a complete elimination of the cytotoxic effect of t-BuOOH in K/VP.5 cells (see below). As can be seen from the EPR spectra of K/VP.5 cells exposed to NOC-15 (Fig. 5, A4 and B4) and Fig. 6, A3 and B3), inhibition of the oxoferryl-Hb free radical species formation by t-BuOOH was accompanied by disappearance of the characteristic PIN, HIN Hb complexes and of non-heme iron dinitrosyl complexes. It is likely that intracellularly chelated iron, capable of decomposing hydroperoxides, catalyzed transfer of electrons from liganded NO to t-BuOOH, an effect earlier observed in in vitro experiments (24).

Nitrosylation of Hb by NOC-15 was obligatory for complete protection against t-BuOOH-induced formation of oxoferryl-Hb species. When a lower concentration of NOC-15 (20 nmol/10⁶ cells) was used, which caused a complete nitrosylation of Hb in the control K/VP.5 cells, no oxoferryl-Hb signal could be detected in the EPR spectra upon subsequent incubation with t-BuOOH (Fig. 6, A2 and A3). In contrast, in Hb-enriched K/VP.5 cells, the lower concentration of NOC-15 (which did not produce the HIN complex but saturated mainly α subunits, yielding PIN complexes) did not prevent the formation of oxoferryl-Hb species upon incubation with t-BuOOH (Fig. 6, B2 and B3), nor did it significantly protect against t-BuOOH-induced cytotoxicity or oxidation of PnA-labeled membrane phospholipids (see below).

Low Temperature EPR Measurements with Low Concentrations of K/VP.5 Cells and Reagents

Similar EPR experiments were conducted using proportionally lower concentrations of reagents (t-BuOOH and NOC-15) and cell suspensions (1 × 10⁶ cells/ml) (Fig. 7). This set of experiments was performed to permit a direct comparison of our EPR results with measurements of cell viability and oxidative stress in phospholipids (see below). To obtain discernible EPR signals under these conditions we had to use multiple spectra acquisitions (10 per sample) and their computer-assisted averaging. While the resultant spectra were still much less resolved than those obtained with proportionally higher concentrations of t-BuOOH, NOC-15, and cells (Fig. 5), the
NOC-15 and/or (56) in control and Hb-enriched K/VP.5 cells exposed to A1, B1 intact K/VP.5 cells or cells exposed to NOC-15 (Fig. 8, nals were observed at published results that intracellular oxy-Hb, nitrosyl-Hb, non-
treated with a lower concentration of nitrosylated heme-iron complexes was observed in the cells
in an environment of high (5). We used low temperature EPR measurements to study the formation of high spin state \(S = \frac{5}{2}, d^6 Fe^{3+} \) iron in an environment of high \( g = 6.0 \) and low \( g = 4.3 \) symmetry (56) in control and Hb-enriched K/VP.5 cells exposed to NOC-15 and/or \( t\)-BuOOH. In the absence of \( t\)-BuOOH, no signals were observed at \( g = 4.3 \) and 6.0 of the EPR spectrum from intact KVP.5 cells or cells exposed to NOC-15 (Fig. 8, A1, B1 and A2, B2, respectively). This is in line with the previously published results that intracellular oxy-Hb, nitrosyl-Hb, non-heme iron \( Fe^{2+} \), and dinitrosyl complexes of non-heme iron \( Fe^{2+} \) (low spin-state of \( d^6 Fe^{2+} \), and of \( d^5 Fe^{2+} \) do not manifest EPR signals in a low magnetic field (48, 55).

Hydroperoxides oxidize oxy-Hb to form met-Hb, oxoferryl-Hb, and decompose heme with subsequent release of iron (33, 56–61). Similarly, hydroperoxides are known to oxidize non-heme iron \( d^6 Fe^{3+} \) to \( d^5 Fe^{3+} \) (57). Therefore, the signal at \( g = 6.0 \) (which is attributed to met-Hb) and the signal at \( g = 4.3 \) (assigned to non-heme Fe\(^{2+}\)) would be expected in the EPR spectra of K/VP.5 cells exposed to \( t\)-BuOOH. EPR spectra of control and Hb-enriched K/VP.5 cells treated with \( t\)-BuOOH are shown in Fig. 8, A3 and B3, respectively. The EPR signals of non-heme Fe\(^{2+}\) at \( g = 4.3 \) were observed in both spectra with higher intensity (by about 50%) in samples from Hb-enriched K/VP.5 cells. No met-Hb signals (at \( g = 6.0 \)) were detected in the samples of either control or Hb-enriched K/VP.5 cells after treatment with \( t\)-BuOOH. This may be due to reduction of met-Hb by intracellular met-Hb reductase (62) or to its further oxidation to oxoferryl-Hb.

In control and Hb-enriched K/VP.5 cells preincubated with NOC-15, the intensity of the EPR signals at \( g = 4.3 \) caused by \( t\)-BuOOH was 1.5-fold greater than those from cells that were not preincubated with NOC-15 (Fig. 8, A4 and B4). This was likely due to liganding and reduction by NO of non-heme complexes of iron with \( t\)-BuOOH-derived radicals rather than to a release of iron as a result of Hb degradation. In fact, our previous data demonstrated that NO protects Hb against oxidative damage by \( t\)-BuOOH (24).

**Effect of NOC-15 on Oxidative Stress Produced by \( t\)-BuOOH**

Cytotoxicity of \( t\)-BuOOH is associated with its ability to induce oxidative stress in cells (19). Using a highly oxidation-sensitive fluorescent fatty acid (cis-parinaric acid, PnA) metabolically integrated into membrane phospholipids, we demon-
strated that interactions of t-BuOOH with Hb were mainly responsible for the oxidation of PnA in major classes of phospholipids (Fig. 9). In agreement with our previous results (32), we found that t-BuOOH produced oxidative stress as measured by oxidation of phospholipid parinaroyl residues (Fig. 10, A and B). Sixty min incubation of the cells with 100 nmol of t-BuOOH/10^6 cells resulted in a loss of 50–70% of PnA in major classes of phospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PEA), phosphatidylinositol (PI), and cardiolipin (CL). This effect was observed in both hemin-treated and non-treated K/VP.5 cells.

We further studied the effects of NOC-15 on t-BuOOH-induced oxidation of PnA in K/VP.5 cells. NOC-15 produced a concentration-dependent antioxidant effect (Fig. 10, A and B). The highest NOC-15 concentration used in the experiments (80 nmol/10^6 cells) completely prevented t-BuOOH-induced oxidation of PnA in all major classes of phospholipids in both non-treated and hemin-treated cells (Fig. 10, A and B, respectively).

The lowest concentration of NOC-15 (20 nmol/10^6 cells) afforded protection of PnA residues in PI and CL in non-treated (control) cells, whereas its antioxidant effect was negligible in PC, PEA, and PS of both non-treated and hemin-treated cells (Fig. 10, A and B, respectively).

Recently, an antioxidant function of NO in cells has been proposed, associated with its ability to scavenge peroxyn and alkoxyl radicals, i.e. to act as a chain breaking antioxidant (18, 19, 22–24). To test whether or not the protective effect of NO in K/VP.5 cells might be due to this mechanism, we studied the effects of NO on peroxidation induced by a lipid-soluble initiator of peroxyl radicals, AMVN, (63). We found that AMVN produced oxidation of PnA in essentially all phospholipid classes in K/VP.5 cells (Fig. 11). Preincubation of cells with NOC-15 did not cause any effect on oxidation of PnA residues by AMVN, which was in contrast to the concentration-dependent protection produced by NOC-15 against t-BuOOH (Fig. 10). A water-soluble homologue of vitamin E, 2,2,5,7,8-pentamethyl-6-hydroxychrome, which reduces peroxyn radicals, however, exerted a concentration-dependent protection against AMVN-induced oxidation of membrane phospholipids (data not shown). Thus, the protective effects of NO against t-BuOOH-induced oxidative stress in K/VP.5 cells are not likely to be associated with NO's antiradical activity via a chain-breaking mechanism.

Effect of NOC-15 on Cytotoxicity Produced by t-BuOOH in K/VP.5 Cells

Alkyl hydroperoxides cause toxicity to various cells (19, 64, 65). This effect was suggested to be mainly due to iron-dependent cleavage of alkyl hydroperoxides and to be proportional to the concentration of alkyl hydroperoxides (19, 63–66). The specific roles that heme- and non-heme-iron centers may play in catalysis of hydroperoxide-mediated oxidative injury is not known. K/VP.5 cells grown in the presence of heme offer a unique opportunity to probe the role of Hb-dependent t-BuOOH-induced oxidative damage in cytotoxicity and protection against it. Since our EPR results demonstrated that K/VP.5 cells grown in the presence of heme responded by increased content of Hb without any significant changes in the level of non-heme iron, increased oxidative stress and cytotoxicity in these cells exposed to t-BuOOH is probably completely due to interactions with increased intracellular Hb. This conclusion is further supported by the results of Wink et al. (18) that showed that low concentrations of t-BuOOH (100 nmol of t-BuOOH/10^6 cells) did not cause significant cytotoxic effects in V79 lung fibroblasts which do not express Hb but which contain significant amount of non-heme iron. We found that the concentration of Hb in K/VP.5 cells was 25 pmol/10^6 cells. In hemin-treated (Hb-enriched) K/VP.5 cells the Hb concentration was proportional to the concentration of heme added to the incubation medium (data not shown). Hb content at 25 μM was 3.5-fold higher (90 pmol of Hb/10^6 cells), as compared with the...
control K/VP.5 cells (Fig. 12). Incubation with t-BuOOH (100 nmol/10^6 cells, 60 min) decreased viability of K/VP.5 cells (Fig. 13). The cytotoxic effects caused by t-BuOOH were greater in hemin-treated cells than in control cells (cell viability was 16 and 41%, respectively) (Figs. 12 and 13). It should be noted that viability of parental K562 cells (with low level of intracellular Hb, 5 pmol/10^6 cells) remained high (about 80%) after incubation with the same concentration of t-BuOOH (100 nmol/10^6 cells, 60 min) (Fig. 12). These results indicate that intracellular Hb was, most likely, a major contributor to iron-mediated toxicity of t-BuOOH to K/VP.5 cells.

To test the effect of NO on Hb-dependent cytotoxicity caused by t-BuOOH, K/VP.5 cells were incubated with t-BuOOH in the absence and in the presence of NOC-15. Both non-treated and hemin-treated K/VP.5 cells were preincubated for 10 min in the presence of various concentrations of NOC-15 after which 100 nmol of t-BuOOH/10^6 cells was added and the incubation continued for another 60 min. NOC-15 protected K/VP.5 cells against t-BuOOH-induced cytotoxicity in a concentration-dependent manner. At low concentrations of NOC-15 (20 nmol/10^6 cells) 70% protection against t-BuOOH-induced cytotoxicity was seen in both control and hemin-treated K/VP.5 cells (Fig. 13, A1 and B1). At higher concentrations of NOC-15 (80 nmol/10^6 cells) 100% protection against t-BuOOH-induced cytotoxicity was observed in both control and hemin-treated K/VP.5 cells (Fig. 13, A2 and B2).

In the absence of t-BuOOH, NOC-15 (20–100 nmol/10^6 cells) did not cause loss of cell viability during 70 min (10–60 min) of incubation (Fig. 13, A2 and B2).

**DISCUSSION**

In the present work, we demonstrated for the first time that nitric oxide can protect cells against oxidative stress and damage induced by organic hydroperoxides via nitrosylation of intracellular heme-iron and non-heme-iron catalytic sites. We chose to use human erythroleukemia K/VP.5 cells, a clone of K562 cells, in which we were able to manipulate the content of endogenous Hb by hemin-activated Hb synthesis (28). By incubation of K/VP.5 cells with either t-BuOOH and/or NOC-15 (a triazene type of NO donor), we demonstrated the relevance of oxoferryl-Hb free radical species to oxidative damage of the cells and the efficacy of NO in the protection of K/VP.5 cells, respectively.

Nitrosylated Heme-Iron Centers Interact with t-BuOOH to Protect Cells against Oxidative Damage—Treatment of both control and Hb-enriched K/VP.5 cells with t-BuOOH produced
an EPR signal of protein-centered free radical species (Fig. 5, A3 and B3) characteristic of oxoferryl-Hb (61). Preincubation of the cells with a high concentration of NOC-15 (80 nmol/10⁶ cells) completely prevented t-BuOOH-dependent formation of oxoferryl free radical species (Fig. 5, A4 and B4) and cytotoxicity (Fig. 13) in the control and Hb-enriched K/V.P.5 cells. This protective effect is likely due to complete nitrosylation of both non-heme and heme iron (HIN complexes characteristic of βNOβNOαNOαNOαNOαNO) in the cells. We suggest that nitrosoyl complexes of non-heme iron and heme iron react with t-BuOOH, resulting in the consumption of both t-BuOOH and liganded NO.

Complete nitrosylation of intracellular Hb seems to be an essential prerequisite for complete protection by nitric oxide for erythroleukemia cells exposed to organic hydroperoxides. Evidence in favor of this interpretation comes from our results with Hb-enriched K/V.P.5 cells (90 pmol/10⁶ cells) exposed to a lower concentration of NOC-15 (20 nmol/10⁶ cells). This resulted in a complete nitrosylation of non-heme iron complexes, as evidenced by saturation of the EPR $g = 2.04$ signal intensity; the magnitude of the signal was not increased upon addition of a higher concentration of NOC-15 (Fig. 2, B2 and B3). In contrast, complete nitrosylation of intracellular Hb by low NOC-15 concentrations did not occur, as evidenced by a significant amount of PIN complexes with a profile of the EPR spectrum characteristic of αNO but not βNO (43, 44, 46) (Fig. 3B). The low concentration of the NO donor caused only partial quenching of oxoferryl-Hb free radical species. Not surprisingly, neither complete inhibition of free radical peroxidation of membrane phospholipids (Fig. 10) nor complete protection against t-BuOOH-induced cytotoxicity (Fig. 13) was observed.

In the control K/V.P.5 cells (25 pmol Hb/10⁶ cells), NOC-15 at 20 nmol/10⁶ cells caused about 80% protection, whereas 80 nmol/10⁶ cells of NOC-15 provided complete protection against t-BuOOH-induced cytotoxicity (Fig. 13). Membrane phospholipid peroxidation was completely prevented by 80 nmol/10⁶ cells of NOC-15 (but not by 20 nmol/10⁶ cells) (Fig. 10A). Total nitrosylation of both non-heme iron and heme iron (yielding HIN complexes) was observed upon exposure of the control cells to a low concentration of NOC-15 (Fig. 2, A2 and A3) which completely prevented appearance of EPR signals of the oxoferryl-Hb free radical species (in cells incubated with t-BuOOH) (Fig. 6, A3).

NO can induce oxidation of oxy-Hb yielding met-Hb and nitrate (6, 67, 68). We did not, however, observe formation of met-Hb as a characteristic EPR signal of ferric (met) form of Hb at $g = 6.0$ in K/V.P.5 cells exposed to NOC-15. Apparently, this might be due to reduction of intracellular met-Hb by the met-Hb reductase and subsequent interaction of ferrous form of Hb with NO to produce heme iron nitrosyl complexes (EPR signal at $g = 2.0$). Formation of such iron-nitrosyl complexes was earlier observed in EPR spectra of erythrocytes, hepatocytes (37), and cardiac tissue (69).

S-Nitrosoylation of oxy-Hb in arterial blood by nitrosylated thiols (70) has recently been demonstrated. If S-nitrosoylation was the predominant reaction of NO with oxy-Hb in K/V.P.5 cells, no EPR signals should be observable. In our experiments, however, exposure to NOC-15 caused an immediate production of EPR signals characteristic of heme-nitrosylated Hb (HIN or PIN complexes depending on the concentration of NO donor...
Nitric Oxide Prevents Oxidative Damage

FIG. 13. Effect of NOC-15 on viability of K/VP.5 cells containing low and high amounts of hemoglobin exposed to tert-butydroperoxide. A, control K/VP.5 cells; B, Hb-enriched K/VP.5 cells (see “Materials and Methods”). I, K/VP.5 cells were preincubated (10 min) with NOC-15, after which 100 nmol of t-BuOOH/10^6 cells was added and the incubation continued for another 60 min. 2, control K/VP.5 cells were incubated with NOC-15 for 70 min in the absence of t-BuOOH. Data are means ± S.E. (n = 5). Cell calculations were performed after staining with trypan blue. Significance was determined by Student’s t test for independent means. * p ≤ 0.05 versus “t-BuOOH-free cells treated with NO.”

relative to the concentration of Hb). This was, most likely, due to the use of NOC-15, which is a triazene type of NO donor. Indeed, thiol/nitrosothiol interactions have been shown to be critical for trans-nitrosylation of Hb (71).

Antioxidant Effects of NO Are Not Due to Radical Scavenging—We applied a newly developed sensitive method to detect oxidative stress in membrane phospholipids of K/VP.5 cells which consisted of metabolically incorporating PnA into the constituent phospholipids and monitoring oxidative processes by fluorescence techniques (31). The method creates membrane phospholipid molecular species labeled with PnA which are markedly more susceptible to oxidation than are molecular species normally found as constituents of the membranes of these cells. In K/VP.5 cells, integrated PnA constituted less than 1% of total lipid fatty acid residues suggesting that membrane structure and characteristics were not significantly altered (31). The oxidation of these PnA-labeled phospholipids was monitored with a high degree of precision by measuring changes in fluorescence intensity of the HPLC peaks corresponding to individual phospholipids. We found that t-BuOOH induced oxidation of PnA in all major classes of phospholipids in K/VP.5 cells. With the exception of phosphatidylserine, the PnA oxidation was not dependent on the content of Hb in the cells. This may indicate that oxidative stress induced by 100 μM t-BuOOH was overwhelmingly strong even in the cells with a lower concentration of Hb. In separate experiments, we observed that when lower t-BuOOH concentrations (20–40 μM) were used (which did not result in a significant cell death over 2-h incubation period) oxidation of PnA-labeled PS was proportional to concentrations of both t-BuOOH and Hb (data not shown). In line with this, paraquat-induced oxidative stress in murine leukemia 32D cells was confined to oxidation of only two phospholipids, phosphatidylserine and phosphatidylglycerol. This site-specific oxidation of the phospholipids preceded paraquat-induced externalization of phosphatidylserine and apoptosis in this cell line (Fabisciak et al. (72)).

Most importantly, NOC-15 inhibited t-BuOOH-induced oxidation of PnA-labeled phospholipids. Significantly higher concentrations of NOC-15 were necessary to completely protect PnA-labeled phospholipids against oxidation in the cells with a higher level of endogenous Hb than in those with a lower level of Hb. This suggests that interaction of NO with t-BuOOH at heme-catalytic sites was involved in protective effects of NOC-15 against oxidative stress in the cells. Surprisingly, the NO donor, NOC-15, was not protective against AMVN-induced phospholipid peroxidation in K/VP.5 cells. In contrast, Goss et al. (73) and Hayashi et al. (74) reported that NOC-15 protected against AMVN-induced oxidation in low density lipoproteins and liposomes, respectively. Moreover, several workers (17–20) presented evidence for protective effects of NO in different cell lines in which oxidative stress was induced by a number of oxidants, including t-BuOOH. An explanation for this apparent contradiction is the specific ability of the erythrocytomecella line used in our experiments (K/VP.5) to express significant amounts of hemoglobin. Hemoglobin is very well known to effectively scavenge NO (67, 68). It is widely used for NO trapping because of the high ratio of NO uptake and release rates for ferrous Hb and paramagnetic properties of heme iron nitrosyl complexes (45, 75). It is likely that effective binding and redox conversions of NO at heme-iron-catalytic sites in both control and hemin-treated K/VP.5 cells decreased the levels of free NO released by NOC-15 to levels insufficient for quenching AMVN-derived radicals. In other cell lines that are not enriched with NO-scavenging hemoproteins, protective effects of NO are readily observed. Indeed, in separate experiments using human leukemia myelotic HL-60 cells, which do not express Hb, NOC-15 acted as an effective inhibitor of AMVN-induced peroxidation of PnA-labeled membrane phospholipids.

What is the mechanism by which the NO-heme iron complex serves as an antioxidant against t-BuOOH in K/VP.5 cells? Several recent studies suggest NO is an antioxidant that can (i) act as a chain-breaking antioxidant (i.e. directly scavenge radicals), and/or (ii) inhibit peroxidation via binding to transition metal centers that are participants in Fenton-type catalysis of oxidation (18, 19, 22, 23).

Since our studies demonstrated that the NO donor exerted no protection against AMVN-induced peroxidation in K/VP.5 cells (Fig. 10), we concluded that radical scavenging mechanisms were not likely involved in NO’s antioxidant protection in Hb-containing K/VP.5 cells. Previous work in model systems has demonstrated that NO can prevent and/or protect against hydrogen peroxide- and/or organic hydroperoxide-induced peroxidation in the presence of hemoproteins (e.g. myoglobin and hemoglobin) (23, 24). Liganding of NO to hemoproteins has been suggested to prevent oxidative stress via hindering the interactions of the heme with hydroperoxides (23). An alternative interpretation is that the protective mechanism of NO is due to direct quenching of both t-BuOOH-derived and protein radicals, and reduction of oxoferryl to ferri-hemoproteins (24).

We found that the t-BuOOH-induced disappearance of iron-
nitrosoyl complexes in NOC-15-treated K/VP.5 cells was accompanied by appearance of the $g = 4.3$ EPR signal of $^{d_{5}}$Fe$^{3+}$ and a lack of met-Hb EPR signal at $g = 6.0$ (Fig. 8). Thus, the reaction evidently proceeds via two-electron reduction of t-BuOOH by NO and heme- and non-heme Fe$^{2+}$ (or iron-nitrosyl complexes) and consumption of intracellular NO as shown in Reaction 1.

$$Fe^{3+} + NO + t-BuOOH + H_2O \rightarrow Fe^{2+} + t-BuOH + HNO_3 + OH^-$$

**REACTION 1**

The lack of the EPR signal at $g = 6.0$ may be due to (i) formation EPR-silent complexes of met-Hb/NO (hemin, Fe$^{2+}$/NO$^{-}$), (ii) reduction of ferric heme iron to ferrous heme iron by ferrihemoprotein reductase, and/or (iii) release of Fe$^{3+}$ from the heme moiety. The last is unlikely since our earlier experiments demonstrated that NO prevented oxidative damage of Hb and release of non-heme Fe$^{3+}$ upon exposure to t-BuOOH (24). Therefore, we concluded that in the case of preincubation of K/VP.5 cells with NOC-15, exposure of K/VP.5 cells to t-BuOOH led to formation of ferric iron (Fe$^{3+}$) mainly due to oxidation of non-heme iron nitrosyl complexes (EPR signal at $g = 2.04$) by t-BuOOH. This suggests involvement of non-heme iron in the mechanism of redox inactivation of t-BuOOH, a pathway which may be important for cells devoid of hemoglobin, as discussed by Wink et al. (18, 19).

In the absence of NO, the reaction between oxy-Hb and t-BuOOH in K/VP.5 cells can proceed via intermediate formation of met-Hb, alkoxyl radicals, and an EPR-silent oxoferryl species which can disproportionate to form an additional pool of met-Hb and “free iron” (34, 57, 60, 61, 65, 77–80). In the presence of excess t-BuOOH, a subsequent reaction of met-Hb and t-BuOOH leads to the formation of oxoferryl free radical species (54, 61, 77) that are the major contributors to EPR signals at $g = 2.0$ obtained from K/VP.5 cells (Figs. 5, A3 and B3, and 7, A2, B2, and B2a). In a separate set of experiments (data not shown), we demonstrated that oxidation of oxy-Hb (300 µM) by excess t-BuOOH (2.5 mM) resulted in the intermediate formation of EPR-detectable alkoxyl and oxoferryl-Hb-derived, protein-centered peroxyl and phenoxyl tyrosyl radicals similar to those observed after 60 min incubation of K/VP.5 cells with t-BuOOH. In addition, excess t-BuOOH caused a $g = 4.3$ EPR signal in K/VP.5 cells over 60 min incubation. This might be due, at least in part, to t-BuOOH-induced oxidative decomposition of Hb heme moiety, degradation of intracellular oxy-Hb, and accumulation of non-heme ferric iron (with a characteristic EPR signal at $g = 4.3$) (34, 60, 65).

**Toxicological and Pharmacological Significance—**Cells containing relatively high concentrations of hemoproteins (e.g. myoglobin, hemoglobin, and cytochrome P-450) are known to be sensitive to hydrogen peroxide or organic peroxides (57, 81). This effect is believed to be largely due to the formation of ferryl-hemoproteins, highly potent oxidants, capable of inducing oxidative stress (20, 50, 79, 82). Oxoferryl myoglobin has been implicated in cardiac damage and failure caused by a variety of oxidative conditions such as reoxygenation (82), acute magnesium deficiency (84), and perfusion with different hydroperoxides and alkylperoxides (85, 86). Oxoferryl hemoglobin is believed to be responsible for damage to red blood cells, endothelial cells, lipoproteins, and oxidative transformations of a variety of drugs (87–89). Ferryl states of cytochrome P-450 can be responsible for cytotoxic effects of peroxides in hepatocytes (90, 91). Antioxidants, capable of reducing oxoferryl-associated free radical species, have been successfully used to protect hemoprotein-rich cells against oxidative damage (91–94).

Conversely, peroxide-induced metalloprotein-dependent oxidative stress has been suggested as a promising tumoricidal strategy (27, 94). While use of “hyperoxygenation therapy” as an “alternative” to proven medical modalities in management of cancer is currently a matter of controversy and significant debate (95), proxidant effects and generation of endogenous peroxides are undoubtedly central to mechanisms of various anticancer drugs (e.g. anthracyclines and bleomycin, platinum derivatives, and the N- and S-mustards) (96). Peroxides (hydrogen peroxide and organic peroxides) have been successfully used as generators of cytotoxic radicals in tumor cells. For example, a combination of γ-inolonic acid and FeSO$_4$ induced lipid peroxidation and cytotoxicity in human breast cancer cells (ZR-75-1) (97). However, optimization of peroxide-based anticancer strategies requires understanding of the specific mechanisms involved. In particular, interaction of peroxides with intracellular metalloproteins may be of significant importance.

Iron is known to have a 2-fold physiological role. It represents an essential element for the growth and viability of all cells including tumor cells (98, 99). Neoplastic cells are believed to have qualitative needs for iron higher than those of normal cells (100). In fact, the role of iron in cell proliferation is thought to represent an important factor in the clonal expansion of cancer cells (101). Intracellular iron may, however, form highly potent oxidants, oxoferryls, and catalyze the generation of deleterious reactive oxygen species, such as superoxide anion, and hydroxyl radicals via the Haber-Weiss and Fenton reactions (76). Under physiological conditions, intracellular iron is seemingly tightly controlled by mechanisms that regulate cellular iron uptake through modulation of transferrin receptors (102) and/or store excess iron (through regulation of ferritin expression) (103). Acute exposure of tumors to iron (hemin, FeSO$_4$) has been shown to increase sensitivity of some cancer cells, particularly those relatively low in endogenous ferritin (e.g. breast cancer cells, BT-20), to oxidant-mediated toxicity. In contrast, repeated, more chronic, exposure increased synthesis and accumulation of the intracellular iron chelator, ferritin, and consequently a marked resistance to H$_2$O$_2$-mediated cytotoxicity was manifested (81).

Heme-iron can be deposited into cells via the hemopexin-dependent pathway during neovascularization and hemorrhage which are common features of malignant tumors (81). This can increase the sensitivity of cancer cells to peroxide-mediated injury or decrease NO-produced cytostasis by liganding NO. In addition, cytotoxic effects of peroxides may depend on modulatory effects of intracellular regulators, such as NO. High tumoricidal activity of NO/hydrogen peroxide in a human ovarian cancer cell line (OVCAR) was found to be due to NO-dependent inhibition of catalase (104). Another potentially important mechanism of NO, hemoglobin-catalyzed redox reaction with peroxides, was investigated in this study.

It is well-known that the prooxidative reactions of organic peroxides are catalyzed by heme- and non-heme iron and are accompanied by generation of oxoferryl and oxygen-derived free radical species (25, 56, 64, 65). The presence of NO completely eliminated the harmful effects of organic peroxides. This suggests that NO operates as a redox cofactor, liganding to intracellular iron to reverse prooxidant reactions catalyzed by heme- and non-heme iron, to antioxidant reactions. Recently, formation of nitrosylated Hb, myoglobin, and cytochrome P-450 complexes was demonstrated in vitro in liver (during ischemia/reperfusion (105) and chronic inflammation (37)) and heart (as a result of ischemia/reperfusion (106) or during cardiac allograft rejection (69)). Since these conditions are known to be accompanied by pronounced oxidative stress, nitrosylation of the hemoproteins may be viewed as a protective mechanism against formation of oxoferryl radical species.
and enhancement of oxidative damage. Conversely, the iron-dependent redox transformation of hydroperoxides, which proceeds with consumption of NO, may be viewed as an important physiological mechanism that can terminate NO's effects on eNOS itself.

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