Mechanisms of Nitric Oxide Protection against *tert*-Butyl Hydroperoxide-Induced Cytotoxicity in iNOS-Transduced Human Erythroleukemia Cells†

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ABSTRACT: We studied nitric oxide-mediated protection against *tert*-butyl hydroperoxide (*t*-BuOOH)-induced cytotoxicity in a subline of human erythroleukemia K562 cells (K/VP.5) and in K/VP.5 cells transduced with a retroviral vector containing the human iNOS gene (K/VP.5-iNOS). K/VP.5-iNOS cells were 2-fold less sensitive to the cytotoxic effects of *t*-BuOOH compared to K/VP.5 cells. A nitric oxide-donor, NOC-15 ((*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate), protected K/VP.5 cells against *t*-BuOOH-induced cytotoxicity and provided an additional increment of protection in K/VP.5-iNOS cells. Under conditions of excess *t*-BuOOH and deficiency of iron catalytic sites (hemoglobin, Hb) in K/VP.5-iNOS cells, the increase of intracellular Hb concentration is the main contributor to enhanced sensitivity of the cells to *t*-BuOOH-induced cytotoxicity (despite the effects of small amounts of endogenously produced nitric oxide). Protection against *t*-BuOOH-induced cytotoxicity in K/VP.5-iNOS cells was diminished by treatment with an iNOS inhibitor, L-N^G-monomethylarginine (L-NMA), but was restored upon addition of NOC-15 to L-NMA-treated cells. Incubation of K/VP.5 cells with NOC-15 resulted in the production of dinitrosyl complexes of non-heme iron and hexacoordinated heme iron nitrosyl complexes based on low-temperature EPR spectra. In K/VP.5-iNOS cells, only a weak EPR signal of dinitrosyl complexes of non-heme iron was observed in the absence of NOC-15. In addition, no heme iron nitrosyl complexes were discernible in the EPR spectra from K/VP.5-iNOS cells. Upon addition of NOC-15 to K/VP.5-iNOS cells, the EPR signal of dinitrosyl complexes of non-heme iron was enhanced, and the EPR signal of nitrosylated heme iron became discernible. It was determined that levels of non-heme and heme (hemoglobin) iron were dramatically decreased in K/VP.5-iNOS cells compared to K/VP.5 cells, thus explaining the decreased intensities of EPR signals of nitrosylated species. In addition, *t*-BuOOH-induced oxoferryl-Hb-associated protein-centered free radical species as well as *t*-BuO• alkoxyl radicals were observed in these two cell lines. These *t*-BuOOH-induced radical species were greatly reduced in K/VP.5-iNOS cells compared to K/VP.5 cells, consistent with a reduction in heme iron levels in the iNOS-expressing cells. Most importantly, the combined action of NOC-15 and *t*-BuOOH resulted in complete elimination of both oxoferryl-associated radical EPR signals as well as those from dinitrosyl complexes of non-heme iron and nitrosylated heme iron in both K/VP.5-iNOS cells and K/VP.5 cells. We conclude that two independent pathways operate in erythroleukemia cells for nitric oxide-mediated protection against *t*-BuOOH-induced cytotoxicity. First, prolonged endogenous production of nitric oxide results in a decreased content of catalytic non-heme iron and heme iron sites through posttranscriptional regulation of iron homeostasis. Second, nitric oxide can chemically reduce *t*-BuOOH-induced oxoferryl and *t*-BuO• alkoxyl radicals.

Non-ferritin-bound catalytically active intracellular non-heme iron and heme iron may be involved in different genotoxic and cytotoxic mechanisms (*1*, *2*). In particular, iron-catalyzed decomposition of hydrogen peroxide and organic hydroperoxides (e.g., lipid hydroperoxides), yielding free radicals (e.g., hydroxyl, alkoxyl, peroxyl radicals), and also formation of oxoferryl-associated radical species are believed to be responsible for oxidative damage to critical biomolecules (*2*−*7*). Therefore, regulation of intracellular iron is deemed to be one of the major mechanisms that determines sensitivity to oxidative stress (*4*, *8*). Expression of several important proteins involved in cellular iron homeostasis has been shown to be regulated by iron regulatory proteins 1 and 2 (IRP-1, IRP-2)[1] binding to specific mRNA sequences—iron responsive elements (IREs) (*9*−*16*). Functional IREs have been identified in the 5′-untranslated regions of ferritin H-chain, ferritin L-chain,

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erythroid aminolevulinate synthase, mitochondrial aconitase, and succinate dehydrogenase as well as in the 3′-untranslated regions of transferrin receptors (*12*, *17*).

There are two distinct pathways through which iron-catalyzed oxidative stress may be regulated by nitric oxide (NO) (*18*−*20*). The first pathway is associated with the ability of NO to interact with IRP-1, thus triggering IRE-dependent responses (*17*, *19*). Two different sites on IRP-1 can be targeted by NO. One target site may be the sulfhydryl group of cysteine 437, which has been shown to regulate the binding of IRP-1 to IREs (*19*, *21*, *22*). The second site is the dynamic Fe-S cluster whose removal from the apoprotein is induced by NO binding (*23*). Hence, NO binding to IRP-1 initiates IRP-1/IRE interactions since only the IRP-1 apoprotein can bind to the IREs (*19*, *21*, *22*). Chronic exposure to NO donors or overproduction of endogenous NO in iNOS-transduced cells results in decreased cellular content of non-heme and heme iron by coordinate translational regulatory mechanisms (*12*, *24*−*27*). The protective role of NO against iron-catalyzed oxidative stress has not been studied in relation to this first pathway in which non-heme and heme iron levels are decreased.

In the second pathway, a direct chemical interaction of NO with hydroperoxides at non-heme and heme iron catalytic sites was demonstrated to prevent generation of different free radical species, thus precluding free radical-induced damage of critical biomolecules (*20*, *28*−*30*). 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 (*29*, *30*) or heme iron catalyzed reactions (*20*, *28*).

To further understand protective mechanisms of NO against oxidative stress in cells, we investigated the cytotoxic effects of *t*-BuOOH in a subline of human erythroleukemia K562 cells (K/VP.5) or K/VP.5 cells retrovirally transduced with iNOS (K/VP.5-iNOS). The cytotoxic effects of *t*-BuOOH in NO-producing versus non-NO-producing cells were correlated with the low-temperature EPR signals from oxoferryl-Hb-associated free radical species and *t*-BuO$^{\bullet}$ alkoxyl radicals, as well as nitrosylated non-heme and heme iron sites in these cells. We report that both decreased levels of heme and non-heme iron and direct reaction of NO at catalytic iron sites protect iNOS-transduced cells against oxidative damage induced by *t*-BuOOH.

MATERIALS AND METHODS

Hemoglobin (Hb), sodium hydrosulfite (dithionite), and *tert*-butylhydroperoxide were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium phosphate (monobasic) was purchashed from Fisher Scientific Co. (Fair Lawn, NJ). NOC-15, (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]-diazen-1-ium-1,2-diolate, was obtained from Cayman Chemical Co. (Ann Arbor, MI). Sephadex G-25 columns were from Pharmacia−LKB (Uppsala, Sweden).

[1] Abbreviations: DNCI, dinitrosyl complexes of non-heme iron; Hb, hemoglobin; HIN, hexacoordinated heme iron nitrosyl complexes; IREs, iron responsive elements; IRP-1, IRP-2, iron regulatory proteins 1 and 2; K/VP.5-iNOS, transduced K/VP.5 cells; L-NMA, L-N^{G}-monomethyl-arginine monoacetate; NOC-15, (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate; PIN, pentacoordinated heme iron nitrosyl complexes; *t*-BuOOH, *tert*-butylhydroperoxide.

Cells and Media. K/VP.5 cells used for most of the experiments described are a subline of human erythroleukemia K562 cells selected for resistance to an anticancer agent, etoposide (*31*). Cells were grown in continuous culture in Dubecco's modified Eagle's medium (DMEM) in the presence of 7.5% iron-supplemented calf serum. K/VP.5 cells were chosen for study because it was found that intracellular Hb was ≈30 pmol/10^6 cells compared to 5 pmol/10^6 cells in parental K562 cells (*20*).

Retroviral Transduction of K/VP.5 Cells. The human hepatocyte iNOS cDNA was subcloned into the MFG retroviral vector as described previously to yield the DFGiNOS retroviral construct (*32*). After transfection into BOSC23 cells and collection of viral supernatants, CRIP cells were infected to generate a stable amphotropic producer cell line as described previously (*32*). Log-phase growth K/VP.5 cells were infected for 8 h with the DFGiNOS viral supernatant followed by selection in G418. K/VP.5 cells were also infected with the previously described BaglacZ viral supernatant (*32*) followed by selection in G418. Complete infection in the surviving populations was confirmed by X-gal staining.

Transduced K/VP.5 cells (K/VP.5-iNOS) were screened for generation of nitrite using the Griess reaction as previously described (*33*) as an indirect measure of NO production. A 20-fold increase in the amount of nitrite production was observed in K/VP.5-iNOS cells compared to parental K/VP.5 cells [68.9 ± 19.8 versus 3.2 ± 1.8 nmol (mg of protein)$^{-1}$ (24 h)$^{-1}$, respectively]. A 24 h incubation with 2.0 mM L-NMA dramatically reduced nitrite production in K/VP.5-iNOS cells to 5.4 ± 2.4 nmol (mg of protein)$^{-1}$ (24 h)$^{-1}$. In separate measurements in K/VP.5 cells and in K/VP.5 cells transduced with BaglacZ, there was no difference in nitrite production [6.9 ± 1.5 and 6.0 ± 2.6 nmol (mg of protein)$^{-1}$ (24 h)$^{-1}$], indicating that transduction of empty vector did not stimulate nitric oxide production.

Manipulation of Endogenous Hemoglobin Content and NO Production in the Cells. To increase the intracellular amount of Hb, K/VP.5 cells or K/VP.5-iNOS cells were incubated with hemin (0−25 nmol/10^6 cells) for 24 h. To manipulate levels of NO production, in some experiments K/VP.5-iNOS cells (and K/VP.5 cells) were incubated for 24 h in the presence of an iNOS inhibitor, L-NMA (2.0 mM).

Cell Incubations. Log-phase cells were separated from growth medium by centrifugation (1500*g* × 5 min). The cell pellet was rinsed twice with a buffer containing 115 mM NaCl, 5 mM KCl, 5 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 25 mM Hepes (pH 7.4). To prevent redox effects of adventitious iron, all aqueous solutions were treated with Chelex-100 (Bio-Rad, Hercules, CA). The cells were incubated for 10 min in the absence or presence of 0.1 mM NOC-15 (20 nmol/10^6 cells), releasing NO with a half-life of 76 min, following which 0.5 mM *t*-BuOOH (100 nmol/10^6 cells) was added to incubate for an additional 60 min.

Cell Viability. Aliquots of cell suspensions (1.0 × 10^6 cells/mL) were taken to assess cell viability using Trypan Blue dye exclusion.

EPR Measurements. Aliquots of cell suspensions (80 × 10^6 cells/mL) were used for EPR experiments. Aliquots of cell suspensions (20 × 10^6 cells in 200 μL) were withdrawn, placed into a Teflon tube (3.7 mm internal diameter), and immediately immersed in liquid nitrogen. Frozen samples

were removed from the tube using a glass rod and stored in liquid nitrogen. The frozen samples were transferred into a liquid nitrogen-filled small (50 mL) Dewar flask mounted in the EPR resonance cavity. EPR measurements were performed on a JEOL-RE1X spectrometer at −170 °C, 320 mT center field, 10 mW power, 0.1 mT field modulation, 25 mT sweep width, 500 receiver gain, 0.1 s time constant. The *g*-factor values were determined relative to external standards, containing Mn^{2+} (in MgO). The spectra were collected using EPRWare software (Scientific Software Services, Bloomington, IL) and transferred as ASCII files to EPR analysis software provided by the Free Radical Metabolite Research Group (NIEHS, Research Triangle Park, NC) for spectral analysis (*34*).

Assay of Intracellular Hb. Ten million cells were incubated (for 10 min at room temperature) in the presence of an amphiphilic channel-forming peptide, alamethicin (0.05 mM), to release intracellular Hb (*20*). After centrifugation (100000*g*, 20 min), the supernatant was collected, and the pellet from cell debris was solubilized in 100 mM phosphate buffer, pH 7.4, containing 1% Triton X-100. Spectra of Hb in the supernatant were recorded in the range 350−700 nm using a Shimadzu 160U UV−Vis spectrophotometer. The concentration of Hb in cells was determined using the molar extinction coefficient for the Hb Soret band (414 nm) of 125 mM^{-1} cm^{-1}. Spectra of the solubilized pellet were also recorded to test for completeness of Hb release into the supernatant. The content of Hb in the solubilized pellet did not exceed 5% of that (in the equal volume) in the supernatant.

Oxyhemoglobin Solutions. Commercial Hb was mainly in the metHb (ferric) form. We reduced metHb (1 mM solution in 100 mM phosphate buffer, pH 7.4) to its ferrous (oxyHb) form using a 4-fold excess of sodium dithionite. Pure oxyHb was obtained by separation on a Sephadex G-25 column preequilibrated with 100 mM phosphate buffer, pH 7.4. The concentrations of oxyHb/metHb were calculated as previously described (*35*) using the molar extinction coefficient for oxyHb of 15.0 mM^{-1} cm^{-1} at 577 nm.

RESULTS AND DISCUSSION

Cytotoxicity of t-BuOOH in K/VP.5 and K/VP.5-iNOS Cells and Additional Effects of an NO Donor. Toxicity of alkyl hydroperoxides to various cells is largely due to their iron-catalyzed decomposition to alkoxyl and/or peroxyl radicals as well as formation of oxoferryl radical species (*29*−*31*). K/VP.5 cells showed high sensitivity to *t*-BuOOH [as compared to a variety of other cell lines tested (*36*)]. This may be due to their ability to express significant amounts of hemoglobin (vide infra) which acts as a catalytic site generating free radical species from *t*-BuOOH (*6*, *20*, *37*, *38*). A similarly high sensitivity to *t*-BuOOH was reported for parental human erythroleukemia K562 cells in which endogenous levels of hemoglobin were increased by growing the cells in hemin-containing medium (*20*, *39*). Cytotoxic effects of *t*-BuOOH on K/VP.5 and K/VP.5-iNOS cells were significantly different (Table 1). One hour incubation with 0.5 mM *t*-BuOOH killed 55% of K/VP.5 cells compared to 25% of K/VP.5-iNOS cells. In contrast, there was no protection against *t*-BuOOH-induced cytotoxicity in K/VP.5 cells infected with the control Baglacz retroviral vector lacking the iNOS gene (results not shown).

Table 1: Protective Effects of Nitric Oxide in *t*-BuOOH-Treated K/VP.5 and K/VP.5-iNOS Cells

drug treatment	% cell viability[a] K/VP.5 cells	% cell viability[a] K/VP.5-iNOS cells
control	91.2 ± 3.0 (6)[b]	90.5 ± 2.5 (6)[b]
t-BuOOH (500 μM)	45.5 ± 5.1 (6)[c]	74.5 ± 4.7 (6)[d]
NOC-15 (80 μM)	89.7 ± 5.7 (3)[e]	88.4 ± 3.5 (3)[e]
NOC-15 + *t*-BuOOH	84.6 ± 4.3 (5)[e]	87.3 ± 3.5 (5)[e]

[a] Cells were incubated for 15 min in the absence or presence of 80 nmol of NOC-15/10^6 cells followed by a further 1 h incubation in the absence or presence of 500 nmol of *t*-BuOOH/10^6 cells. Trypan blue exclusion was used to assess % cell viability. Results are expressed as the mean ± SEM. Numbers in parentheses represent the number of replicate experiments performed on separate days. Statistical significance was determined using an All Pairwise Comparison Procedure (Tukey Test). [b] Significantly different compared to *t*-BuOOH treatment in both K/VP.5 and K/VP.5-iNOS cells; $p < 0.02$. [c] Significantly different compared to all other groups/conditions; $p < 0.02$. [d] Significantly different compared to *t*-BuOOH treatment in K/VP.5 cells and compared to controls in both K/VP.5 and K/VP.5-iNOS cells; $p < 0.02$. [e] Significantly different compared to *t*-BuOOH treatment in K/VP.5 cells; $p < 0.02$.

Our previous work has demonstrated that the level of endogenous Hb in K/VP.5 cells may be a critical determinant of the cell's sensitivity to *t*-BuOOH-induced cytotoxicity (*20*). Therefore, we assayed the content of Hb in K/VP.5 cells and K/VP.5-iNOS cells. Concentrations of Hb were dramatically lower in K/VP.5-iNOS cells compared to K/VP.5 cells: 4.0 and 32.0 pmol of Hb/10^6 cells, respectively. Since incubation with hemin can cause a substantial increase of Hb in K/VP.5 cells (*20*), we decided to use this approach to test whether increased intracellular Hb content will result in an increased sensitivity of K/VP.5-iNOS cells to *t*-BuOOH. When K/VP.5 cells or K/VP.5-iNOS cells were incubated with different concentrations of hemin (0−25 μM, for 24 h), an increased content of Hb was detected in both types of cells (Figure 1). In K/VP.5 cells, the Hb content increased from 32.0 to ≈70 pmol of Hb/10^6 cells. In K/VP.5-iNOS cells, this effect was more dramatic: the initial very low level of hemoglobin (≈4 pmol of Hb/10^6 cells) was increased by more than an order of magnitude (up to ≈55 pmol of Hb/10^6 cells). Thus, despite continuous production of NO, K/VP.5-iNOS cells responded by a very pronounced increase in the content of Hb upon 24 h incubation with hemin. Importantly, in both K/VP.5 cells and K/VP.5-iNOS cells, hemin-induced elevation of Hb content was accompanied by an increased sensitivity to *t*-BuOOH-induced cytotoxicity. A sharp increase in Hb content in K/VP.5-iNOS cells incubated with 5−10 μM hemin produced a drastic decrease in cell viability. Interestingly, when the hemin-induced endogenous Hb content in K/VP.5-iNOS cells reached the level of Hb in K/VP.5 cells (≈32 pmol of Hb/10^6 cells), the viability of K/VP.5-iNOS cells in the presence of *t*-BuOOH was the same as that of K/VP.5 cells (in the absence of hemin). These results clearly indicate that under conditions of excess *t*-BuOOH and a deficiency of iron catalytic sites (Hb) in K/VP.5-iNOS cells, the increase of intracellular Hb concentration is the main contributor to the enhanced sensitivity of the cells to *t*-BuOOH-induced cytotoxicity (despite the effects of small amounts of endogenously produced nitric oxide).

Previously it was demonstrated that NO donors were able to protect different cell lines against *t*-BuOOH-induced

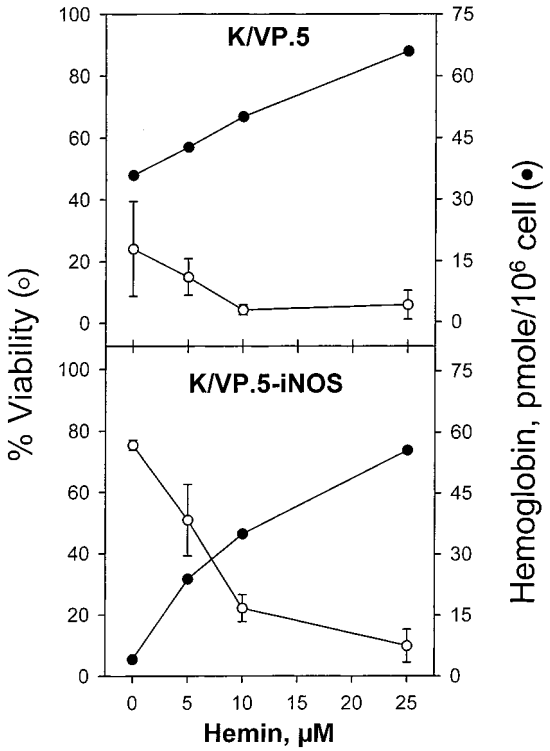

FIGURE 1: Effects of hemin on hemoglobin content and *t*-BuOOH-induced cytotoxicity in K/VP.5 and K/VP.5-iNOS cells. Cells were incubated for 24 h with hemin (0−25 μM) following which hemoglobin content was measured. Cells were incubated for 1 h in the presence of 500 μM *t*-BuOOH, and viability was assessed by trypan blue exclusion. For viability, results shown are the mean ± SEM from 3−4 experiments performed on separate days.

toxicity (*29*). Hence, we suggested that endogenously formed NO might be responsible, at least in part, for protection against *t*-BuOOH in K/VP.5-iNOS compared to K/VP.5 cells In accord with this, we found that preincubation of K/VP.5 cells with an excess of NO donor, NOC-15 ((*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate), 80 μM, 15 min), resulted in nearly complete protection against *t*-BuOOH-induced cytotoxicity (Table 1). In addition, NOC-15 provided an additional component of protection against *t*-BuOOH in K/VP.5-iNOS cells (Table 1) though this additional protection did not achieve statistical significance ($p = 0.063$). Thus, either endogenously or exogenously produced NO protected K/VP.5 cells against *t*-BuOOH-induced toxicity.

To further our understanding of interactions of endogenously formed nitric oxide with iron proteins in *t*-BuOOH-induced cytotoxicity, we manipulated levels of NO production in K/VP.5-iNOS cells (and K/VP.5 cells) using an iNOS inhibitor, L-NMA, and determined cell viability upon exposure to *t*-BuOOH. We determined the effectiveness of L-NMA in inhibiting iNOS activity and performed measurements of nitrite formation in the cells incubated for 24 h in the presence and in the absence of L-NMA (2.0 mM). We found that extremely low levels of nitrite generated in K/VP.5 cells [<5 nmol (mg of protein)$^{-1}$ (24 h)$^{-1}$ were not changed by incubation in the presence of L-NMA. In contrast, high levels of nitrite produced by K/VP.5-iNOS cells [>60.0 nmol (mg of protein)$^{-1}$ (24 h)$^{-1}$] were dramatically decreased by L-NMA [down to <7−8.5 nmol (mg of protein)$^{-1}$ (24 h)$^{-1}$]. We then compared the sensitivity of L-NMA-treated cells

Table 2: Effects of L-NMA on Nitric Oxide-Mediated Protection against *t*-BuOOH in K/VP.5 and K/VP.5-iNOS Cells

	% cell viability[a]	
drug treatment	K/VP.5 cells	K/VP.5-iNOS cells
control	89.7 ± 2.4 (6)[b]	90.1 ± 1.5 (6)[b]
t-BuOOH (500 μM)	46.1 ± 5.1 (6)[c]	44.0 ± 9.2 (6)[d]
NOC-15 (80 μM)	87.7 ± 5.7 (2)	90.9 ± 8.0 (2)
NOC-15 + *t*-BuOOH	87.8 ± 2.2 (5)[e]	82.6 ± 3.2 (5)[e]

[a] Cells were incubated for 24 h in the presence of 2 mM L-N^G-monomethylarginine monoacetate (L-NMA). Cells were then incubated for 15 min in the absence or presence of 80 nmol of NOC-15/10^6 cells followed by a further 1 h incubation in the absence or presence of 500 nmol of *t*-BuOOH/10^6 cells. Trypan blue exclusion was used to assess % cell viability. Results are expressed as the mean ± SEM or as mean ± range. Numbers in parentheses represent the number of replicate experiments performed on separate days. Statistical significance was determined using an All Pairwise Comparison Procedure (Tukey Test). [b] Significantly different compared to *t*-BuOOH treatment in both K/VP.5 and K/VP.5-iNOS cells; $p < 0.02$. [c] Significantly different compared to all other groups/conditions except *t*-BuOOH treatment in K/VP.5-iNOS cells; $p < 0.02$. [d] Significantly different compared to controls in both K/VP.5 and K/VP.5-iNOS cells; $p < 0.02$. [e] Significantly different compared to *t*-BuOOH treatment in K/VP.5 cells; $p < 0.02$.

to *t*-BuOOH. As expected, the cytotoxicity of *t*-BuOOH to K/VP.5 cells was not changed by L-NMA. Also, the protective effect of NOC-15 in K/VP.5 cells was not affected by L-NMA treatment as well (Table 2). The protection against *t*-BuOOH-induced cytotoxicity in K/VP.5-iNOS cells compared to K/VP.5 cells (Table 1) was diminished by L-NMA (Table 2) but was restored upon addition of NOC-15 (80 μM) to the L-NMA-treated K/VP.5-iNOS cells (Table 2). In separate experiments, we determined whether L-NMA treatment caused any changes in the content of endogenous Hb in the treated cells. We found that no significant changes of Hb content were produced by L-NMA treatment in either K/VP.5-iNOS cells or K/VP.5 cells. These results indicate that the level of NO production is one of the critical factors that determines the sensitivity of K/VP.5 cells or K/VP.5-iNOS cells to *t*-BuOOH-induced cytotoxicity. Excess NO (achieved by the addition of NOC-15) eliminates the dependence of the cells on the endogenous source of NO (in K/VP.5-iNOS cells) and results in maximal protection of the cells (both K/VP.5 cells and K/VP.5-iNOS cells) against *t*-BuOOH-induced cytotoxicity.

Low-Temperature EPR Spectroscopy of Nitrosylated Iron Proteins and Free Radical Species in K/VP.5 and K/VP.5-iNOS Cells. Two different pathways may be involved in NO-mediated protection against *t*-BuOOH-induced cytotoxicity: (i) posttranscriptional regulation of non-heme and heme iron proteins (*18*−*20*, *33*); and (ii) direct interaction of NO with catalytic iron sites, preventing iron-induced generation of radical species from *t*-BuOOH (*6*, *20*, *28*, *29*). To determine whether one or both of these pathways are responsible for NO-mediated cyto-protection against *t*-BuOOH toxicity, we used low-temperature EPR spectroscopy to assess *t*-BuOOH-dependent formation of free radical species and NO-induced nitrosylation of non-heme and heme proteins in K/VP.5 and K/VP.5-iNOS cells. Our previous work has demonstrated that in K/VP.5 cells with different endogenous levels of Hb, the magnitude of HIN/PIN EPR signals as well as that of *t*-BuOOH-induced EPR signals was proportional to Hb concentrations (*20*). This is in line with

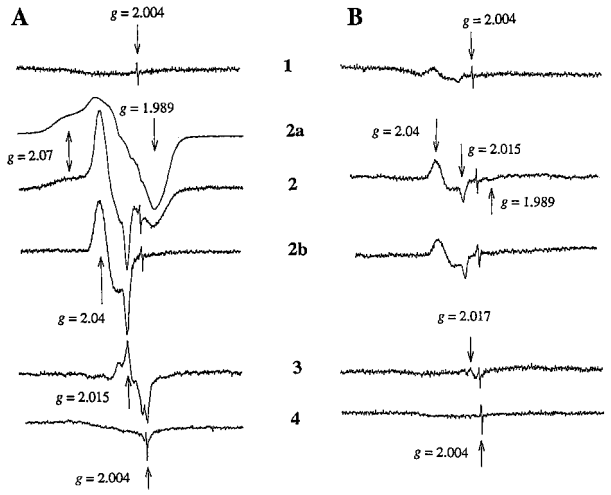

FIGURE 2: Low-temperature EPR spectra obtained from erythroleukemia K/VP.5 cells (A) and K/VP.5-iNOS cells (B), exposed to an NO donor, NOC-15, and/or *t*-BuOOH. Spectra shown are from the following: control intact cells (A1, B1); cells incubated 10 min with NOC-15 (20 nmol/10^6 cells) (A2, B2); cells incubated 60 min with *t*-BuOOH (100 nmol/10^6 cells) (A3, B3); cells preincubated for 10 min with NOC-15 (20 nmol/10^6 cells) and subsequently incubated for 60 min with *t*-BuOOH (100 nmol/10^6 cells) (A4, B4). An EPR spectrum of hexacoordinated complexes of nitrosylated Hb (see Materials and Methods) is shown in A2a. The EPR spectrum of non-heme iron dinitrosyl complexes in K/VP.5 cells (A2b) was obtained by subtraction of 10% of spectrum A2a from spectrum A2. The EPR spectrum shown in B2b is a 33% reduction of spectrum A2b representing EPR signals of non-heme iron dinitrosyl complexes in K/VP.5 cells for comparison with B2, the EPR spectrum representing non-heme iron dinitrosyl complexes in K/VP.5-iNOS cells. Spectrometer conditions were as follows: modulation amplitude, 0.2 mT; microwave power, 10 mW; receiver gain, 500 (except A2a, where receiver gain was 50); time constant, 0.3 s; scan rate, 6.25 mT/min. The spectrometer was operated at 9.05 GHz with a 100 kHz modulation frequency. All spectra presented are computerized averages of 3 acquisitions.

the commonly recommended use of nitric oxide as a probe for EPR detection of metalloenzymes in cells (*40*−*43*).

Iron−*Nitrosyl Complexes in K/VP.5 and K/VP.5-iNOS Cells.* EPR spectra of K/VP.5 cells displayed only nonspecific free radical signals at $g = 2.004$ (Figure 2A1), likely produced by components of the mitochondrial respiratory chain (*36*, *44*). An additional anisotropic signal with $g_{||} = 2.04$ and $g_{\perp} = 2.015$ was observed in K/VP.5-iNOS cells (Figure 2B1). The features at $g_{||} = 2.04$ and $g_{\perp} = 2.015$ were earlier observed in K562 cells exposed to NO (*20*, *45*) and were assigned to dinitrosyl complexes of non-heme iron (DNCI) (*18*, *19*, *21*). Apparently, formation of DNCI in K/VP.5-iNOS cells was caused by endogenously produced NO which reacted with intracellular non-heme iron.

Previously, we demonstrated that Hb-rich K/VP.5 cells produced characteristic signals of nitrosylated Hb in EPR spectra upon addition of NOC-15 (*20*). Therefore, it was expected that endogenously generated NO in K/VP.5-iNOS cells would similarly result in detectable nitrosylated Hb signals. Suprisingly, these signals were not found in the EPR spectra of K/VP.5-iNOS cells (Figure 2B1). This result can be explained by a fast conversion of nitrosylated Hb into metHb, by low levels of Hb expression (and consequently low levels of nitrosylated Hb), or by a combination of these two mechanisms.

To distinguish between these possibilities, we performed EPR measurements using K/VP.5 or K/VP.5-iNOS cells incubated with NOC-15 to generate NO levels in excess of cellular Hb concentrations. First, we incubated K/VP.5 cells with NOC-15 (20 nmol/10^6 cells) and observed anisotropic EPR spectra with (i) principal features at $g_{||} = 2.04$ and $g_{\perp} = 2.015$; (ii) a free radical signal at $g = 2.004$ indicative of DNCI (Figure 2A2). Importantly, spectral excursions at $g = 2.07$ (a maximum) and $g = 1.989$ (a trough) were detected in the spectrum (Figure 2A2) that could be assigned to the EPR signal of hexacoordinated heme iron nitrosyl (HIN) complexes of nitrosylated Hb. Indeed, similar HIN nitrosyl complexes of Hb have been shown previously in model systems (*46*−*48*). Essentially the same spectra were observed in erythrocytes upon exposure to NO (*49*, *50*). To confirm this assignment of features $g = 2.07$ and $g = 1.989$ to the signal of nitrosylated Hb in K/VP.5 cells, we compared EPR signals from the cells with those obtained from pure Hb upon addition of NO (vide infra). HIN complexes are characterized by a large unresolved symmetrical spectrum centered at $g = 2.026$ and a shoulder at $g = 2.07$ (*47*−*49*). The HIN complexes can be observed in Hb when NO ligands to the hexacoordinated heme iron of both α and β subunits (αNOβNOαNOβNO). In our experiments, this type of spectrum was observed after 1 min of exposure of 0.02 mM Hb (50 mM phosphate buffer, pH 7.4) to NO gas in anaerobic conditions (Figure 2A, 2a). A shoulder at $g = 2.07$ was clearly discernible.

We next incubated K/VP.5-iNOS cells with NOC-15 (20 nmol/10^6 cells) and observed EPR spectra qualitatively similar to those observed with K/VP.5 cells incubated with NOC-15. There was a weak signal with the feature at $g = 1.989$ (a trough) characteristic of HIN complexes of nitrosylated Hb (*47*−*49*) (Figure 2B2). In addition, there was a pronounced (3.5-fold) increase in the intensity of the features at $g_{||} = 2.04$ and $g_{\perp} = 2.015$ (i.e., appearance of additional amounts of DNCI; Figure 2B2) as compared to K/VP.5-iNOS cells not treated with NOC-15 (Figure 2B1). Overall our results indicate that endogenously produced NO in K/VP.5-iNOS cells did not saturate all heme and non-heme iron sites, thus accounting for the lack of detectable nitrosylated Hb signals (Figure 2B1).

To quantitatively compare the intensities of DNCI and HIN signals in the EPR spectra, we performed a computer-assisted subtraction of the EPR signal from nitrosylated pure Hb (Figure 2A, 2a, at a gain of 50) from the EPR signal of K/VP.5 cells exposed to NOC-15 (Figure 2A2, at a gain of 500). This resulted in a reconstructed spectrum with $g = 2.04$ and $g = 2.015$ that is typical of DNCI complexes of non-heme iron (Figure 2A, 2b). The best fit of the spectra was obtained when 10% of the EPR spectrum of nitrosylated Hb (Figure 2A, 2a, at a gain of 50) was subtracted from the spectrum in Figure 2A2. This procedure demonstrated that DNCI complexes represented a dominating signal in the EPR spectrum from K/VP.5 cells exposed to NOC-15.

A 33% reduction in the EPR spectrum from Figure 2A, 2b yielded the spectrum shown in Figure 2B, 2b. This spectrum is similar to the spectrum in Figure 2B2 obtained from K/VP.5-iNOS cells treated with NOC-15. Comparison of these spectra indicated that the DNCI signal (related to non-heme iron) was predominant compared to the HIN signal (related to heme iron) in the spectrum of K/VP.5-iNOS cells treated with NOC-15 (Figure 2B2). Consistent with this observation, the concentration of intracellular Hb in K/VP.5-

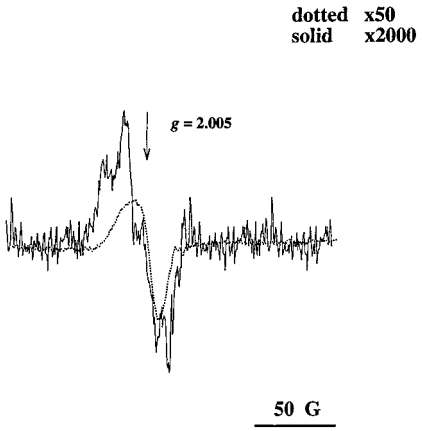

FIGURE 3: EPR spectrum (solid) of oxoferryl free radical species in K/VP.5 cells obtained by subtraction of the 20% EPR spectrum of *t*-BuO• alkoxyl radicals (dotted line) from the EPR spectrum (Figure 2A3) recorded after incubation of K/VP.5 cells for 60 min with *t*-BuOOH (100 nmol/10^6 cells). The EPR spectrum of alkoxyl radicals was obtained by incubating ferrous iron (0.1 mM) and cysteine (0.5 mM) at pH 7.4 in the presence of 0.5 mM *t*-BuOOH. Spectrometer conditions were the same as in the legend to Figure 2 except that the receiver gains were 50 (dotted spectrum) and 2000 (solid spectrum), respectively.

iNOS cells was one-eighth of that found in K/VP.5 cells (4 pmol/10^6 versus 32 pmol of Hb/10^6, respectively). Thus, levels of both endogenous non-heme and heme iron catalytic sites were dramatically decreased in K/VP.5-iNOS cells.

Apparently, decreased levels of intracellular iron proteins in K562-iNOS cells are due to chronic endogenous production of NO triggering the modulation of IRP-1 binding to IREs (*12*, *17*, *24*−*27*). Similar observations have recently been reported in K562 cells retrovirally transduced with murine NOS-2 (*24*, *25*). Posttranscriptional regulation of iron protein expression by NO-dependent mechanisms has also been demonstrated in parental K562 cells (*51*, *52*) and endothelial cells (*18*) after addition of NO donors.

Oxoferryl-Hb Free Radical Species in K/VP.5 and K/VP.5-iNOS Cells Exposed to t-BuOOH. Exposure of K/VP.5 cells to 0.5 mM *t*-BuOOH gave rise to two partially resolved EPR signals: oxoferryl-Hb free radical species and alkoxyl radicals of *t*-BuOOH (Figure 2A3). This became apparent after subtraction of 10% of the characteristic *t*-BuO• alkoxyl radical signal shown in Figure 3 (dashed) from the spectrum found in Figure 2A3. The typical EPR spectrum of the oxoferryl-Hb protein-centered free radicals became pronounced (Figure 3, solid). Similar signals were identified by Shiga et al. (*53*): an unresolved quintet centered at $g = 2.005$. Several recent reports indicate that more than one radical species may be sequentially produced on different amino acid residues (e.g., on Tyr and on Trp) upon addition of H_2O_2 or organic hydroperoxides to different heme proteins (*54*−*57*). Since the goal of the present study did not specifically involve characterization of these transient radical species, we will denote all different *t*-BuOOH-induced protein-associated free radical species as "protein-centered radicals". In addition, iron-dependent cleavage of *t*-BuOOH in K/VP.5 cells was accompanied by oxidation of intracellular iron. This was detected as the appearance of a characteristic EPR signal of the ferric (d^5 Fe^{3+}) form of iron at $g = 4.3$ (Figure 4A2) (*58*).

Exposure of K/VP.5-iNOS cells to the same concentration of *t*-BuOOH (100 nmol/10^6 cells) produced a weak signal

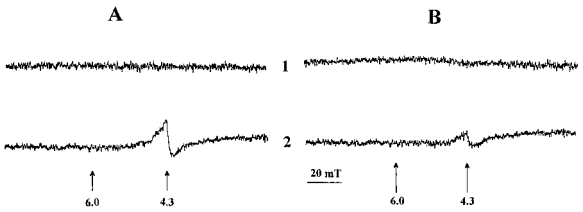

FIGURE 4: Low-temperature EPR spectra of high-spin iron complexes obtained from K/VP.5 cells (A) and K/VP.5-iNOS cells (B) before and after incubation with *t*-BuOOH. Spectra shown are from intact cells (1) and from intact cells after incubation with *t*-BuOOH (100 nmol/10^6 cells) for 60 min (2). Spectrometer conditions were the same as in the legend to Figure 1 except that the modulation amplitude was 0.5 mT. The spectra presented are computerized averages of 3 acquisitions.

in the EPR spectrum (Figure 2B3) whose magnitude was at least one-seventh of that in K/VP.5 cells. Due to very low intensity and poor resolution, the $g = 2.017$ signal can only be tentatively assigned to the major component of the oxoferryl-Hb free radical species mentioned above. Accordingly, the $g = 4.3$ signal (assigned to the ferric d^5 Fe^{3+} form of iron) from K/VP.5-iNOS cells was significantly reduced as compared to K/VP.5 cells (compare Figure 4A2 and 4B2). In separate spectrophotometric experiments, we demonstrated that 1 h incubation of K/VP.5 cells with 0.5 mM *t*-BuOOH (100 nmol/10^6 cells) results in complete oxidative degradation of hemoglobin in the cells. Together these results indicate that incubation of K/VP.5 cells with *t*-BuOOH results in formation of oxoferryl-Hb-associated protein-centered free radical species as well as *t*-BuO• alkoxyl radicals. These free radical species were dramatically reduced in K/VP.5-iNOS cells likely due to NO-induced down-regulation of intracellular iron proteins. These results correlate well with the significantly attenuated cytotoxic effect of *t*-BuOOH in K/VP.5-iNOS cells compared to K/VP.5 cells (Table 1).

Combined Effects of t-BuOOH and NOC-15. If interaction of NO with *t*-BuOOH-induced radical species at iron catalytic sites is an important mechanism of protection, one would expect that excess NO should completely eliminate both the EPR signals from free radical species generated upon addition of *t*-BuOOH to the cells and the cytotoxicity of *t*-BuOOH (*20*). Indeed, as shown in Figure 2A4 and 2B4, only nonspecific free radical signals at $g = 2.004$ were observed in both K/VP.5 cells and K/VP.5-iNOS cells preincubated with NOC-15 and subsequently exposed to *t*-BuOOH. Neither signals from *t*-BuOOH-induced free radical species nor signals from nitrosyl complexes of heme or non-heme iron complexes were detected in the EPR spectra.

We have previously suggested that in NOC-15-pretreated K/VP.5 cells, addition of *t*-BuOOH oxidizes non-heme iron nitrosyl complexes, resulting in loss of EPR signals (*20*). Under these conditions, there is also a two-electron reduction of *t*-BuOOH by iron−nitrosyl complexes of heme- and non-heme Fe^{2+} resulting in the loss of both oxoferryl-Hb-associated radical species and *t*-BuO• alkoxyl radicals with accompanying oxidation to Fe^{3+} (*20*). As expected, exogenously added NO completely eliminated the cytotoxic effects of *t*-BuOOH in both K/VP.5 cells and K/VP.5-iNOS cells (Table 1). It should also be mentioned that NO can directly interact with reactive radicals, such as alkoxyl and peroxyl radicals (*59*, *60*), that may participate in propagation

of *t*-BuOOH-induced peroxidative damage of critical biomolecules, thus protecting cells against oxidative damage (*61*). While this mechanism may not be very efficient in cells with high endogenous levels of hemoproteins (due to wasteful interactions of NO with hemoproteins) (*20*), it may be quite important under conditions when the content of the hemoproteins is lowered, e.g., in K/VP.5-iNOS cells.

In conclusion, this study demonstrates for the first time that NO can protect cells against oxidative stress induced by hydroperoxides acting via two independent pathways: by decrease of catalytic heme- and non-heme iron sites and by direct chemical interaction with hydroperoxide-induced free radical species at the iron catalytic sites to reduce oxyferryl and alkoxyl radical species. The former pathway may function as an adaptive mechanism that becomes operational upon long-term exposure of cells to NO; the latter pathway represents a fast reaction that may be considered an emergency mechanism to protect cells from the consequence of acute and intensive oxidative stress.

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