ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 3, 2004

Forum Original Research Communication

Factors Influencing Nitroxide Reduction and Cytotoxicity *In Vitro*

YUVAL SAMUNI,* JANET GAMSON,* AYELET SAMUNI, KENICHI YAMADA, ANGELO RUSSO, MURALI C. KRISHNA, and JAMES B. MITCHELL

ABSTRACT

Nitroxides have been shown to be effective antioxidants, radiation protectors, and redox-active probes for functional electron paramagnetic resonance (EPR) imaging. More recently, the nitroxide 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-*N*-oxyl (Tempol) has been shown to exert differential cytotoxicity to tumor compared with normal cell counterparts. Nitroxides are readily reduced in tissues to their respective hydroxylamines, which exhibit less cytotoxicity *in vitro* and do not provide radiation protection or an EPR-detectable signal for imaging. In order to better understand factors that influence nitroxide reduction, the rate of reduction of Tempol in mouse and human cell lines and in primary cultures of tumor cells was measured using EPR spectroscopy. Additionally, the cytotoxicity of high concentrations of Tempol and the hydroxylamine of Tempol (Tempol-H) was evaluated in wild-type and glucose-6-phosphate dehydrogenase (G6PD)-deficient Chinese hamster ovary cells. The results show that in general Tempol was reduced at a faster rate when cells were under hypoxic compared with aerobic conditions. Neither depletion of intracellular glutathione nor treatment of cells with sodium cyanide influenced Tempol reduction rates. G6PD-deficient cells were found to reduce Tempol at a significantly slower rate than wild-type cells. Likewise, Tempol-induced cytotoxicity was markedly less for G6PD-deficient cells compared with wild-type cells. Tempol-H exhibited no cytotoxicity to either cell type. Tempol-mediated cytotoxicity was enhanced by glutathione depletion and inhibition of 6-phosphogluconate dehydrogenase in wild-type cells, but was unaltered in G6PD-deficient cells. Collectively, the results indicate that while the bioreduction of Tempol can be influenced by a number of factors, the hexose monophosphate shunt appears to be involved in both nitroxide reduction as well as cytotoxicity induced by high levels of exposure to Tempol. *Antioxid. Redox Signal.* 6, 587–595.

INTRODUCTION

NITROXIDES are effective antioxidants and radiation protectors in both *in vitro* and *in vivo* models (12, 17, 26, 28, 29). Nitroxides have also been used in functional electron paramagnetic resonance (EPR) imaging studies as a novel means to assess tissue redox capacity non-invasively (27, 45). The majority of studies utilizing nitroxides as antioxidants or radiation protectors have used either low to high concentrations with short exposure times (*in vitro*) or bolus infusions (*in vivo*). Though nitroxides administered in this fashion exhibit little toxicity (28, 29), recently it has been shown that prolonged administration of high concentrations of nitroxides can lead to significant cytotoxicity (14, 15).

The metabolism of nitroxides occurs in cells (and tissues) and yields primarily the hydroxylamine (7, 16), which can be oxidized back to nitroxides (6, 13, 23). Both the nitroxides and, to a much lesser extent, their respective hydroxylamines are effective antioxidants in various cell and animal model systems (30). Interestingly, nitroxides, though not

Radiation Biology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD.
*These authors contributed equally.

hydroxylamines, are effective radioprotectants (29, 44) and have been found to confer greater radioprotection to normal tissue than to tumor tissue (17, 19). It has been postulated that the basis for such selective radioprotection is the lowered partial pressure of O_2 in tumor tissue, which results in enhanced bioreduction of nitroxides. The bioreduction of nitroxides depends on their structure, temperature, and oxygen concentration and on cell type, and it is difficult to anticipate the rate of reduction of nitroxides in different cell types, or to compare between rates of normal and tumor cells. Normal and tumor tissues differ in their oxygen concentration, redox status, intracellular pH, and vasculature. The bioreduction of nitroxides in normal and tumor tissues can take place in several ways, including enzymatically in the mitochondria at the ubiquinone level (8), or in the microsomal portion of the cell (21, 24). However, the difference in bioreduction of nitroxides by normal versus tumor tissues has been attributed to inherent differences in biochemical characteristics such as glutathione (GSH) content (10) and expression of genes usually associated with oxidative stress. With respect to cytotoxicity, nitroxides at high concentrations over prolonged exposures have been shown to exhibit more cytotoxcity to tumor versus normal cell counterparts (14). Thus it is important to better understand the factors that influence nitroxide reduction, to shed light on both imaging and cytotoxicity studies.

The present study uses EPR spectrometry and cytotoxicity assays to compare the rate of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempol) reduction and Tempol-mediated cytotoxicity using rodent and human cell lines. In addition to hypoxia, the results show that glucose-6-phosphate dehydrogenase (G6PD) activity is important in nitroxide reduction and cytotoxicity.

MATERIALS AND METHODS

Reagents

Tempol, sodium cyanide (NaCN), 6-aminonicotinamide (6-AN), desferrioxamine (DF), DNase, and collagenase were purchased from Sigma-Aldrich (Milwaukee, WI, U.S.A.), and L-buthionine-*S*,*R*-sulfoximine (BSO) was purchased from Schweizerhall Inc. (South Plainfield, NJ, U.S.A.). The hydroxylamine of Tempol (Tempol-H) was obtained from Molecular Probes (Eugene, OR, U.S.A.).

Cell culture

Cells—Parental wild-type Chinese hamster ovary (CHO) cells (CHO K1); mutant CHO cells (CHO 16B), which are deficient in G6PD activity (37) (kindly provided by Dr. T. Stamato); and human skin fibroblast 1522 cells—were grown in F12 medium, supplemented with 10% (or 15% for human skin fibroblast 1522 cells) fetal calf serum (FCS), penicillin, and streptomycin. Human breast cancer (MCF7) cells, adriamycin-resistant human breast cancer (MCF7-ADR) cells, and mouse radiation-induced fibrosarcoma (RIF) cells were grown in RPMI-1640, supplemented with 10% FCS, penicillin, and streptomycin. Stock cultures of exponentially growing cells were trypsinized, rinsed, plated into 100-mm-diameter dishes, and incubated overnight prior to experimental protocols. Tempol or Tempol-H (final concentration, 100 m*M*) was added to the cells in full media and incubated as a function of time (in min). For some studies cells were preincubated with DF (final concentration, 500 μ*M*; 2 h), 6-AN (final concentration, 50 μ*M*; 2 h), or BSO (final concentration, 5 m*M*; 20 h) prior to treatment with Tempol (drugs remained on the cells during the Tempol treatment). Following treatment, cells were trypsinized, rinsed, counted, and plated for macroscopic colony formation. After appropriate incubation periods, colonies were fixed, stained, and counted. The plating efficiency ranged from 60% to 80%. For EPR studies cells were plated as described above, and following overnight incubation were incubated for 30 min (unless stated otherwise) with one or more of the following drugs (final concentrations): 10 m*M* NaCN and/or 5 m*M* BSO (for 18 h). Following incubation with the drugs, cells were rinsed, trypsinized, rinsed, counted, resuspended to 10^8/ml in the presence of 10 μ*M* Tempol, and taken for EPR measurements.

Measurement of GSH

Total GSH content was determined using a 5,5′-dithiobis(2-nitrobenzoic acid) GSH reductase recycling method (41). Protein determinations for GSH measurements and for EPR reduction rates (see below) were done by the method of Bradford (4).

Oxygen consumption assay

A YSI Clark electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) and YSI amplifier were used to follow cellular oxygen consumption. For each experiment a single-cell suspension of 8×10^6 cells/ml (total volume, 2.5 ml) of either CHO K1 or CHO 16B cells in full medium with HEPES buffer was maintained at 37°C and followed for approximately 20 min. After the cell suspension had equilibrated for 5 min at 37°C, Tempol was added to the cell suspension to a final concentration of 10 m*M*. Cyanide-resistant respiration was evaluated by adding NaCN (final concentration, 10 m*M*) during the study. The rates of oxygen consumption of control cells were compared with those of treated cells, and the respiration rate is expressed as a percent of control.

Preparation of RIF cell suspensions from intact tumor

Female C3H mice, 6 weeks of age, were supplied by the Frederick Cancer Research and Development Center Animal Production Area (Frederick, MD, U.S.A.). The animals were housed five mice per cage in climate-controlled, circadian rhythm-adjusted rooms, and allowed food and water *ad libitum*. The animals were 2–4 months of age at the time of tumor implantation. The tumor line was RIF, which was a gift from Dr. Theodore L. Phillips. On the day of implantation, stock tumors were resected, minced, and suspended in digest media (2% collagenase/0.2% DNase in Dulbecco's phosphate-buffered saline [DPBS] with Ca^{2+} and Mg^{2+}). A single-cell suspension was collected, and the enzymes were neutralized with 5% FCS. The red blood cells were lysed with ACK

lysing buffer (ammonium chloride, 155 m*M*; potassium bicarbonate, 10 m*M*; EDTA, 0.12 m*M*). The cells were then washed, centrifuged, and resuspended (~10^7 cells/ml) in DPBS without Ca^{2+} and Mg^{2+}. One million cells (100 µl) were injected subcutaneously into the right lower thigh of each animal. All animal studies were carried out under the aegis of a protocol approved by the National Cancer Institute Animal Care and Use Committee and were in compliance with the *Guide for the Care and Use of Laboratory Animals* (32).

On the day of the experiment, tumors (~1 cm in diameter) were removed, minced, and suspended in digest media (2% collagenase/0.2% DNase in DPBS with Ca^{2+} and Mg^{2+}). A single-cell suspension was collected, and the enzymes were neutralized with 5% FCS. Red blood cells were lysed with ACK lysing buffer. The cells were washed, centrifuged, and resuspended in RPMI (+10% FCS) at a final concentration of 20×10^6/ml. The cells were aliquoted into 1-ml samples in Eppendorf micro-centrifuge tubes and incubated for 30 min at 4°C with 10 µg of rat anti-mouse CD 45 antibody (PharMingen, San Diego, CA, U.S.A.). Avidin-labeled magnetic beads were simultaneously incubated for 30 min at 4°C with 10 µg of biotin-labeled goat anti-rat antibody (Pierce Biotechnology, Inc., Rockford, IL, U.S.A.). The tube containing the beads was placed in a magnetic rack, the supernatant was aspirated, and the cells that had been incubated with CD 45 were added to the beads and incubated at 4°C for 30 min. The tube containing the suspension was once again placed in the magnetic rack, and the supernatant, which contained CD 45-negative cells, was collected and counted. The remaining cells, which were coated with magnetic beads, were incubated with 2 m*M* biotin (in DPBS) for approximately 1 min and put back in the magnetic rack. CD 45-positive cells, which had been released from the beads, were collected from the supernatant. This procedure was repeated four times. CD 45-negative tumor cells were pelleted and resuspended in RPMI at a final concentration of 2×10^7/ml.

EPR measurements

EPR measurements at 37°C were performed for different cell densities incubated with 10 µ*M* Tempol. Cells at a density of 5×10^7 cells/ml (low cell density) and at 10^8 cells/ml (high cell density) were incubated with Tempol prior to EPR measurements. The reaction mixture was transferred to a gas-permeable Teflon capillary (Zeus Industries, Orangeburg, SC, U.S.A.) having an inner diameter of 0.81 mm, a wall thickness of 0.38 mm, and a length of 15 cm. Each capillary was folded twice, inserted into a narrow quartz tube that was open on both ends (2.5 mm inner diameter), and placed within the EPR cavity. The EPR cavity was continuously flushed at 37°C with N_2 alone or N_2/O_2 at various gas mixtures containing 5% CO_2, which maintain the pH in physiological range. EPR spectra were recorded using a Varian E-9 X-band spectrometer with the following instrument settings: modulation amplitude, 1 G; time constant, 0.25 s; modulation frequency, 100 kHz; microwave power, 10 mW; the magnetic field was held constant at 3,352 G, and the decay of the nitroxide EPR signal was monitored. The initial (6–12 min) rate of decay of signal intensity was measured. Since cells, especially from different species, vary in size, the rates of Tempol reduction (µ*M*/min) were standardized per total protein content in the cells, rather the number of cells as described above.

RESULTS

EPR spectroscopy was used to study the change in concentration of Tempol in cells as a function of time of incubation at two different cell densities under aerobic conditions at 37°C. The rate of reduction was higher at 37°C as compared with the rate at room temperature (data not shown). A representative profile of EPR signal of Tempol for two cell densities as a function of time is shown in Fig. 1. Tempol reduction rates were determined from these profiles and are normalized for protein content, and the results are shown in the inset of Fig. 1. These experiments suggest that Tempol is reduced as a function of time and that the reduction rates are dependent on cell density. The reduction was faster at higher cell densities compared with lower cell densities. From these data Tempol reduction rates were determined. To study the dependence of nitroxide reduction on cell type and oxygen content, EPR experiments were carried out in normal and malignant cells incubated with 10 µ*M* Tempol under aerobic or hypoxic conditions. Figure 2 and Table 1 show the reduction rates of Tempol from the various cell lines tested. The results show that the nitroxide reduction rates are dependent on cell type. For a given cell type, the reduction rates were generally higher under hypoxic conditions. Earlier studies have suggested that mitochondrial pathways are in part responsible for reduction of

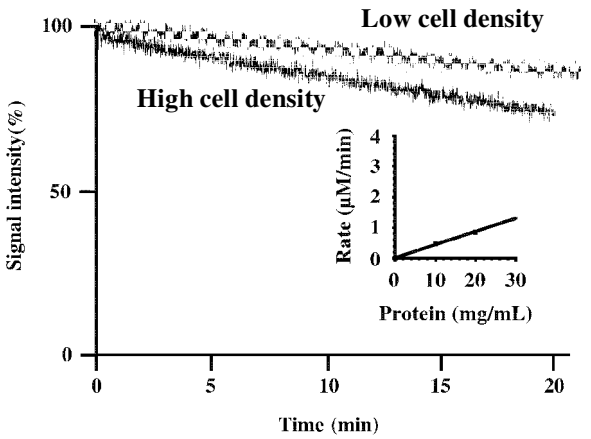

FIG. 1. Decay of EPR signal of Tempol. CHO cells were incubated with 10 µ*M* Tempol. The reaction mixture was transferred to a gas-permeable Teflon capillary and placed within the EPR cavity. The EPR cavity was continuously flushed at 37°C with air containing 5% CO_2, and the decaying signal was monitored. EPR signals were recorded using a Varian E-9 X-band spectrometer with the following instrument settings: modulation amplitude, 1 G; time constant, 0.25 s; modulation frequency, 100 kHz; microwave power, 10 mW; the magnetic field was held constant at 3,352 G. **Inset:** Reduction rates calculated from the initial slopes of the decaying signal and standardized for protein content. Low cell density was 5×10^7 cells/ml, and high cell density was 10^8 cells/ml.

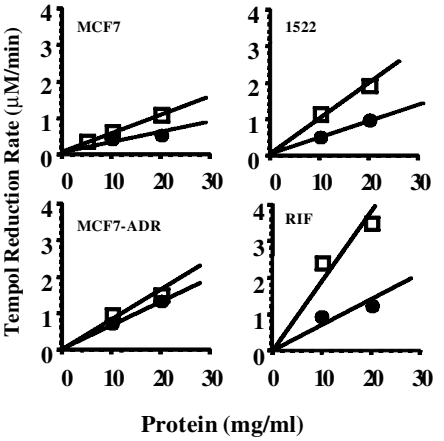

FIG. 2. Oxygen-dependent rate of Tempol reduction: anoxia (squares) and under air (circles). In representative experiments, human skin fibroblasts (1522), human breast cancer cells (MCF7), and MCF7-ADR cells were trypsinized, counted, and taken for EPR measurements. The EPR cavity was continuously flushed with N_2 at 37°C containing 5% CO_2 or air. The initial slope (6–12 min) of the decaying signal was measured and used for calculation of the reduction rate.

nitroxides, at the level of ubiquinone in the electron transport chain (38). However, for the human skin fibroblasts incubated with cyanide (10 m*M*) to block the electron transport through the respiratory chain, no changes in the reduction rates were noted in either aerobic or hypoxic conditions (Fig. 3). Experiments using cyanide and hypoxia suggest a pathway other than mitochondrial-mediated as responsible for the reduction of Tempol. Three of the four cell lines exhibited significantly higher Tempol reduction rates under hypoxic as opposed to aerobic conditions.

Earlier studies on Tempol reduction in erythrocytes found that the hexose monophosphate (HMP) shunt was an important determinant on the nitroxide reduction (5). In normal erythrocytes, nitroxide reduction rates exhibited first-order kinetics, whereas in G6PD-deficient cells, the reduction rates were slower and exhibited second-order kinetics. Correlations of nitroxide reduction rates with GSH levels as well as pentose phosphate shunt activity were noted, consistent with the dependence of reduced thiol levels on the HMP activity.

TABLE 1. RATES OF REDUCTION OF TEMPOL BY VARIOUS CELL LINES

Cell line or primary culture	N_2	*Air*
1522	100 ± 5.6 (2)	59 ± 2.3 (7)*
MCF7	58 ± 9.2 (6)	29 ± 3.2 (9)*
MCF7-ADR	80 ± 5 (4)	68 ± 8 (10)
RIF	179 ± 39.3 (3)	70 ± 6.8 (4)*

Mean rates (n*M* × min^{-1} × mg of $protein^{-1}$ × ml) are presented with SEM. Number of experiments is provided in parentheses.

*$p < 0.05$ as compared with the rate in anoxia.

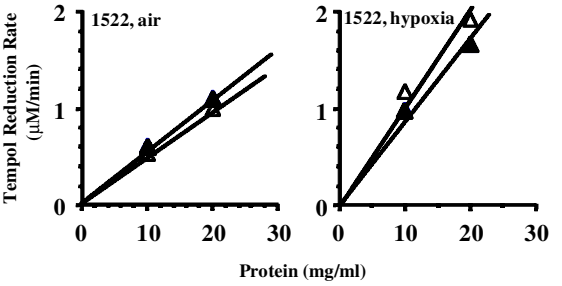

FIG. 3. Oxygen-dependent rate of reduction of Tempol in the presence of NaCN: control (solid triangles) and treated for 30 min with 10 m*M* NaCN (open triangles). Typical experiments where human skin fibroblasts (1522) were incubated with 10 m*M* NaCN for 30 min and then trypsinized, counted, and taken for EPR measurements. The EPR cavity was continuously flushed at 37°C with various N_2/O_2 gas mixtures and 5% CO_2. The initial slope (6–12 min) of the decaying signal was measured and used for calculation of the reduction rate.

In order to obtain additional insight into the metabolism of nitroxides in non-erythrocytic cells, a pair of epithelial-derived mammalian cell lines differing in the G6PD activity were tested. Wild-type CHO cells (CHO K1) and CHO cells that are null mutants for G6PD (CHO 16B) were exposed to Tempol (10 μ*M*), and the nitroxide level was monitored by EPR under aerobic conditions. Figure 4 and Table 2 show the reduction rates of Tempol in CHO K1 and CHO 16B cells. The wild-type CHO K1 cells reduced Tempol faster than the CHO 16B cells, the mutant for G6PD. The reduction product of Tempol was found to be the corresponding one-electron reduced hydroxylamine, Tempol-H (data not shown). Depletion of thiols by treatment with BSO had no effect on nitroxide reduction in CHO K1 cells. However, in the G6PD-deficient CHO 16B cells, the rate in reduction was increased approximately 40% (data not shown). Oxygen consumption studies

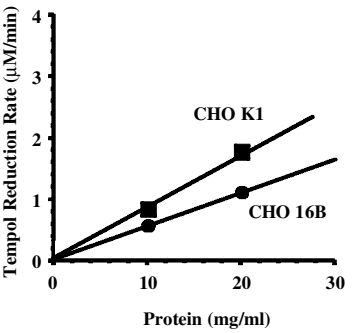

FIG. 4. The rate of reduction of Tempol by CHO cell lines: CHO K1 (solid squares) and CHO B16 mutant (solid circles). In representative experiments, CHO K1 and mutant CHO B16 cells were trypsinized, counted, and taken for EPR measurements. EPR measurements were performed in mixtures containing 0.5, 1, and 2 × 10^6 cells/ml and 10 μ*M* Tempol. The EPR cavity was continuously flushed at 37°C with air containing 5% CO_2. The initial slope (6–12 min) of the decaying signal was measured and used for calculation of the reduction rate.

TABLE 2. RATES OF REDUCTION OF TEMPOL BY CHO CELL LINES UNDER AIR

Cell line	*Air*
CHO-K1 (wild type)	57 ± 4.4 (25)
CHO-B16	39 ± 2.8 (23)*

Mean rates (nM × min^{-1} × mg of protein^{-1} × ml) are presented with SEM. Number of experiments is provided in parentheses.

*$p < 0.05$ as compared with the rate of wild-type CHO.

in these cells showed that in the presence of Tempol, cyanide-independent respiration in CHO K1 cells proceeded, but there was no cyanide-resistant respiration in CHO 16B cells (data not shown).

To examine the cytotoxic effects of exposure to high concentrations of Tempol and Tempol-H in CHO K1 and CHO 16B, clonogenic cell survival experiments were carried in these cells after exposure to Tempol (100 mM) and Tempol-H (100 mM) (Fig. 5). Exposure to Tempol at a concentration of 100 mM caused significant cytotoxicity in the CHO K1 cell line, whereas no cytotoxic effects were observed in the CHO 16B cell line under identical conditions (Fig. 5A). However, the one-electron reduced Tempol-H did not elicit cytotoxic effects in both CHO K1 and CHO 16B cell lines (Fig. 5B). Depletion of intracellular thiols by pretreatment with BSO sensitized the CHO K1 cells to Tempol exposure, whereas CHO 16B cells were not affected by thiol depletion. The influence of thiol level on Tempol reduction rates in CHO K1 and the mutant CHO 16B was examined by EPR on cells pretreated with BSO to decrease the intracellular thiol levels (Fig. 5C). Again the CHO K1 cells reduced the nitroxides faster than the G6PD mutant CHO 16B cells. However, the nitroxide reduction rates were enhanced in BSO-treated CHO K1 cells. It is known that BSO treatment creates a more oxidizing environment. We next pretreated both cell lines with 6-AN, an inhibitor of 6-phosphogluconate dehydrogenase (1), and assessed survival of cells to Tempol (Fig. 5D). 6-AN pretreatment had no effect on the survival of CHO 16B cells against Tempol, but clearly sensitized CHO K1 cells to Tempol. Lastly, to determine the potential role for metals in Tempol-mediated cytotoxicity, cells were pretreated with DF 2 h prior to exposure to Tempol (Fig. 6). No significant changes were observed in both CHO K1 and CHO 16B cells exposed to Tempol in the presence of DF, which suggests that redox-active transition metal complexes do not play a significant role in nitroxide-mediated cytotoxicity.

DISCUSSION

Nitroxides are being used in a variety of studies as antioxidants (ischemia/reperfusion injury, inflammation, shock, *etc.*) (18, 20, 33, 40), radiation protectors (12, 17, 29), antihypertensive agents (36, 43), animal weight and tumorigenesis control (31), chemoprevention (34), anti-tumor agents (15), and functional probes in EPR imaging (27, 45). The diversity of applications for nitroxides speaks perhaps to the importance of free radicals in treatment, diagnosis, and disease processes. The focus of the present study was to determine factors that influence the bioreduction of the nitroxide, Tempol. This is important for several reasons. First, there are a number of preclinical studies supporting the premise that Tempol is a selective radiation protector for normal tissues as

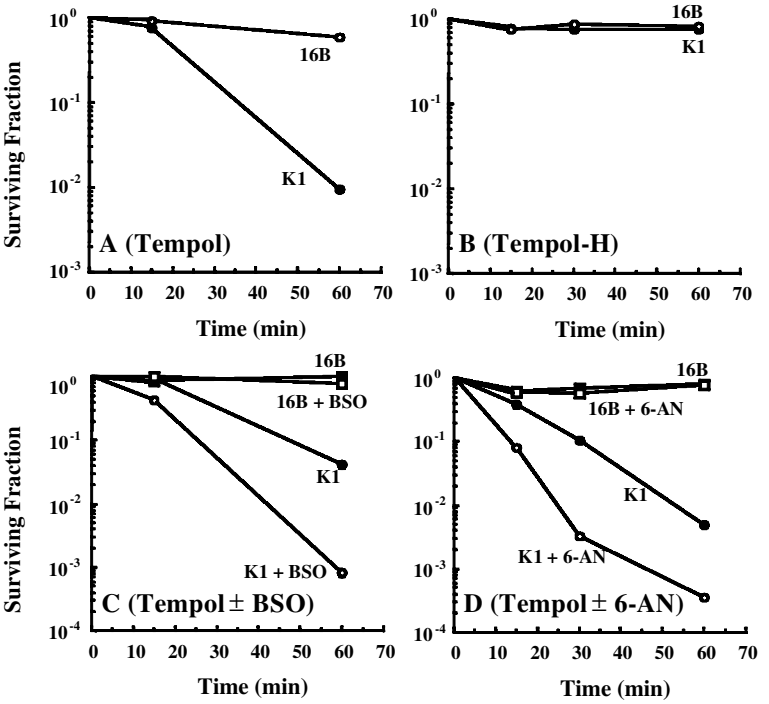

FIG. 5. Clonogenic cell survival of CHO K1 and CHO 16B cells treated with (A) 100 mM Tempol, (B) 100 mM Tempol-H, (C) 100 mM Tempol with BSO pretreatment, and (D) 100 mM Tempol with 6-AN pretreatment as a function of time.

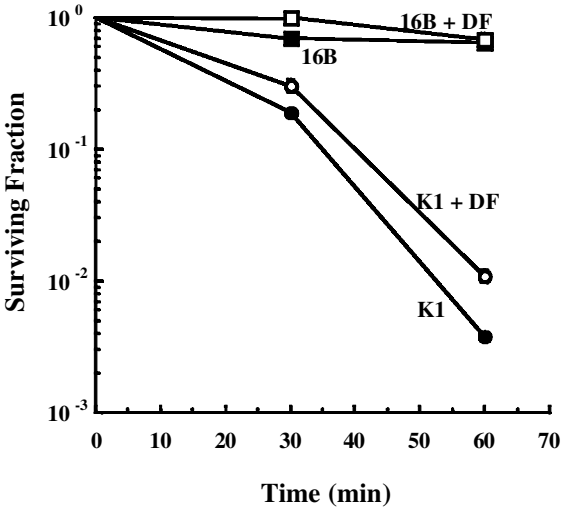

FIG. 6. Clonogenic cell survival of CHO K1 and CHO 16B cells treated with 100 m*M* Tempol with DF pretreatment as a function of time.

opposed to tumor (17, 19). Hence it is important to understand those factors that facilitate Tempol bioreduction given that only the oxidized form of Tempol is radioprotective (29, 44). Second, nitroxides are being developed and used as probes for EPRI to non-invasively determine tissue redox characteristics (27, 45). The basis of this type of novel imaging depends on the reduction/conversion of Tempol to the hydroxylamine, Tempol-H. Lastly, Tempol at high concentrations and prolonged exposures has been shown to exert significant anti-tumor effects (14, 15). A better understanding of Tempol bioreduction might be useful in each condition listed above in selecting among hundreds of nitroxide analogues the best molecule for specific applications.

The *in vivo*/*in vitro* rate of reduction of nitroxides depends on various factors such as the structure of the nitroxides, pharmacokinetics, temperature, oxygen concentration, and cell type. In general, six-membered ring nitroxides are reduced faster than the corresponding five-membered ring nitroxides. In the present study, Tempol was selected primarily because of its hydrophilic, uncharged nature, which ensures permeation and distribution of the nitroxide in the cell. Further, the cells were maintained in a constant atmosphere by placing the cell suspension in a gas-permeable Teflon tube in the EPR cavity, which was continuously flushed with various gas mixtures. Thus, oxygen concentrations were controlled at all times, and undesired changes in signal intensity due to modulation of relaxation time and line-broadening were avoided (25). Since the rate of reduction was found to be temperature dependent (data not shown), temperature was kept constant at 37°C.

The cell lines selected were established human tumor cell lines, normal human skin fibroblasts, and primary cultures of RIF mouse tumors. The O_2 dependency of the rate of reduction was apparent for MCF7, 1522, and RIF but not for MCF7-ADR (Fig. 2). This agrees with previous reports of mixed thymus and bone marrow mouse cells, which reduced Tempol irrespective of O_2 levels (39), and of hepatocytes, which reduced Tempol more efficiently in anoxia (9, 21). This difference in the effect of O_2 on Tempol reduction by various cell types could be explained by the differences in levels of mitochondrial and microsomal enzymes, since it is known that reduction occurs at these sites (8, 22, 24). The non-enzymatic reduction of nitroxide is also dependent on oxygen concentration since the oxidation of hydroxylamine is partially inhibited by superoxide dismutase (2). Furthermore, superoxide oxidizes the nitroxide to oxoammonium cation, which in turn can accept two electrons from reducing agents such as NADPH to yield the hydroxylamine (26). Thus, both reduction and oxidation can occur, and these processes are pH-dependent since as the pH decreases nitroxides remove superoxide more efficiently than hydroxylamines produce oxidation (46). Further, nitroxides can be reduced directly by ascorbate but not by NADPH, NADH, or GSH. The reduction of Tempol has recently been studied *in vivo* and in cell homogenates and was found to occur in mitochondria, cytosol, and microsomes (42). These observations are consistent with the small size, neutral charge, and hydrophilic nature of Tempol.

Oxygen levels were shown to have a significant influence on Tempol reduction in all the cell lines examined (with exception to MCF7-ADR cells), with the general observation that Tempol reduction was faster under hypoxic conditions. Tempol reduction under both aerobic and hypoxic conditions was more efficient in the freshly isolated RIF-1 tumor cells compared with tumor cell lines maintained in culture for extended periods. Tempol reduction rates in these freshly isolated tumor cells may more accurately reflect what might be expected *in vivo* as compared with the more established tumor lines studied (MCF7). Inhibition of the respiratory chain enzymes using cyanide did not influence the reduction rates. While earlier studies demonstrated a dependence of intracellular thiols on nitroxide reduction in *in vivo* imaging studies (27, 45), in the present study with cells in suspension no such behavior was observed. In these studies, thiol depletion in tumor showed slower nitroxide reduction rates compared with control. Depletion of thiols *in vivo* may exert secondary effects that influence nitroxide reduction. We are currently in the process of studying this effect in more detail.

A general determinant of the efficiency of cellular reduction of nitroxide is the cellular redox status, which in turn is dependent on oxygen, thiol status, HMP activity, *etc.* (35). The availability of cell lines with well-characterized differences in HMP activity resulting from G6PD deficiency helped in identifying a general pathway responsible for the reduction of nitroxide as well as in nitroxide-mediated cytotoxicity. Tempol cytotoxicity studies with CHO K1 and CHO 16B cell lines demonstrated significant cytotoxicity to wild-type CHO K1 cells, but not to the mutant CHO 16B cells. However, the hydroxylamine Tempol-H had no cytotoxic effects on either cell line. Intracellular thiol depletion by pretreatment with BSO was found to sensitize wild-type CHO K1 cells and not the mutant CHO 16B cells to Tempol challenge. Similar selective sensitization of Tempol cytotoxicity was observed in CHO K1 cells in the presence of 6-AN, which inhibits 6-phosphogluconate dehydrogenase (1). Studies with preincubation of CHO K1 and CHO 16B cells with DF exhibited slight protection of CHO K1 and no effect on CHO 16B cells, indicating a minor role for redox-active tran-

sition metal complexes in Tempol-induced cytotoxicity. Since Tempol-mediated cytotoxicity could be modulated in CHO K1 cells by GSH depletion, by inhibition of 6-phosphogluconate dehydrogenase, and slightly by DF, it could be concluded that the conversion of Tempol to Tempol-H involves an oxidative component. CHO 16B cells, which convert Tempol to Tempol-H at a slower rate, were less susceptible to Tempol-mediated cytotoxicity. Based on these results, the following general conclusions can be drawn: (a) Tempol-mediated cytotoxicity is associated with the conversion of the nitroxide to hydroxylamine; (b) the pathways responsible for the conversion of nitroxide to hydroxylamine are in part dependent on the pentose cycle; (c) the hydroxylamine, which is the product of nitroxide reduction, does not exert cytotoxic effects; and (d) nitroxides, in the process of being converted to the hydroxylamine, consume/deplete essential factors or possibly activate/promote toxic intermediates, presumably via the oxoammonium intermediate (26).

In the pentose phosphate cycle, both NADPH (oxidative) and ribose-5-phosphate (non-oxidative), which is necessary for DNA and RNA synthesis, are generated. While compromised HMP activity has been shown to confer enhanced sensitivity to various forms of oxidative stress (3), our results show that cells with deficiency in the HMP activity were resistant to exposure to nitroxide radicals at high concentration. A possible explanation may be based on observations made in a recent study, where inhibition of pathways in the oxidative pathway of the pentose cycle was lethal in tumor cells (11). Wild-type CHO cells have more non-mitochondrial oxygen utilization and can potentially produce more reducing equivalents to produce reactive oxygen species. These oxidants, such as superoxide, can react with Tempol and produce the oxioammonium cation, which is oxidative and potentially cytotoxic. Since CHO 16B cells have less non-mitochondrial respiration potential, their overall capability to produce reactive oxygen species is less and is expected to generate less oxoammonium, and thereby less cytotoxicity results from Tempol exposure at high concentrations. It is also possible that Tempol, at high concentrations, may competitively inhibit these enzymes to inflict damage in wild-type CHO cells (CHO K1), which have a functioning HMP, whereas the mutant cells without the HMP (CHO 16B) lack these enzymes. This hypothesis is further supported by experimental evidence that nitroxides are reduced more efficiently in wild-type cells than the G6PD mutant cells. Further studies may help in a better understanding of the roles the HMP pathways play in the cytotoxic effects of nitroxides as well as the cellular reduction of nitroxides.

ABBREVIATIONS

6-AN, 6-aminonicotinamide; BSO, L-buthionine-*S*,*R*-sulfoximine; CHO, Chinese hamster ovary; DF, desferrioxamine; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; EPR, electron paramagnetic resonance; G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; HMP, hexose monophosphate; MCF7-ADR cells, adriamycin-resistant human breast cancer cells; RIF, radiation-induced fibrosarcoma; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl; Tempol-H, hydroxylamine of Tempol.

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Address reprint requests to:
James B. Mitchell, Ph.D.
Radiation Biology Branch
National Cancer Institute
Building 10, Room B3-B69
Bethesda, MD 20892

E-mail: jbm@helix.nih.gov

Received for publication June 26, 2003; accepted February 19, 2004.

This article has been cited by:

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