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# Ascorbic acid decreases oxidant stress in endothelial cells caused by the nitroxide tempol

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**Abstract**
Stable nitroxide radicals have been considered as therapeutic antioxidants because they can scavenge more toxic radicals in biologic systems. However, as radicals they also have the potential to increase oxidant stress in cells and tissues. We studied the extent to which this occurs in cultured EA.hy926 endothelial cells exposed to the nitroxide Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl). Tempol was rapidly reduced by the cells, as manifest by an increase in the ability of the cells to reduce extracellular ferricyanide and by disappearance of the Tempol EPR signal. Cells loaded with ascorbic acid, which directly reacts with Tempol, showed increased rates of Tempol-dependent ferricyanide reduction, and a more rapid loss of the Tempol EPR signal than cells not containing ascorbate. In this process, intracellular ascorbate was oxidized, and was depleted at lower Tempol concentrations than was GSH, another important intracellular low molecular weight antioxidant. Further evidence that Tempol concentrations of 100–1000 μM induced an oxidant stress was that it caused an increase in the oxidation of dihydrofluorescein in cells and inhibited ascorbate transport at concentrations as low as 50–100 μM. The presence of intracellular ascorbate both prevented dihydrofluorescein oxidation and spared GSH from oxidation by Tempol. Such sparing was not observed when GSH was depleted by other mechanisms, indicating that it was likely due to protection against oxidant stress. These results show that whereas Tempol may scavenge other more toxic radicals, care must be taken to ensure that it does not itself induce an oxidant stress, especially with regard to depletion of ascorbic acid.

**Keywords:** *Ascorbic acid, GSH, tempol, dehydroascorbic acid, ferricyanide, oxidant stress*

## Introduction

Stable nitroxide compounds have long been of interest as potential antioxidants in experiments involving both cultured cells [1–3] and animals [4,5]. The proposed mechanisms for these beneficial effects include: function as superoxide dismutase mimics [6–8], direct reduction of potentially toxic quinone radicals [6], and oxidation of redox-active trace metal ions [2,6]. Protective effects of nitroxides have been most evident in cells under acute oxidant stress. For example, nitroxides improved cultured cell survival following treatment with superoxide or $H_2O_2$ [9] and irradiation [1]. On the other hand, when cancer cell lines were treated over long periods in culture without additional oxidant stress, nitroxides increased cell death due to apoptosis [10,11], an effect probably mediated through impairment of mitochondrial function [12].

Acute treatment of cells with moderate concentrations of cell-penetrant nitroxides, such as Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), does not appear to cause significant toxicity [13]. In human erythrocytes, treatment with Tempol results in a first-order decay in the electron paramagnetic resonance (EPR) signal of the free radical [14,15], which is considered to reflect reduction to the non-toxic hydroxylamine [15,16]. This reduction was likely due to cellular thiols [17], since it correlated with decreases in cellular GSH concentrations [15], which

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in turn were dependent on the pentose phosphate cycle activity [13,15]. In addition to GSH, Tempol is also readily reduced by ascorbic acid present in cells. In fact, the reaction with ascorbate in erythrocytes can be carried out under conditions in which ascorbate is depleted, but there is little change in intracellular GSH concentrations [18]. This has prompted the use of sequential acute treatments with Tempol as a means to acutely deplete endogenous ascorbate in human erythrocytes [19,20]. Ascorbate is also consumed before cellular GSH in response to several oxidants including ferricyanide [21] and free radical initiators [21] in erythrocytes, and menadione [22] and nitrite [23] in endothelial cells.

If ascorbate is more sensitive than GSH to oxidant stress in cells, the question arises as to whether ascorbate in turn can protect the cells against oxidant stress, and especially whether it can spare cellular GSH. Such protection has been observed *in vivo* in newborn rats [24] and in aortic endothelial cells in culture without added oxidant stress [25], but has not been a consistent finding in cultured endothelial cells under significant oxidant stress. For example, although GSH was preserved by ascorbate in response to *tert*-butylhydroperoxide [26] and by the intracellular redox cycling agent menadione [22], it afforded little protection of human umbilical vein endothelial cells against lipid peroxidation initiated by exogenous $H_2O_2$ [27]. It remains to be seen whether ascorbate will protect cells against nitroxide-induced oxidant stress and loss of GSH.

Since nitroxides considered as therapeutic agents would reach target tissues through the bloodstream, they would necessarily interact with endothelial cells. Further, dysfunction of endothelial cells is one of the earliest features of atherosclerotic and hypertensive vascular disease [28]. Therefore, we chose to study the acute antioxidant responses of EA.hy926 endothelial cells to the nitroxide Tempol, especially with regard to interactions with ascorbic acid. EA.hy926 cells are an immortalized hybridoma cell line derived originally from human umbilical vein endothelial cells that retain many of the features expected of endothelial cells, including expression of factor VIII antigen [29], formation of capillary-like tubes in culture [30], oxidative modification of human LDL [31], and calcium-dependent stimulation of eNOS [31]. In the present studies, we found that low millimolar concentrations of Tempol induced an oxidant stress in the cells, and that ascorbate was effective in preventing this stress and in preserving cellular GSH.

## Materials and methods

### *Materials*

Dihydrofluorescein diacetate was from Molecular Probes, Inc. (Eugene, OR, USA) and was initially dissolved in dimethylsulfoxide, and the final dimethylsulfoxide concentration in the incubation was 0.2%. Perkin–Elmer Life and Analytical Sciences, Inc. (Boston, MA) supplied the L-[1-$^{14}$C] ascorbic acid, which was dissolved in deionized water containing 0.1 mM acetic acid and stored in multiple aliquots at −20°C. Media for cell culture was prepared by the Cell Culture Core of the Vanderbilt Diabetes Research and Training Center. Dehydroascorbic acid (DHA), Tempol and other analytical reagents were from Sigma/Aldrich Chemical Co. (St. Louis, MO).

### *Cell culture and preparation for assays*

EA.hy926 cells were kindly provided by Dr Cora Edgell (University of North Carolina, Chapel Hill, NC). Cells were cultured in Dulbecco's minimal essential medium that contained 20 mM D-glucose, 10% (v/v) fetal bovine serum, 5 mM hypoxanthine, 20 μM aminopterin and 0.8 mM thymidine (HAT media supplement, Sigma/Aldrich Chemical Co.). The cells were cultured to confluence for 18–24 h in 6- or 12-well plates and rinsed 3 times in Krebs–Ringer Hepes (KRH) buffer at 37°C before use in an experiment. KRH consisted of 20 mM Hepes, 128 mM NaCl, 5.2 mM KCl, 1 mM $NaH_2PO_4$, 1.4 mM $MgSO_4$ and 1.4 mM $CaCl_2$, pH 7.4.

### *Ferricyanide reduction*

EA.hy926 cells cultured to confluence on 12-well plates were rinsed three times in KRH to remove culture medium and incubated with or without 0.2 mM DHA as noted for 15 min at 37°C in KRH containing 5 mM D-glucose. Tempol was added to the indicated concentration, followed by 1 mM ferricyanide and the incubation continued at 37°C. After 30 min, duplicate aliquots of the supernatant were sampled for assay of ferrocyanide by the method of Avron and Shavit [32] using *ortho*-phenanthroline as the detecting agent. Correction was made in each assay for the absorbance generated by cells alone.

### *EPR studies*

Confluent EA.hy926 cells were cultured in 10 cm culture dishes for 18 h in the presence or absence of 0.2 mM ascorbate. The cells were rinsed three times in medium without serum, and then detached from the plate by treatment with 0.15% trypsin in EDTA. After removal from the plate, the cells were centrifuged, the trypsin solution was removed, and the cells were suspended to a 50% packed cell volume in culture medium containing serum. Just before use, the cells were treated with 10 μM Tempol and placed into 50 μl glass capillary tubes for EPR spectroscopy at 37°C, as described previously for erythrocytes [33],

except that the spectrometer magnetic field was fixed on the position of the Tempol low-field resonance line and the amplitude was followed with time.

### *Measurement of intracellular ascorbic acid and GSH*

After incubations as indicated in 6-well plates, the medium was aspirated, and the cells were gently rinsed three times with 2 ml of ice-cold KRH. After removal of the last rinse the monolayer was treated with 0.1 ml of 25% metaphosphoric acid (w/v), followed by 0.35 ml of a buffer containing 0.1 M $Na_2HPO_4$ and 0.05 mM EDTA, pH 8.0. The cells were scraped from the plate and cell debris was removed by centrifugation at 3°C. Aliquots of the supernatant were taken for assay of ascorbic acid and GSH. Ascorbic acid was measured by high performance liquid chromatography with electrochemical detection as previously described [19], except that tetrapentylammonium bromide was used as the ion pair reagent. GSH was assayed by the method of Hissin and Hilf [34]. Intracellular concentrations of ascorbate and GSH were calculated based on the intracellular water space present in EA.hy926 cells of 3.6 ± 1.2 μl/mg protein, measured as recently described [35].

### *Assay of ascorbate transport*

Following incubations as indicated, confluent EA.hy926 cells in 12-well plates were rinsed three times in KRH and incubated at 23°C in KRH that contained 5 mM D-glucose and 0.05 μCi of 6–9 μM L-[1-$^{14}$C]ascorbic acid. After 30 min, the cells were rinsed twice in 2 ml of ice-cold KRH and the cell monolayer was solubilized with the addition of 1 ml of 0.05N NaOH. The cells were scraped from the plate and the lysate was added to 5 ml of Ecolume liquid scintillation fluid (ICN, Costa Mesa, CA) with mixing. After 60 min, to allow decay of chemiluminescence, the radioactivity of the samples was measured in a Packard CA-2200 liquid scintillation counter.

### *Measurement of intracellular oxidant stress as oxidation of dihydrofluorescein*

Reactive oxygen species generated within cells were measured as oxidation of intracellular dihydrofluorescein, as previously described in a fluorescence microtiter plate reader [22]. Briefly, following treatments as indicated, cells in 96-well plates were rinsed twice in KRH to remove medium, and incubated in 0.2 ml of KRH that contained 5 mM glucose and 20 μM dihydrofluorescein diacetate. Cells were then incubated for 30 min at 37°C in the dark, rinsed three times with 0.2 ml of KRH, followed by treatment with the indicated concentration of Tempol. Fluorescence was measured every 4 min in a microtiter plate reader (Fluostar Galaxy, BMG Labtechnologies, Cork, Ireland) during incubation at 37°C for 36 min. The excitation wavelength was 480 nm and the emission wavelength was 520 nm. The slope of the linear portion of the time course (0–750 s) was calculated and divided by the slope of a sample that did not contain Tempol.

### *Statistics*

Results are shown as mean vs. standard error. Statistical comparisons were made using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). Differences between treatment groups were assessed by two-way analysis of variance with *post-hoc* testing using Dunnett's test.

## Results

The ability of EA.hy926 cells to reduce Tempol to its hydroxylamine was estimated as an increase in ferricyanide reduction induced by Tempol. As shown in Figure 1, basal rates of ferricyanide reduction were increased by concentrations of 0.5 mM Tempol and higher. Cells that had been loaded with ascorbate by pre-incubation with DHA had 50% greater rates of ferricyanide reduction in the absence of Tempol, and these rates were progressively increased by Tempol, beginning at 0.2 mM Tempol. Further, loading the cells with ascorbate significantly increased the area under the curve above basal in ascorbate-treated cells compared to control cells ($p = 0.04$, by paired $t$-testing, $N = 3$). These results

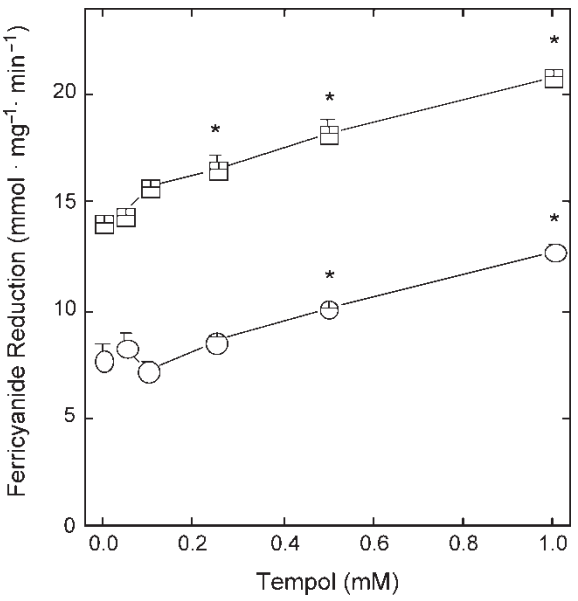


Figure 1. Tempol effects on ferricyanide reduction by EA.hy926 cells. Ferricyanide reduction was measured as described in the "Materials and methods" section in control cells (circles) and in cells that had been loaded with ascorbate by incubation with 0.2 mM DHA for 15 min (squares). Results shown are from three experiments, with an asterisk indicating $p < 0.05$ compared to the sample that did not receive Tempol.

suggest that the cells reduce Tempol to its hydroxylamine, which in turn reduces ferricyanide, either directly or indirectly. Also, ascorbate-loaded cells have a higher rate of Tempol reduction than cells not containing ascorbate. This likely reflects direct reduction of intracellular Tempol by ascorbate, which was measured next.

The ability of EA.hy926 cells to reduce Tempol was measured directly by following the amplitude of the high-field peak in the Tempol EPR signal (see insert to Figure 2). In the absence of cells, medium alone caused a small decrease in the amplitude of the EPR signal generated by 10 μM Tempol. In the presence of cells at about a 50% packed cell volume, however, there was a progressive decrease to zero in the Tempol EPR signal over 20 min (Figure 2). Cells that had been cultured for 18 h with 0.2 mM ascorbate showed a much more rapid decline in the amplitude of the Tempol EPR signal, such that the signal had already decreased significantly before the cells and Tempol solution could be loaded into the capillary tube and placed in the EPR microwave cavity (Figure 2). These results show the high capacity of the cells to reduce Tempol, especially when they contained ascorbate.

An ascorbate-induced increase in Tempol reduction should be accompanied by a decrease in intracellular ascorbate concentrations. To assess this, EA.hy926 cells were loaded with ascorbate to an intracellular concentration of 0.5 ± 0.06 mM by pre-incubation with 0.2 mM DHA, followed by exposure to increasing concentrations of Tempol for 30 min. As shown in Figure 3, there was a 50% decrease in ascorbate concentrations at Tempol concentrations of 0.2 mM and higher. On the other hand, GSH concentrations were decreased only at a Tempol concentration of 2 mM. Despite that fact that Tempol oxidized GSH in roughly equimolar amounts in KRH (results not shown), little GSH was lost in the cells. Together, these results show that ascorbate was more sensitive to oxidation by Tempol than was GSH.

To determine whether Tempol induces an oxidant stress in EA.hy926 cells beyond that related to ascorbate oxidation, the rate of intracellular oxidation of dihydrofluorescein was measured. As shown in Figure 4, incubation of cells with increasing concentrations of Tempol resulted in a progressive increase in the appearance of fluorescein in the cells and incubation medium, although the maximal increase was variable from experiment to experiment, accounting for the large error bars. This increase was almost completely prevented in cells loaded with ascorbate before exposure to Tempol. These results indicate that intracellular ascorbate protects the cells against oxidant stress due to Tempol.

The ascorbate transporter of EA.hy926 cells is sensitive to acute modification by oxidant stress [36], so its activity was used to determine whether Tempol

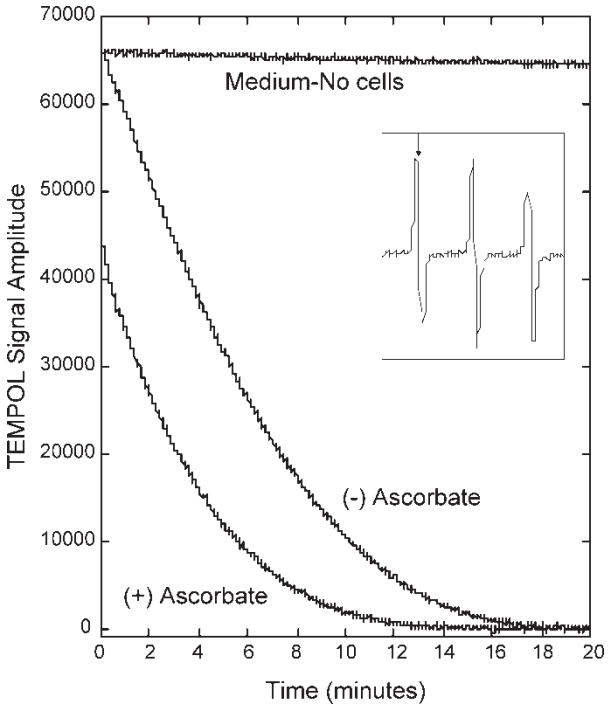


Figure 2. Time-dependent reduction of the Tempol radical by EA.hy926 cells as measured by EPR spectroscopy. The inset shows the EPR spectrum of Tempol with an arrow indicating the low-field line used for measurement of signal amplitude.

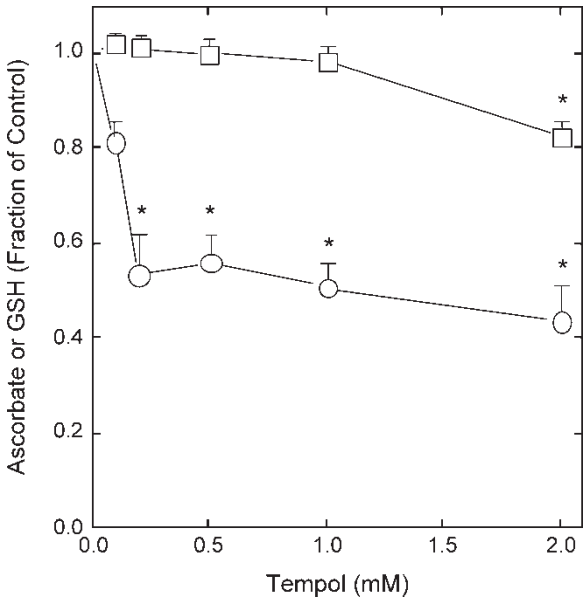


Figure 3. Loss of ascorbate and GSH due to Tempol. EA.hy926 cells were rinsed three times in KRH to remove medium and incubated at 37°C in KRH that contained 5 mM D-glucose and 0.2 mM DHA. After 15 min, Tempol was added to the indicated concentration, and the incubation was continued for an additional 30 min before the cells were rinsed twice in KRH and taken for measurement of intracellular ascorbate (circles) and GSH (squares) concentrations. Results from three or more experiments are expressed as a fraction of the concentration of ascorbate or GSH measured in the absence of Tempol. For ascorbate this was 0.5 ± 0.06 mM, and for GSH it was 1.4 ± 0.07 mM. An asterisk (*) indicates $p < 0.05$ compared to the value without Tempol.

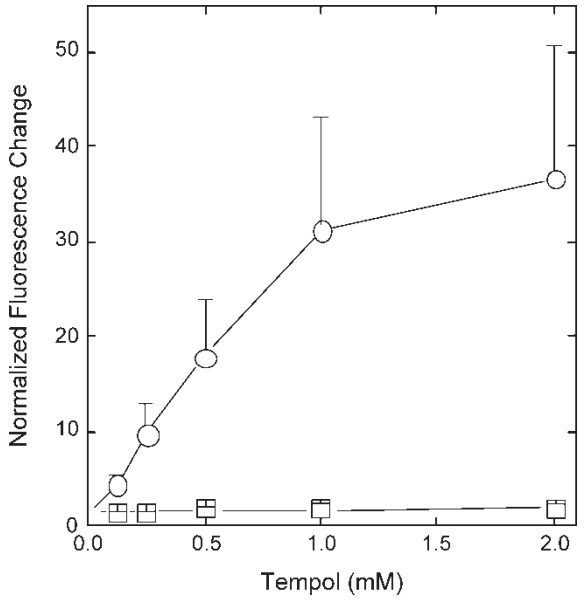


Figure 4. Generation of intracellular oxidant stress by Tempol. EA.hy926 cells were incubated in the absence (circles) or presence (squares) of 0.5 mM DHA during loading of the cells with dihydrofluorescein diacetate, with subsequent treatment with the indicated concentration of Tempol and measurement of fluorescence as described in the "Materials and methods" section. The slope of the linear portion of the time course, normalized to the initial slope at time zero, is plotted as a function of the Tempol concentration. Results are shown as averages of quadruplicate measurements from six experiments.

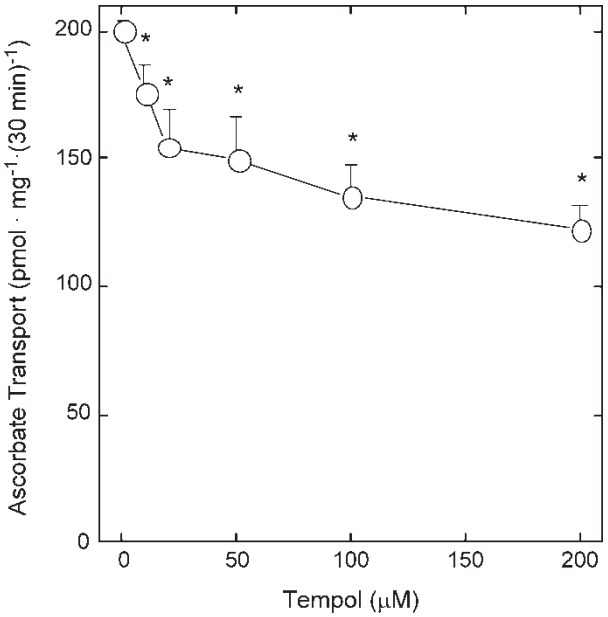


Figure 5. Inhibition of ascorbate transport by Tempol. EA.hy926 cells, in which intracellular ascorbate is not detected in culture, were rinsed free of culture medium and incubated at 37°C in KRH that contained 5 mM D-glucose and the indicated concentration of Tempol. After 30 min, the cells were rinsed three times in KRH and used for assay of ascorbate transport as described in the "Materials and methods" section. Results are shown from four experiments, with an asterisk indicating $p < 0.05$ compared to cells without Tempol.

has functional consequences for the cells. As shown in Figure 5 ascorbate transport was decreased by pre-treatment of cells with concentrations of Tempol 10-fold lower than those shown to generate significant oxidant stress. A significant inhibition was observed at a Tempol concentration of 10 μM and a 40% decrease occurred at 200 μM Tempol. It should be noted that after Tempol treatment, the cells were rinsed three times to remove excess Tempol, and that the incubation medium contained 0.5 mM GSH to protect radiolabeled extracellular ascorbate from oxidation by any remaining Tempol.

If ascorbate is more sensitive to Tempol than GSH, the question arises as to whether ascorbate can spare GSH in response to Tempol. To enhance the ability of Tempol to deplete GSH, cells were treated with the agent in the absence of glucose. As shown by the circles in Figure 6A, treatment of cells in the absence of glucose with 2 mM Tempol significantly decreased GSH. When cells had been pre-loaded with ascorbate, Tempol did not decrease GSH at 2 mM Tempol (Fig. 6A, squares). To assess the specificity of this effect, cells were treated by agents known to lower GSH by different non-oxidative mechanisms. As shown in Fig. 6B, overnight treatment with L-buthionine-[*S*,*R*]-sulfoxime, an inhibitor of GSH biosynthesis, decreased GSH, and there was no effect of loading the cells with even four times the concentration of DHA used in the experiments shown in Figure 6A. Of note is the finding that ascorbate loading alone increased GSH by 13%, an effect not observed during acute incubations. When GSH was depleted by inhibiting glutathione reductase with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), or by conjugating it with diethylmaleate, there was no protection afforded by pre-loading the cells with ascorbate (Figure 6C). These results show that the sparing of GSH by intracellular ascorbate is limited to Tempol, and not observed with agents that deplete GSH by non-oxidative mechanisms.

## Discussion

In this work, we evaluated the interaction of the stable nitroxide Tempol with endothelial cells, targeting its interactions with the major low molecular weight antioxidants in the cells, GSH and ascorbate. The results show that Tempol is reduced by the cells, that elevations in intracellular ascorbate concentration accelerate this reduction, that Tempol induces an oxidant stress in the cells, and that this oxidant stress can be mitigated by adequate concentrations of intracellular ascorbate. These findings have relevance to the potential use of such nitroxides as therapeutic agents in conditions associated with oxidant stress.

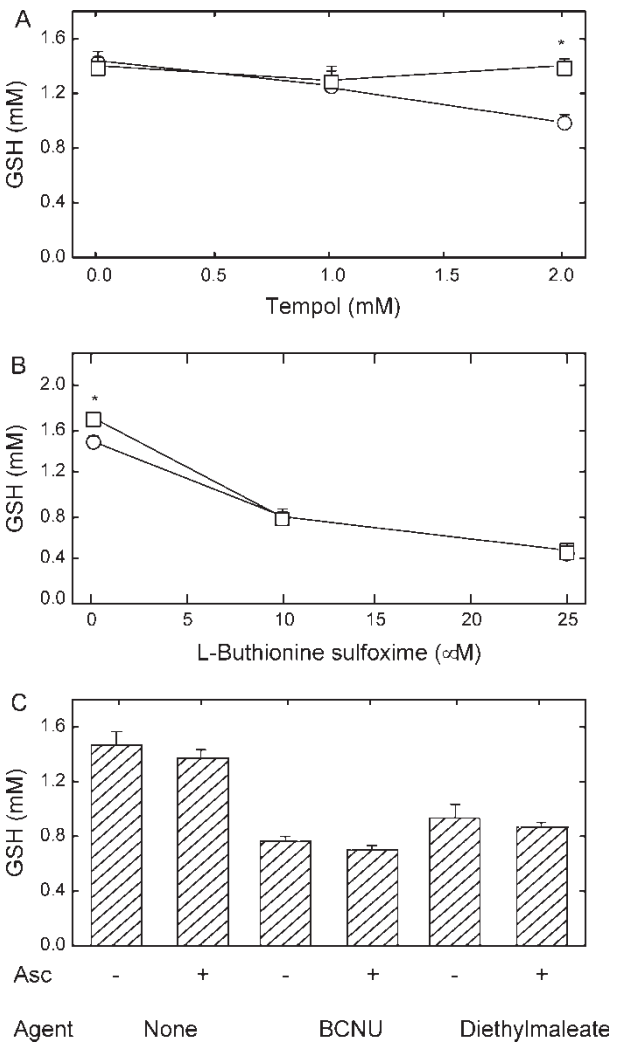


Figure 6. Ascorbate sparing of GSH from oxidation by Tempol. **Panel A.** EA.hy926 cells were incubated for 15 min at 37°C in KRH that contained 5 mM D-glucose in the absence (circles) and presence (squares) of 0.2 mM DHA, rinsed three times and incubated for 30 min at 37°C in KRH without D-glucose and with the indicated concentration of Tempol, and taken for assay of GSH ($N = 4$ experiments). **Panel B.** EA.hy926 cells were cultured 16 h in the presence of the indicated concentration of L-buthionine sulfoxime and in the absence (circles) and presence (squares) 0.8 mM DHA before three rinses to remove serum and assay of intracellular GSH ($N = 6$ experiments). **Panel C.** EA.hy926 cells were loaded where indicated (ASC) by treatment with DHA as described for Panel A, rinsed three times in KRH, then incubated at 37°C in KRH that contained 5 mM D-glucose and no additions (None), 0.1 mM (BCNU), or 0.1 mM diethylmaleate. After 30 min of incubation the cells were rinsed and taken for assay of GSH ($N = 4$ experiments). In the top two panels, an asterisk indicates $p < 0.05$ compare to the corresponding sample not treated with ascorbate.

Reduction of Tempol by EA.hy926 endothelial cells was confirmed by a Tempol-dependent increase in ferricyanide reduction in plated cells, and by disappearance of the Tempol radical in suspended cells. Ferricyanide has been described to oxidize the hydroxylamine of Tempol and thus regenerate Tempol [37]. We used the corresponding increase in ferrocyanide to quantify this effect. Since neither ferrinor ferrocyanide enters cells, the hydroxylamine of Tempol either exited the cells to react directly with ferricyanide or served as a substrate on the inner face of the plasma membrane for a trans-membrane oxidoreductase activity that appears to have ascorbate as its major intracellular electron donor [38,39]. As expected from its role as a donor to the ferricyanide reductase, ascorbate enhanced ferricyanide reduction. The finding that it also significantly increased Tempol-dependent ferricyanide reduction and loss of the Tempol EPR signal confirms the prominent interaction of ascorbate and Tempol within the cells. Further, rates of ferricyanide reduction induced by low millimolar concentrations of Tempol were comparable to those observed for basal- and ascorbate-dependent ferricyanide reduction. This attests to the relatively high capacity of the cells to reduce Tempol to its hydroxylamine.

Reduction of low millimolar concentrations of Tempol nonetheless caused an oxidant stress in the cells, which was detected by oxidation of dihydrofluorescein to fluorescein, by inhibition of redox-sensitive ascorbate transport into the cells, and by oxidative loss of ascorbate and GSH. Dihydrofluorescein oxidation is considered a marker of intracellular oxidant stress, although the reactive species are not well defined [40]. With regard to Tempol, however, there could also be direct oxidation by Tempol of intracellular dihydrofluorescein [41]. Perhaps a better indicator of oxidant stress generated in the cells due to Tempol was the finding that ascorbate transport was quite sensitive to acute inhibition by Tempol under conditions designed to eliminate direct reaction of Tempol with tracer ascorbate used to measure transport. We have recently found that ascorbate transport in EA.hy926 cells is also sensitive to a variety of sulfhydryl reagents, mild oxidants, and even to changes in intracellular GSH concentrations [36]. In contrast to oxidation of dihydrofluorescein and inhibition of ascorbate transport, which were evident at low micromolar Tempol concentrations, oxidation of ascorbate and GSH required higher concentrations. This probably relates to the presence of robust mechanisms for recycling both low molecular weight antioxidants in these cells. For example, bovine arterial endothelial cells have redundant mechanisms for recycling ascorbate from its oxidized products, including direct and enzyme-dependent reduction of DHA by GSH [42]. The finding that ascorbate was more sensitive to increasing concentrations of Tempol than was GSH likely reflects a greater reactivity of Tempol with ascorbate than with GSH, as well as a greater ability of the cells to recycle GSH via glutathione reductase and the pentose phosphate cycle. Although Tempol induces an intracellular oxidant stress, the cells handle the low micromolar concentrations expected *in vivo* quite well.

Whilst GSH clearly recycles ascorbate from DHA, two findings in the present work support previous studies cited in the "Introduction" section that ascorbate can also spare GSH. This was evident in cells acutely treated with Tempol (but not in cells depleted of GSH by other mechanisms), as well as in cells cultured overnight with ascorbate. Sparing of GSH in culture is likely due to ascorbate acting as a primary antioxidant to scavenge reactive oxygen species, whether this sparing of GSH occurs directly, or by sparing L-cysteine needed for GSH biosynthesis. The latter mechanism was observed for a similar effect of L-lipoic acid to spare GSH [43]. Most nucleated cells studied to date contain low millimolar concentrations of ascorbate [44], which in many instances are similar to or even greater than those of GSH [45,46]. Thus, in situations of acute oxidant stress, the presence of ascorbate could protect GSH and crucial sulfhydryls (such as those on transport proteins like the SVCT2 ascorbate transporter) from oxidation or disulfide formation and improve cell function and survival.

In conclusion, whilst low concentrations of Tempol and other stable nitroxide radicals have beneficial effects due to their ability to act as catalysts in the dismutation of superoxide, they are scavenged within cells by ascorbate, GSH, and probably protein thiols. This will impair their antioxidant efficacy, and could lead to functional inhibition of proteins that are sensitive to thiol modification. These considerations indicate a need for caution in its use as an antioxidant in clinical settings.

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