The carbonylation and covalent dimerization of human superoxide dismutase 1 caused by its bicarbonate-dependent peroxidase activity is inhibited by the radical scavenger tempol

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Tempol (4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl) reduces tissue injury in animal models of various diseases via mechanisms that are not completely understood. Recently, we reported that high doses of tempol moderately increased survival in a rat model of ALS (amyotrophic lateral sclerosis) while decreasing the levels of oxidized hSOD1 (human Cu,Zn-superoxide dismutase) in spinal cord tissues. To better understand such a protective effect in vivo, we studied the effects of tempol on hSOD1 oxidation in vitro. The chosen oxidizing system was the bicarbonate-dependent peroxidase activity of hSOD1 that consumes H2O2 to produce carbonate radical, which oxidizes the enzyme. Most of the experiments were performed with 30 μM hSOD1, 25 mM bicarbonate, 1 mM H2O2, 0.1 mM DTPA (diethylenetriaminepenta-acetic acid) and 50 mM phosphate buffer at a final pH of 7.4. The results showed that tempol (5–75 μM) does not inhibit hSOD1 turnover, but decreases its resulting oxidation to carbonylated and covalently dimerized forms. Tempol acted by scavenging the carbonate radical produced and by recombining with hSOD1-derived radicals. As a result, tempol was consumed nearly stoichiometrically with hSOD1 monomers. MS analyses of turned-over hSOD1 and of a related peptide oxidized by the carbonate radical indicated the formation of a relatively unstable adduct between tempol and hSOD1-Trp32. Tempol consumption by the bicarbonate-dependent peroxidase activity of hSOD1 may be one of the reasons why high doses of tempol were required to afford protection in an ALS rat model. Overall, the results of the present study confirm that tempol can protect against protein oxidation and the ensuing consequences.

Key words: bicarbonate-dependent peroxidase activity of hSOD1, cyclic nitroxide, hSOD1 carbonylation, hSOD1 covalent dimerization, non-native hSOD1 form, tempol.

INTRODUCTION

Cyclic nitroxides are stable free radicals that are potential therapeutic agents because of their pronounced antioxidant properties and low toxicity [1–6]. In vivo, the most investigated nitroxide is tempol (4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl), which reduces tissue injury in diverse animal models of oxidative stress and inflammation [7]. The mechanisms of protection are not completely understood, but have mainly been attributed to the ability of nitroxides (TPNO+) to act as multifunctional antioxidants in vivo [1–6]. Indeed, nitroxides can react with several biological oxidants and reductants while being recycled through oxammonium cation (TPNO2-) and hydroxylamine (TPNOH) derivatives respectively. This cycling enables nitroxides to deactivate several redox active species before they are metabolized and/or consumed by recombination reactions with specific radicals, such as tyrosyl and thyl radicals [4–6].

been characterized by immunological-based techniques. Because these techniques provide limited structural information, possible structural overlaps among the non-native hSOD1 proteins cannot be excluded. In addition, the so-called non-native hSOD1 forms may result from inherent properties of the ALS-hSOD1 mutants under physiological conditions [11–16], but may also form by the oxidation of wild-type hSOD1 [17,18,20,22,24–28].

In investigating the effects of tempol on the evolution of ALS in hSOD1G93A rats, we showed that the moderate extension of survival in these animals was accompanied by decreased levels of neuron loss and of non-native forms of hSOD1 in spinal cord tissues [8]. To better understand the protective action of tempol in vivo, it was important to determine whether tempol inhibited the oxidation of wild-type hSOD1 in vitro. Oxidation of hSOD1 in vitro is usually accomplished by treating the enzyme with high concentrations of H$_2$O$_2$ for several hours [17,18,20,25,28]. In the present study we examined the bicarbonate-dependent peroxidase activity of hSOD1 because the enzyme acts as the source and the target of attacking oxidants, in particular the carbonate radical [24,26,27]. As a consequence, hSOD1 is oxidized to non-native hSOD1 forms, such as carbonylated and covalently dimerized hSOD1 [24,26–30].

**EXPERIMENTAL**

**Materials**

Unless stated otherwise, all chemicals were purchased from Sigma–Aldrich, Merck or Fisher and were analytical grade or better. The H$_2$O$_2$ solutions were prepared just before use, and the concentrations were determined spectrophotometrically with horseradish peroxidase to produce compound I ($A_{532}$ = 5.4 × 10$^5$ M$^{-1}$ cm$^{-1}$) [31]. The tempol concentration was determined spectrophotometrically ($\varepsilon_{280} = 1.44 \times 10^5$ M$^{-1}$ cm$^{-1}$) [32]. Mass spectrometry grade trypsin (Gold) was purchased from Promega. DHR (dihydrorhodamine 123) was purchased from Molecular Probes. Orthodianisidine was obtained from Merck. hSOD1 (bovine SOD1) was purchased from Alexis Biochemicals. Anti-SOD1 and goat anti-sheep antibodies were obtained from Calbiochem. Bovine liver catalase was purchased from Boehringer. The peptide KVWGS (100% purity) was obtained from Promexon Consultoria S/A. The perchlorate salt of the carbonatotetrammine cobalt(III) complex ([Co(NH$_3$)$_4$CO$_3$]ClO$_4$) was a gift from B. Kalyanaraman (Medical College of Wisconsin, Milwaukee, WI, U.S.A.) [26]. All solutions and buffers were prepared with distilled water purified in a Milli-Q system and treated with Chelex-100® resin (Sigma–Aldrich).

**Recombinant hSOD1 expression and purification**

The enzyme was expressed in *Escherichia coli*, purified and analysed as previously described [30]. Typically, hSOD1 preparations have copper and zinc contents $\geq$0.70 ion per monomer and a specific activity of 4.3 × 10$^3$ units/mg (mg of protein normalized to the copper content). In the present study, the concentrations of hSOD1 are always expressed as the monomer concentration, which is redox active. Indeed, hSOD1 is a homodimeric protein containing one copper and one zinc ion per monomer.

**Enzymatic incubations**

Unless otherwise stated, the reaction mixtures contained hSOD1 (30 μM in terms of monomer units), 1 mM H$_2$O$_2$, 25 mM sodium bicarbonate, tempol (at the specified concentrations), 0.1 mM DTPA (diethylenetriaminepenta-acetic acid) and 50 mM phosphate buffer adjusted to a final pH of 7.4; the mixtures were incubated at 37°C for 1 h.

**H$_2$O$_2$ consumption**

H$_2$O$_2$ consumption was monitored by measuring the remaining oxidant at different incubation times via the orthodianisidine method [30]. Aliquots were taken from the incubation mixtures at the specified times and added to a sampling buffer consisting of 50 mM phosphate buffer, 0.3 mM orthodianisidine hydrochloride and 1 μM horseradish peroxidase (40 μg/ml), pH 7.4. The residual H$_2$O$_2$ was measured spectrophotometrically at 550 nm based on a calibration curve obtained with known concentrations of H$_2$O$_2$.

**Enzymatic production of the carbonate radical**

The production of the carbonate radical by hSOD1 activity was monitored by DHR and DMPO (5,5-dimethylpyrroline-N-oxide) oxidation [30]. In the first case, low concentrations of hSOD1 (2 μM in terms of monomer units) were employed to limit carbonate radical formation to concentrations much lower than that of DHR (100 μM). In aqueous solutions, DHR should be employed at concentrations ≤100 μM to avoid self-quenching of its absorbance. DHR oxidation to rhodamine was monitored spectrophotometrically at 500 nm ($\varepsilon_{500} = 7.88 \times 10^4$ M$^{-1}$ cm$^{-1}$) [33]. When 100 mM DMPO was used as a probe for the carbonate radical, the hSOD1 concentration was 30 μM (in terms of monomer units). At the specified incubation times, aliquots (50 μl) were transferred to capillary tubes and EPR spectra were recorded at room temperature (25 ± 2°C) on a Bruker EMX spectrometer equipped with a high-sensitivity cavity. The concentration of the DMPO/•OH adduct in the sample was estimated by double integration of the EPR spectrum and comparison with that of a standard tempol solution scanned under the same conditions. To estimate the second-order rate constant of the reaction of tempol with the carbonate radical we employed the competitive kinetic approach [31,34] to the data obtained in the experiments with DHR. The rate of DHR oxidation was calculated as half of the rate of rhodamine production due to the stoichiometry (two DHR molecules produce one rhodamine). The second-order rate constant of the reaction between tempol and the carbonate radical ($k_{\text{tempol}}$) was obtained by plotting the fraction of inhibition of DHR oxidation ($F / (1 − F)$) against the tempol concentration that caused it (eqns 1–3) [31,34]:

\[
\text{DHR} + \text{CO}_3^{2−} \overset{k_{\text{DHR}}}{\longrightarrow} \text{DHR}^+ + \text{HCO}_3^{−} \quad (1)
\]

\[
\text{tempol} + \text{H}^+ + \text{CO}_3^{2−} \overset{k_{\text{tempol}}}{\longrightarrow} \text{TPNO}^+ + \text{HCO}_3^{−} \quad (2)
\]

\[
(F / (1 − F)) \times k_{\text{DHR}} \times [\text{DHR}] = k_{\text{tempol}} \times [\text{tempol}] \quad (3)
\]

**Analysis of hSOD1 carbonylation**

Protein carbonyl contents were determined as previously described [35] with minor modifications. After 1 h of incubation, the reactions were stopped by the addition of catalase (100 μg/ml). Aliquots containing 1 mg/ml of hSOD (50 μl) were removed and incubated in the dark with 1 mM FTC (fluorescein 5-thiosemicarbazide) in 50 mM phosphate buffer, pH 6.0, at 37°C for 2 h. The protein was then precipitated by the addition of a 10-fold excess of ice-cold acetone (−20°C), and the mixture was kept overnight at −20°C. After centrifugation (16 000 g for
10 min at 4°C), the protein pellets were broken up and washed twice with ice-cold acetone. Protein pellets were resuspended in water, and the protein concentration was determined using a Bradford protein assay (Bio-Rad Laboratories). Protein aliquots (10 μg) were diluted in Laemml buffer and submitted to SDS/PAGE (15% gels). Fluorescent protein images from the gel were captured with a Typhoon 9400 imager using an excitation wavelength of 488 nm and an emission filter at 520 nm (40 nm bandpass). A relative quantification of the bands was performed with ImageJ 1.44p software. The gels were then fixed with methanol (50%) and acetic acid (10%) for 10 min, followed by an overnight staining with Coomassie Blue.

**Analysis of hSOD1 covalent dimerization**

After 1 h incubation, the reactions were stopped by the addition of catalase (100 μg/ml). Aliquots corresponding to 5 μg of protein were resuspended in Laemml buffer, submitted to SDS/PAGE (15% gel) and transferred on to nitrocellulose membranes (GE Healthcare) using a Bio-Rad Laboratories semi-dry transfer system according to the manufacturer’s instructions. Blocking was performed with 5% (w/v) non-fat dried skimmed milk powder in TBST (TBS with 0.05% Tween 20) for 2 h. Immunodetection was performed using sheep anti-SOD (1:5000 dilution, 2 h) antibody with an ECL detection kit (Pierce SuperSignal West Pico). Densitometric analysis was performed with ImageJ 1.44p software.

**Analysis of tempol consumption by EPR**

Incubations containing hSOD1 or bSOD1 (30 μM in terms of monomer units) were performed as described above. Aliquots were removed at specified times and analysed by EPR at room temperature on a Bruker EMX spectrometer equipped with a high sensitivity cavity. Before scanning the second EPR spectra, the reactions were stopped by the addition of catalase (100 μg/ml). The remaining nitroxide concentrations in the samples were estimated by a double integration of the EPR spectrum and a comparison with that of a standard tempol solution scanned under the same conditions.

**Analysis of tempol consumption by HPLC UV–visible**

After 1 h incubation at 37°C, the reactions were stopped by the addition of catalase (100 μg/ml). The samples were then submitted to ultrafiltration (Microcon; cut-off 5 kDa; Millipore) to retain hSOD1 and catalase. Control experiments showed that 15% of the tempol was lost during ultracentrifugation, most likely because it interacted with the filter. The remaining tempol was estimated by integration of the corresponding HPLC peak and comparison with those of a standard tempol solution.

**Digestion of hSOD1**

After a 1 h incubation at 37°C, the samples were dried in a vacuum centrifuge and solubilized in denaturing buffer (10 mM Tris/HCl, 6 M guanidine hydrochloride, 0.1 mM DTPA and 30 mM DTT, pH 8.0) under reduced oxygen tension to reduce the disulfide bonds. Iodoacetamide (150 mM) was then added, and the samples were incubated for 3 h in the dark at 37°C to alkylate free thiols. After the alkylation step, the samples were desalted against water by ultrafiltration (Amicon Ultra, cut-off 3 kDa, Millipore) and dried [24]. Digestion with trypsin was performed in 50 mM ammonium bicarbonate containing 2 mM calcium chloride, pH 8.0, for 20 h at 37°C. A substrate/trypsin ratio of 50:1 was employed for all samples. The hydrolysates were analysed by MALDI (matrix-assisted laser-desorption ionization) MS.

**Peptide oxidation**

The peptide corresponding to hSOD1 residues 30–34 (KVWGS) was oxidized by the carbonate radical produced from the photolysis of a carbonatotetramine cobalt(III) complex \{[Co(NH₄)₄CO₃][ClO₄] \} [26]. The peptide (2–4 mM) was mixed with the cobalt complex (2–4 mM) in the presence of tempol (0.5–2 mM) in bicarbonate buffer (10 mM) adjusted to pH 7.4 with carbon dioxide (100%) or in deaerated phosphate buffer (20 mM), pH 7.4. The mixtures were transferred to EPR flat cells and irradiated for 1 min at 254 nm in a Photoreactor ICH-2 (Luzchem) (energy incident in the sample of approximately 6.3 mW/cm²). The samples were submitted to electrospray and MALDI MS analyses.

**MS and MS/MS (tandem MS) analysis by MALDI–TOF/TOF (tandem time-of-flight)**

The samples of hSOD1 hydrolysates and oxidized peptide were analysed on a Bruker Daltonics UltraflexTM MALDI–TOF/TOF instrument in a reflector mode using a Smartbeam II laser™. Mass spectra were collected in the positive ion mode and ions were extracted with a 90 ns delay and accelerated to 25 kV. Each spectrum obtained was a sum of 1500 laser shots. All samples were desalted and concentrated with a ZipTipC₁₈ (Millipore) before analysis. The samples were premixed with matrix using 1 μl of sample solution and 4 μl of matrix solution, spotted on to the MALDI polished steel target plate and air-dried. The matrix solution employed was 20 mg/ml 2,5-dihydroxybenzoic acid in 70% acetonitrile, 0.1% trifluoroacetic acid and 29.9% water. The instrument was externally calibrated with a mixture of peptide ions over the m/z range of 757–3174 (peptide calibration standard: tube 4 from Bruker starter kit for MALDI–TOF-MS). Data were analysed with the software FlexAnalysis (version 3.3).

**MS and MS/MS analysis by electrospray**

The hSOD1 samples for electrospray analysis were incubated for 30 min at 37°C and were immediately desalted and concentrated with a ZipTipC₁₈. Turned-over hSOD1 and oxidized peptide were analysed on a UHR-ESI-Q-TOF Bruker Daltonics MaXis 3G spectrometer with CaptiveSpray source in the positive mode. The nano captive electrospray source and the electrospray source were used for the peptide and hSOD1 respectively. The samples were injected through direct infusion using a spraying solution (50:50, v/v water/acetonitrile) pumped at 30 and 240 μl/h for the oxidized peptide and hSOD1 respectively. MS data were recorded in the first 1 min of injection and MS/MS spectra were recorded from 1.0 to 30 min. The interface conditions for the nano captive source were: capillary, 1.8 kV; dry heater, 1500°C; dry gas, 6 l/min. The ESI (electrospray ionization) conditions...
were: capillary, 2.5 kV; dry heater, 1800 °C; dry gas, 4.0 l/min; end plate, −500 V. Nitrogen was used as collision gas and the CID (collision-induced dissociation) energy was optimized from 5 to 40 eV. The selected collision energy was 30 eV. The instrument was externally calibrated using an ESI low concentration tuning mix over the m/z range of 100 to 2000. The Bruker DataAnalysis software (version 4.0) was employed for data acquisition and processing. Under the experimental conditions, mass errors were below 2 p.p.m.

Statistical analysis

All of the data are expressed as means ± S.D. Statistical significance was calculated using one-way ANOVA with Tukey post-test using GraphPad 4.00 software.

RESULTS

Effects of tempol on the bicarbonate-dependent peroxidase activity of hSOD1

In addition to efficiently dismutating superoxide anion to H2O2 and molecular oxygen, SOD1 consumes H2O2 in a very slow process that leads to the inactivation of the enzyme [36]. In the presence of the bicarbonate/CO2 pair, the bicarbonate buffer, SOD1 turnover increases and the enzyme consumes considerable amounts of H2O2 to produce the carbonate radical by mechanisms that are under debate [26,27,30,37,38]. The relevant aspect of this reaction with regard to neurodegenerative processes is that the carbonate radical diffuses away from the active site and oxidizes distant targets, such as other proteins [27,39] and, in the case of hSOD1, its own Trp32 residues [24,26]. Before examining whether tempol affects the oxidation of hSOD1 that results from its bicarbonate-dependent peroxidase activity, it was necessary to investigate the effects of tempol on hSOD1 turnover. The latter process involves the redox cycling of the copper ion at the active site of hSOD1, and tempol has previously been shown to interact with copper ions [40]. However, tempol (5–75 μM) had marginal effects on the rate of H2O2 (1 mM) uptake by hSOD1 (30 μM in terms of monomer units) in the presence of bicarbonate (25 mM) (Figure 1A). In contrast, these concentrations of tempol strongly inhibited the ability of myeloperoxidase to consume H2O2 in the presence of chloride [32,41]. Therefore, the results shown in Figure 1A argue against a direct interaction between tempol and the copper ion in the hSOD1 active site.

Although it did not inhibit the peroxidase activity of hSOD1, tempol was expected to decrease the yield of carbonate radical because nitroxides are rapidly oxidized by it to produce the corresponding oxammonium cation and bicarbonate [42]. The effect of tempol on carbonate radical production by hSOD1 was first examined by monitoring DHR oxidation to rhodamine [33]. In these incubations, lower concentrations of hSOD1 (2 μM in terms of monomer units) were employed because in aqueous solutions DHR should be employed at concentrations ≤100 μM to avoid absorbance self-quenching. In the absence of tempol, hSOD1 oxidized DHR producing rhodamine at a rate of 1 μM/min (Figure 1B) [30]. Tempol inhibited the rate of rhodamine formation in a concentration-dependent manner (Figure 1B). We next applied the competitive kinetic approach [31,34] to determine the apparent second-order rate constant of the reaction between tempol and the carbonate radical (k1) (eqns 1–3). Despite the linear fit of the data to eqn (3), the obtained value of k1 was 3.4×109 M−1·s−1 at pH 7.4, 37°C (Figure 1C) higher than the precise value determined by Goldstein et al. [42] at pH 10.3, 25°C (k1=4.0×109 M−1·s−1). Nevertheless, the values are close enough to indicate that tempol competes with DHR for the carbonate radical. The discrepancy in the value is probably due to the differences in pH and temperature, as well as the fact that the enzymatic system possesses targets other than DHR and tempol for the carbonate radical, including hSOD1 and H2O2. In fact, the competitive kinetic approach can be employed to determine a second-order rate constant when known concentrations of two targets compete for the same species and the second-order rate constant of one target is known (eqns 1–3). If other targets are present, the fraction of the inhibition of target oxidation by a given concentration of target1 should be higher than that expected from the product of k1×[target2] because of the contribution of targets that are unaccounted for.

To confirm further that tempol deactivates part of the carbonate radical that is produced, we performed enzymatic incubations in the presence of DMPO (100 mM). This spin trap is oxidized by the carbonate radical producing the DMPO•− radical adduct, which can be detected by EPR [26,30]. As previously reported, the bicarbonate-dependent activity of SOD1 generates a considerable yield of DMPO•− radical adduct [26,30], which was inhibited by tempol in a concentration-dependent manner (Figure 2). The percentage of inhibition for the initial oxidation rate caused by 75 μM tempol was approximately 38%. This value is consistent with the concentration of DMPO (100 mM) employed and with the second-order rate constant values for the reaction of the carbonate radical with tempol (k=4.0×109
Tempol inhibits hSOD1 carbonylation and dimerization

Effects of tempol on oxidative hSOD1 modifications

Human SOD1 residues, in particular Trp$^{52}$ and histidine residues, are targets of the carbonate radical that is produced by its bicarbonate-dependent peroxidase activity [26,27,44] where they are oxidized to the corresponding hSOD1-Trp$^*$ and hSOD1-His$^*$ radicals. These short-lived species decay to carbonylated products and to a covalent dimer [26,29], which we recently characterized as hSOD1-Trp$^{52}$-Trp$^{52}$-hSOD1 [24]. In the present study, we first examined the effects of tempol on the total yield of carbonylated hSOD1 residues that were produced after a 1-h incubation by trapping them with a fluorescent probe (FTC) and then performing SDS/PAGE separation as described in the Experimental section [35]. As shown in Figure 3, the bicarbonate-dependent peroxidase activity of hSOD1 resulted in hSOD1 carbonylation, particularly of the native hSOD1 dimer, which appears as a monomer on the SDS/PAGE gel at \( \sim 20 \) kDa. In the region of the hSOD1 covalent dimer (\( \sim 40 \) kDa), the levels of carbonylation/protein were apparently lower, in agreement with the notion that hSOD1-Trp$^*$ decays to either the hSOD1-Trp$^{52}$-Trp$^{52}$-hSOD1 covalent dimer or to N-formyl-kyurenine and kynurenine [24]. Tempol at concentrations \( \geq 15 \) μM inhibited the levels of hSOD1 carbonylation/protein by approximately 50% (Figures 3A and 3B). Tempol was even more effective in inhibiting dimer production, as shown by parallel experiments in which hSOD1 products were analysed by Western blotting with an anti-SOD1 antibody (Figure 4A). Tempol decreased the formation of hSOD1 covalent dimers in a concentration-dependent manner with almost no dimer being produced in the presence of tempol concentrations of approximately 30 μM; these are nearly stoichiometric with that of hSOD1 monomers (Figure 4B). Although inefficiently, tempol also protected hSOD1 from inactivation, which is attributed to oxidation of histidine residues that co-ordinate the copper ion in the hSOD1 active site (Figure 4C).

Taken together, these results suggest that, in addition to deactivating a portion of the produced carbonate radicals (Figures 1B, 1C and 2) [43], tempol reacts with hSOD1-derived radicals [4–6,45–47]. Actually, it is reasonable to suppose that tempol cannot efficiently compete with all of the possible targets within the hSOD1 structure for the carbonate radical that is generated in the active site. As a consequence, hSOD1-derived radicals are produced and may react with tempol and consume it [4–6,45–47]. Therefore we examined whether tempol was consumed by the bicarbonate-dependent peroxidase activity of hSOD1.

Tempol consumption

Analysis by EPR of standard incubations in the presence of different concentrations of tempol showed that the nitroxide is consumed in a concentration-dependent manner up to approximately 30 μM after 1 h (Figure 5A). In contrast, 50 μM tempol, when incubated under the same experimental conditions except for the substitution of hSOD1 by bSOD1, was marginally consumed. To further confirm the amount of

Figure 2  Effect of tempol on CO$_3$•$^-$ production by the bicarbonate-dependent peroxidase activity of hSOD1 monitored by DMPO oxidation

(A) Representative EPR spectra. The samples contained hSOD1 (30 μM in terms of monomer units), 1 mM H$_2$O$_2$, 25 mM HCO$_3^-$, 100 mM DMPO, 0.1 mM DTPA, tempol (at the specified concentrations) and 50 mM phosphate buffer adjusted to pH 7.4, and were incubated at 37 °C. At the specified times, aliquots were subjected to EPR analysis. The cross and black boxes mark the first and second spectral line of tempol and DMPO•OH respectively. The EPR spectra shown are representative of three independent experiments. (B) Production of DMPO•OH radical adduct with time: without tempol (Δ); plus tempol (30 μM) (○); and plus tempol (75 μM) (■). The concentrations of the radical adduct were determined as described in the Experimental section. The values shown are the means ± S.D. obtained in three independent experiments. The instrumental conditions were: microwave power, 10 mW; modulation amplitude, 1.0 G; sweep time, 41 s; time constant, 41 ms; and modulation amplitude, 1 G.

M$^{-1}$s$^{-1}$) [43] and with DMPO (\( k = 1.5 \times 10^9 \) M$^{-1}$s$^{-1}$) [43]. However, the actual inhibition value was higher than the expected value (approximately 20%). Again, the discrepancy can be attributed to the fact that hSOD1 (30 μM in terms of monomer units) and H$_2$O$_2$ are also targets for the carbonate radical. Taken together, these results show that tempol marginally affects hSOD1 turnover during its bicarbonate-dependent peroxidase activity, but decreases the total yield of carbonate radical by reacting with the radical and protecting other targets from it (Figures 1 and 2).

Figure 3  Effect of tempol on hSOD1 carbonylation resulting from its bicarbonate-dependent peroxidase activity

The samples contained hSOD1 (30 μM in terms of monomer units), 1 mM H$_2$O$_2$, 25 mM HCO$_3^-$, 0.1 mM DTPA, tempol (at the specified concentrations) and 50 mM phosphate buffer adjusted to pH 7.4, and were incubated for 1 h at 37 °C. The reactions were stopped by adding catalase, and hSOD1 carbonylation was analysed as described in the Experimental section. (A) Representative fluorescence image of SDS/PAGE of carbonylated hSOD1 forms stained with FTC; (B) The same as (A), but stained with Coomassie Blue. The arrow indicates catalase; (C) Carbonylated hSOD1 fluorescence normalized to protein content. Each lane of the gels similar to those shown in (A) and (B) was quantified. The values shown in (C) correspond to the means ± S.D. obtained in three independent experiments. *P < 0.05 compared with control in the absence of tempol; #P<0.05 compared with 50 μM tempol.

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The samples contained hSOD1 (30 μM in terms of monomer units) or hSOD1 (30 μM in terms of monomer units), 1 mM H2O2, 25 mM HCO3−, 0.1 mM DTPA, tempol (at the specified concentrations) and 50 mM phosphate buffer adjusted to pH 7.4, and were incubated for 1 h at 37 °C. The reactions were stopped by adding catalase, and hSOD1 covalent dimerization and inactivation were analysed as described in the Experimental section. (A) Representative Western blot visualized with an anti-SOD1 antibody. (B) Relative quantification of the bands at approximately 40 kDa in gels similar to the one shown in (A). (C) Remaining hSOD1 dismutase activity. The values shown in (B) and (C) are the means ± S.D. obtained in three independent experiments; *P < 0.01 compared with control without tempol.

Figure 5 Tempol consumption during the bicarbonate-dependent peroxidase activity of hSOD1

(A) EPR analysis of tempol consumption. The samples contained hSOD1 (30 μM in terms of monomer units) or hSOD1 (30 μM in terms of monomer units), 1 mM H2O2, 25 mM HCO3−, 0.1 mM DTPA, tempol (at the specified concentrations) and 50 mM phosphate buffer adjusted to pH 7.4, and were incubated for 1 h at 37 °C. The spectra shown are representative of two independent experiments and were scanned at the specified times. Catalase (100 μg/ml) was added to the 1 h incubation samples before the second scan. The instrumental conditions were: microwave power, 10 mW; modulation amplitude, 1 G; sweep time, 41 s; time constant, 41 ms; modulation amplitude, 1 G. (B) and (C) Quantification of tempol consumption by HPLC UV–visible. Reaction mixtures such as those in (A) were: microwave power, 10 mW; modulation amplitude, 1.0 G; sweep time, 41 s; time constant, 41 ms; modulation amplitude, 1 G. (B) and (C) Quantification of tempol consumption by HPLC UV–visible. The values shown in (C) are the means ± S.D. obtained in three independent experiments; *P < 0.01 compared with control without tempol.

Figure 4 Effect of tempol on hSOD1 covalent dimerization and inactivation resulting from its bicarbonate-dependent peroxidase activity

The samples contained hSOD1 (30 μM in terms of monomer units), 1 mM H2O2, 25 mM HCO3−, 0.1 mM DTPA, tempol (at the specified concentrations) and 50 mM phosphate buffer adjusted to pH 7.4, and were incubated for 1 h at 37 °C. The reactions were stopped by adding catalase, and hSOD1 covalent dimerization and inactivation were analysed as described in the Experimental section. (A) Representative Western blot visualized with an anti-SOD1 antibody. (B) Relative quantification of the bands at approximately 40 kDa in gels similar to the one shown in (A). (C) Remaining hSOD1 dismutase activity. The values shown in (B) and (C) are the means ± S.D. obtained in three independent experiments; *P < 0.01 compared with control without tempol.

tempol that is consumed by hSOD1-containing incubations, parallel experiments were performed with 50 μM tempol. After incubation for 1 h, the samples were filtered to retain hSOD1 and analysed by HPLC/UV at 240 nm to determine the amount of remaining tempol (Figures 5B and 5C). In agreement with the EPR data (Figure 5A), approximately 30 μM tempol was consumed in incubations containing 30 μM hSOD1 (in terms of monomer units). These results indicate a nearly stoichiometric consumption of one tempol per one hSOD1 monomer, suggesting that tempol is recombining mainly with the solvent-exposed hSOD1-Trp* residue. This finding agrees with the fact that tempol was not consumed in incubations containing hSOD1, which has a bicarbonate-dependent peroxidase activity similar to that of hSOD1 with regard to H2O2 consumption and carbonate radical production. Additionally, similarly to hSOD1, hSOD1 possesses histidine residues that are oxidized during its bicarbonate-dependent peroxidase activity [44]. However, bSOD1 does not have a tryptophan residue and is therefore not oxidized to an enzyme-derived tryptophyl radical [26].

Attempts to characterize a hSOD1–tempol adduct

The ability of nitroxides to recombine with protein radicals is widely accepted, but the stability of the produced adducts remains uncertain [45–47]. To investigate whether tempol recombines with hSOD1-Trp* to produce a stable adduct, we first attempted to detect it in the hydrolysates of turned-over hSOD1 [45]. Standard incubations were performed in the absence and presence of 100 μM tempol, and the turned-over samples were digested with trypsin and analysed by MADI-TOF/TOF-MS, as described in the Experimental section. The MS spectra of the samples in the m/z range of tryptic hSOD1 peptides containing the Trp32 residue confirmed that the bicarbonate-dependent peroxidase activity of hSOD1 promotes the oxidation of its Trp32 residue (V31WGSIK36; m/z 689.4) to kynurenine (V31KynGSIK36; m/z 693.5), N-formylkynurenine (V31NKFynGSIK36; m/z 721.5) [26] and to a Trp32–Trp32 cross-linked dimer [(E36SNGPVKVGSIK36); m/z 2797.6], which is characterized by a loss of a trypsin cleavage and a partial cleavage of the cross-link during MS analysis [E36SNGPVKVGSIK36; m/z 1400.8 and E36SNGPVKVV(−2H)GSIK36; m/z 1398.8] (Supplementary Figure S1 at http://www.biochemj.org/bj/455/bj4550037add.htm) [24]. In agreement with the results shown in Figures 3 and 4, tempol inhibited all oxidation products of the Trp32 residue of hSOD1 (Supplementary Figure S1). However, the presence of an adduct of tempol with the tryptic peptides containing Trp32 [V31W(tempol)GSIK36; m/z 860.5] and E36SNGPVKVV(tempol)GSIK36; m/z 1571.9] was not clear (Supplementary Figure S2 at http://www.biochemj.org/bj/455/bj4550037add.htm). For instance, in addition to its detection in the control samples, the peak at m/z 1571.6 in the samples that were incubated with tempol did not provide consistent MS/MS spectra. One possibility to explain tempol inhibition without the detection of the corresponding adduct could be the instability of the
Tempol inhibits hSOD1 carbonylation and dimerization

Figure 6 Characterization of the covalent adduct KVW(tempol)GS by ESI-Q-TOF (quadrupole–time-of-flight)-MS/MS analysis

The samples contained 2 mM KVWGS, 4 mM [Co(NH3)4CO3]+ and 1.0 mM tempol in 10 mM ammonium bicarbonate buffer adjusted to pH 7.4 with CO2 (100 %). The samples were irradiated for 1 min and analysed by UHR-ESI-Q-TOF-MS with CaptiveSpray source as described in the Experimental section. (A) MS data of the sample recorded in the first 1 min of injection; the insert shows a 100× magnification of the specified region. (B) MS/MS data of the peak at m/z 747.4 recorded from 1 to 30 min. The fragments of the series of a, b, c, x, y and z that were identified are labelled and are also shown with the accurate mass measurements in Supplementary Table S1 (at http://www.biochemj.org/bj/455/bj4550037add.htm).

protein–tempol adduct under the conditions required for protein reduction, alkylation and hydrolysis. Therefore we tried to detect the hypothetical adduct in the whole protein [45]. In this case, the turned-over samples were immediately desalted, concentrated in a ZipTipC18 and analysed by ESI-MS, as described in the Experimental section. However, the complexity of the ESI spectra with many charged hSOD1 forms (with and without metals, oxidized and non-oxidized) precluded the detection of potential hSOD1–tempol adducts (Supplementary Figure S3 at http://www.biochemj.org/bj/455/bj4550037add.htm).

Another method of avoiding the long procedures that are required for protein hydrolysis for MS analysis is the replacement of the protein residues of interest by a related peptide. Therefore, we took advantage of previous studies from our laboratories with the peptide that corresponds to hSOD1 residues 30–34 (KVWGS). This peptide, when subjected to the carbonate radical that is generated during photolysis of the [Co(NH3)4CO3]+ complex (see the Experimental section) [26], is oxidized to products similar to those produced from the oxidation of hSOD1, such as the corresponding KVW(•)GS radical and the Trp-Trp cross-linked dimer [(KVWGS)2] (J. N. Dos Prazeres, V. Paviani and O. Augusto, unpublished work). In the present study we irradiated the [Co(NH3)4CO3]+ complex in the presence of the peptide and tempol and submitted the samples to ESI-MS analysis, as described in the Experimental section. The MS spectra of the samples that were irradiated in the presence of tempol clearly showed the native peptide (KVWGS; m/z 576.3) and a small peak of its tempol adduct [KVW(tempol)GS; m/z 747.4] (Figure 6A). This adduct was submitted to MS/MS analysis, which proved that tempol was bound to the tryptophan residue because several fragments containing both moieties were identified (Figure 6B and Supplementary Table S1 at http://www.biochemj.org/bj/455/bj4550037add.htm). Also, the adduct KVW(tempol)GS cleaved under MS/MS conditions to the parent peptide (m/z 576.3) and an oxidized form of tempol (m/z 172.1), most likely its oxammonium cation [48]. Even under the MS conditions, the adduct apparently loses water, as is indicated by the peak at m/z 729.4 [KVW(tempol)GS-H2O] (Figure 6A). Accordingly, the MS/MS analysis of peptide samples by MADI-TOF/TOF provided reasonable sequencing only of the m/z 729.4 peak (Supplementary Figure S4 at http://www.biochemj.org/bj/455/bj4550037add.htm). These results show that tempol recombines with the peptide-derived tryptophanyl radical to produce an adduct that is stable enough to be sequenced by MS/MS. Therefore the non-detection of the adduct in the hydrolysates of turned-over hSOD1 was most likely due to its instability under the conditions required for protein reduction, alkylation and hydrolysis.

DISCUSSION

Tempol and other cyclic nitroxides inhibit the peroxidase activity of haem proteins either by reacting with and deactivating higher oxidation intermediates produced during the turnover of these enzymes with H2O2, such as compounds I and II [32,41,46], or by deactivating substrate-derived radicals [49]. Previously,
Even lymphocytes of sporadic ALS patients [22]. However, the hSOD1 reported that carbonylated wild-type hSOD1 is present in the and a subset of sporadic ALS. Indeed, it has been recently dependent toxic mechanism to account for both familial ALS from an attack of hSOD1 by carbonate radicals [24,26,29,44]. (Figure 3) and covalent dimerization (Figure 4), which are partially responsible for the inhibition of hSOD1 carbonylation with hSOD1-derived radicals [4–6,45–47]. These reactions are monomers (Figure 5); this result indicates that tempol reacts tempol was consumed nearly stoichiometrically with hSOD1 case of the bicarbonate-dependent peroxidase activity of hSOD1.

Our study showed that the tested concentrations of tempol marginally affected hSOD1 turnover in the presence of H2O2, and bicarbonate (Figure 1A), but it decreased the yield of the attacking carbonate radical by reacting with it. Such a deactivation of the carbonate radical was evident because tempol competed for the radical with its well-known targets, DHR (Figures 1B and 1C) [33] and DMPO (Figure 2) [30,43]. In deactivating part of the carbonate radical, tempol inhibited the oxidative modifications of hSOD1, such as carbonylation (Figure 3) and covalent dimerization (Figure 4), that resulted from its peroxidase activity. These inhibitions, however, were not exclusively due to scavenging of the carbonate radical. Indeed, in this case tempol should have acted nearly catiolytically because its reaction with the carbonate radical produces bicarbonate and the oxammonium cation, which in turn oxidizes H2O2 to a superoxide anion, regenerating the nitroxide [49]. However, in the case of the bicarbonate-dependent peroxidase activity of hSOD1, tempol was consumed nearly stoichiometrically with hSOD1 monomers (Figure 5); this result indicates that tempol reacts with hSOD1-derived radicals [4–6,45–47]. These reactions are partially responsible for the inhibition of hSOD1 carbonylation (Figure 3) and covalent dimerization (Figure 4), which are preceded by the formation of hSOD1-derived radicals that result from an attack of hSOD1 by carbonate radicals [24,26,27,29].

The interest in the carbonylation of hSOD1 is increasing because this oxidative modification may provide a hSOD1-dependent toxic mechanism to account for both familial ALS and a subset of sporadic ALS. Indeed, it has been recently reported that carbonylated wild-type hSOD1 is present in the lymphocytes of sporadic ALS patients [22]. However, the hSOD1 residues that are carbonylated in vivo have yet to be characterized. Even in vitro, hSOD1 carbonylation remains poorly characterized, except for the Trp12 residue, which was shown to be oxidized to N-formyl-kyurenine and kynurenine during the bicarbonate-dependent peroxidase activity of hSOD1 (Supplementary Figure S1) [26,27]. The oxidation of His48, His80, His110 and His120 has been demonstrated in incubations of hSOD1 with H2O2 and EDTA, but the products were not fully characterized [28].

Among the amino acids, tryptophan is a preferred target of the carbonate radical \( k = 7 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \), and the production of hSOD1-Trp\(^*\) during the bicarbonate-dependent peroxidase activity of hSOD1 has been unequivocally demonstrated [26]. The hSOD1-Trp\(^*\) is responsible for the covalent dimerization of hSOD1 [24], which tempol inhibited in a concentration-dependent manner (Figure 4). Tempol was consumed in the process (Figure 5), indicating that it recombined with hSOD1-Trp\(^*\) [3–6,45–47]. However, the attempts to obtain an MS characterization of a hSOD1-Trp–tempol adduct in hSOD1 hydrosylates and in the whole protein failed (Supplementary Figures S1–S3). Nevertheless, the oxidation of the peptide corresponding to hSOD1 residues 30–34 (KVWGGS) by the carbonate radical produced from [Co(NH\(_3\))\(_4\)CO\(_3\)]\(^+\) photolysis in the presence of tempol led to the full MS and MS/MS characterization of the KVW(tempol)GS adduct \([m/z \ 747.4]\) (Figure 6, and Supplementary Table S1). To the best of our knowledge, this is the first MS/MS characterization of an adduct of tempol with a peptide-derived radical [45–47].

The KVW(tempol)GS adduct partially decomposed under MS/MS conditions to the native peptide (KVWGGS) and the oxammonium cation of tempol (TPNO\(^+\); \(m/z \ 172.1\)) (Figure 6, and Supplementary Figure S4), indicating a heterolytic cleavage that repairs the peptide-derived radical. Because the adduct was not detectable in hSOD1 hydrosylates, it was apparently cleaved under the reducing and acidic conditions that were required to denature proteins and to desalt and concentrate protein hydrosylates respectively. An investigation into the stability of the adduct under different conditions should be pursued in future studies. The important point to emphasize is that the adduct of tempol with a peptide-derived tryptophanyl radical is relatively unstable, but can be fully characterized by MS/MS.

In conclusion, our results showed that tempol does not inhibit the bicarbonate-dependent peroxidase activity of hSOD1, but decreases the consequence of this activity, that is, hSOD1 oxidation. Tempol acts by partially scavenging the carbonate radical produced and by recombining with hSOD1-derived radicals. The latter process most likely occurs because tempol cannot efficiently compete with targets within the hSOD1 molecule for a radical that is generated at the active site and that diffuses through the enzyme into the solution [26,27,44]. In reacting with hSOD1-derived radicals, tempol protects the enzyme from oxidation to non-native forms, but is consumed in the process. Such consumption may be one of the reasons why high doses of tempol are required to observe a moderate protective effect in an ALS rat model that overexpresses hSOD1\(^{G93A}\) with regard to evolution of the symptoms, neuronal losses and non-native hSOD1 forms [8]. This possibility should be further evaluated in the future, although the participation of oxidative processes in the production of non-native hSOD1 forms in ALS disease remains to be unambiguously demonstrated [12–18,25,28]. Overall, the present study further supports the view that tempol and related cyclic nitroxides are protective against protein oxidation and the ensuing consequences [1–6,47,50].

**AUTHOR CONTRIBUTION**

Raphael Queiroz designed and performed most of the experiments and analysed the data. Veronica Paviani, Emerson Marques and Paolo Di Mascio designed, performed and analysed the MS experiments. Fernando Coelho expressed and purified recombinant hSOD1 and analysed it. Ohara Augusto designed the research, analysed the data and wrote the paper; all of the authors were involved in discussions and approved the paper.

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SUPPLEMENTARY ONLINE DATA

The carbonylation and covalent dimerization of human superoxide dismutase 1 caused by its bicarbonate-dependent peroxidase activity is inhibited by the radical scavenger tempol

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Figure S1 MALDI–TOF/TOF-MS analysis of the oxidation products of Trp32 of turned-over hSOD1

(A) The samples contained hSOD1 (30 μM in terms of monomer units), 25 mM bicarbonate and 0.1 mM DTPA in 50 mM phosphate buffer adjusted to pH 7.4. (B and C) The same as (A) plus: 1 mM H2O2 (B); 1 mM H2O2 and 75 μM tempol (C). All samples were incubated for 1 h at 37 °C. The samples were then reduced, denatured, alkylated, digested with trypsin and analysed by MALDI–TOF/TOF as described in the Experimental section of the main text. The spectra shown are representative of three independent experiments in the specified m/z regions. As a result of the bicarbonate-dependent peroxidase activity, the Trp 32 residue of native hSOD1 (V 31WGSIK36; m/z 689.4) is oxidized to kynurenine (V 31KynGSIK36; m/z 693.5), N-formylkynurenine (V 31NfKGSIK36; m/z 721.5) and to a Trp 32–Trp32 cross-linked dimer [(E 26SNGPVKVWGSIK36)2; m/z 2797.6], which is characterized by loss of a trypsin cleavage and a partial cleavage of the cross-link during MS analysis [E 26SNGPVKVWGSIK36; m/z 1400.8 and E26SNGPVKVW−2H(GSIK36); m/z 1398.8].

Figure S2 MALDI–TOF/TOF-MS analysis of turned-over hSOD1 in m/z region expected for tempol adducts

(A) The samples contained hSOD1 (30 μM in terms of monomer units), 25 mM bicarbonate and 0.1 mM DTPA in 50 mM phosphate buffer adjusted to pH 7.4. (B and C) The same as (A) plus: 1 mM H2O2 (B); 1 mM H2O2 and 75 μM tempol (C). All samples were incubated for 1 h at 37 °C. The samples were then reduced, denatured, alkylated, digested with trypsin and analysed by MALDI–TOF/TOF as described in the Experimental section of the main text. The spectra shown are representative of three independent experiments in the specified m/z regions. Putative adducts of tempol with the tryptic peptides of hSOD1 containing Trp32 could be expected at m/z 860.5 [V31W(tempol)GSIK36] and m/z 1571.9 [E26SNGPVKVW(tempol)GSIK36]. In the samples incubated with tempol, a reasonable peak at m/z 1571.6 was observed, but it did not provide consistent MS/MS spectra (results not shown).

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The samples contained hSOD1 (40 μM in terms of monomer units), 50 mM bicarbonate and 0.1 mM DTPA in 50 mM phosphate buffer adjusted to pH 7.4; (B and C) the same as (A) plus: 1 mM H₂O₂ (B); 1 mM H₂O₂ and 100 μM tempol (C). All samples were incubated 30 min at 37°C and were immediately desalted and concentrated with a ZipTipC18 before ESI-MS analysis as described in the Experimental section of the main text. The deconvoluted spectra were obtained by using the maximum entropy algorithm.

### Table S1 Sequencing of the peptides KVW(tempol)GS and KVWG

The m/z values of the fragments identified in the MS/MS analysis of the specified peptides by ESI-MS/MS. The spectra of both peptides were obtained under the same experimental conditions as those of Figure 6(B) of the main text. To facilitate analysis, the KVW(tempol)GS peptide and the Roepstorff–Fohlmann–Biemann nomenclature (fragments of the series a, b, c and x, y, z) are shown below.

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<th>Tryptophan(tempol)</th>
<th>Glycine</th>
<th>Serine</th>
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<th>Valine</th>
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