The Mechanism by which 4-Hydroxy-2,2,6,6-tetramethylpiperidene-1-oxyl (Tempol) Diverts Peroxynitrite Decomposition from Nitrating to Nitrosating Species

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Received October 9, 2001

Tempol is a stable nitroxide radical that has been shown to protect laboratory animals from the injury associated with conditions of oxidative and nitrosoactive stress. Tempol's protective mechanisms against reactive oxygen species have been extensively studied, but its interactions with reactive nitrogen species remain little explored. Recently, it has been shown that tempol is a potent inhibitor of peroxynitrite-mediated phenol nitration while it increases phenol nitrosation by a complex mechanism [Carrol et al. (2000) *Chem. Res. Toxicol.* **13**, 294]. To obtain further mechanistic insights, we reexamined the interaction of peroxynitrite with tempol in the absence and presence of carbon dioxide. Stopped-flow kinetic studies confirmed that tempol does not react directly with peroxynitrite but levels off the amount of oxygen (monitored with an oxygen electrode) and nitrite (monitored by chemiluminescence) produced from peroxynitrite in the presence and absence of carbon dioxide to about 30% and 70% of the initial oxidant concentration at pH 5.4, 6.4, and 7.4. Tempol inhibited phenol nitration while increasing the amounts of 4-nitrosophenol, that attained yields close to 30% of the peroxynitrite in the presence of carbon dioxide at pH 7.4. Fast-flow EPR experiments showed detectable changes in the instantaneous tempol concentration (maximum of 15%) only in the presence of carbon dioxide. Under these conditions, the instantaneous concentration of the carbonate radical anion was reduced by tempol in a concentration-dependent manner. The results indicate that tempol is oxidized by peroxynitrite-derived radicals ($^{\bullet}OH$ and $CO_3^{\bullet-}$, in the absence and presence of carbon dioxide, respectively) to the oxoammonium cation which, in turn, is reduced back to tempol while oxidizing peroxynitrite to oxygen and nitric oxide. The latter reacts rapidly with peroxynitrite-derived nitrogen dioxide to produce the nitrosating species, dinitrogen trioxide. Overall, the results support a role for peroxynitrite and its derived radicals in the tissue pathology associated with inflammatory conditions.

Introduction

Stable nitroxide radicals are membrane-permeable, low molecular weight antioxidants that have long been known to protect laboratory animals and bacterial and mammalian cells from the injury associated with oxidative stress conditions (*1*, *2*). Nitroxide antioxidant mechanisms have been shown to include dismutation of superoxide anion, detoxification of ferryl-heme species, and termination of radical chain reactions (*1*, *2*). More recently, the nitroxide tempol[1] has been shown to protect experimental animals from injuries associated with excessive nitric oxide production such as that resulting from multiple organ failure (*3*), transient cerebral ischemia (*4*), carrageenan-induced pleurisy (*5*), and dinitrobenzenesulfonic acid-induced colitis (*6*). In all of these models, tempol decreased the levels of 3-nitrotyrosine-protein residues in the injured tissues.

Although several physiological mechanisms can be responsible for the nitration of protein-tyrosine residues in vivo, formation of peroxynitrite in the presence of carbon dioxide is a particularly efficient one (*7*, *8*). It is noteworthy that recent in vitro studies have shown that tempol is a potent catalytic inhibitor of peroxynitrite-mediated phenol nitration while it increases phenol nitrosation (*9*). The mechanism proposed to explain the effects of tempol, however, was based on unprecedented reactions and on an excess production of carbonate radical anion over nitrogen dioxide from peroxynitrite (*9*).

Today, the yield of peroxynitrite decomposition to radicals in the absence (eqs 1 and 2) and presence of

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[1] Abbreviations: peroxynitrite and PN, sum of peroxynitrite anion [$ONOO^-$, oxoperoxonitrate(−1)] and peroxynitrous acid (ONOOH, hydrogen oxoperoxonitrate) unless specified; HCO_3^-/CO_2, sum of HCO_3^- and CO_2 unless specified; tempol and TP-NO$^{\bullet}$, 4-hydroxy-2,2,6,6,-tetramethyl-1-piperidinyloxy.

10.1021/tx015571z CCC: $22.00
Published on Web 03/06/2002

carbon dioxide (eq 3) has been established to be close to 30% (*10*−*20*).

$$ONOO^- + H^+ \rightleftarrows ONOOH \quad (1)$$

$$ONOOH \rightarrow 0.71\ NO_3^- + 0.71\ H^+ + 0.29\ {}^{\bullet}NO_2 + 0.29\ {}^{\bullet}OH \quad (2)$$

$$ONOO^- + CO_2 \rightarrow [ONOOCO_2^-] \rightarrow 0.65\ NO_3^- + 0.65\ CO_2 + 0.35\ {}^{\bullet}NO_2 + 0.35\ CO_3^{\bullet -} \quad (3)$$

In addition, by taking into account the secondary reactions of the radicals produced from peroxynitrite decay in the absence of carbon dioxide (eqs 1 and 2), Coddington and co-workers (*16*) simulated a net equation that was able to predict the long-known oxygen evolution from peroxynitrite at different pH values (*21*). In a simplified view, oxygen yield was shown to parallel hydroxyl radical yield because this species oxidizes peroxynitrite to nitric oxide and oxygen (see, for instance, eq 8, obtained by the addition of eqs 1, 2, and 4−7) (*16*). Since nitric oxide reacting with nitrogen dioxide produces the nitrosating agent dinitrogen trioxide (eq 5) (*22*), we hypothesized that a similar mechanism could operate during the interaction of tempol with peroxynitrite. In this work, this hypothesis is substantiated and a new mechanism is proposed to explain the effects of tempol in diverting peroxynitrite decomposition from nitrating to nitrosating species.

$$0.29\ {}^{\bullet}OH + 0.29\ ONOO^- \rightarrow 0.29\ {}^{-}OH + 0.29\ {}^{\bullet}NO + 0.29\ O_2 \quad (4)$$

$$0.29\ {}^{\bullet}NO_2 + 0.29\ {}^{\bullet}NO \rightleftarrows 0.29\ N_2O_3 \quad (5)$$

$$0.29\ N_2O_3 + 0.29\ H_2O \rightarrow 0.58\ NO_2^- + 0.58\ H^+ \quad (6)$$

$$0.29\ H^+ + 0.29\ {}^{-}OH \rightarrow 0.29\ H_2O \quad (7)$$

$$ONOOH + 0.29\ ONOO^- \rightarrow 0.29\ O_2 + H^+ + 0.58\ NO_2^- + 0.71\ NO_3^- \quad (8)$$

Experimental Procedures

Materials. Tempol was from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical grade or better. Peroxynitrite was synthesized from sodium nitrite (0.6 M) and hydrogen peroxide (0.65 M) in a quenched-flow reactor; excess hydrogen peroxide was used to minimize nitrite contamination. To eliminate excess hydrogen peroxide, the peroxynitrite solution was treated with manganese dioxide (*19*). Synthesized peroxynitrite contained low levels of contaminating hydrogen peroxide (<1%) and nitrite (10−30%) that were determined as previously described by the titanyl method and by absorbance measurements at 354 nm (ϵ = 246 $M^{-1}{\cdot}cm^{-1}$) (*23*). In some instances, nitrite and nitrite plus nitrate levels were measured by chemiluminescence (see below). The concentration of peroxynitrite stock solutions was determined spectrophotometrically at 302 nm (ϵ = 1670 $M^{-1}{\cdot}cm^{-1}$) (*19*, *23*). 2- and 4-nitrophenol were purified by sublimation and recrystallization, respectively. 4-Nitrosophenol was synthesized by incubating 5 mM phenol, 10 μM tempol, and 3 mM peroxynitrite at pH 10.5 for 48 h; the compound was purified by HPLC as described below and characterized by its UV−visible spectrum (ϵ_{400nm} = 3054 $M^{-1}{\cdot}cm^{-1}$) in methanol/potassium hydroxide (*24*). Concentrations of carbon dioxide were calculated from the added bicarbonate concentrations by using pK_a = 6.4 (*19*). Buffers were pretreated with Chelex-100 to remove contaminant metal ions. All solutions were prepared with distilled water purified with a Millipore Milli-Q system.

Stopped-Flow Studies. The rate of peroxynitrite decomposition in the presence and absence of tempol was monitored with a stopped-flow spectrophotometer (Applied Photophysics SX-18 MV) at 302 nm (*11*). Constant temperature was maintained at 25 ± 0.2 °C, and the pH of the reaction mixtures was determined at the outlet.

Oxygen Evolution. Oxygen evolution studies were performed using an oxygen monitor (Gilson 5/6 oxygraphy) at 25 ± 1 °C. The saturation oxygen concentration at this temperature was taken as 250 μM.

Chemiluminescent Assay of Nitrogen-Derived Products. The concentrations of nitric oxide, nitrite, and nitrate were determined with a nitric oxide analyzer (NOA^{TM280}, Sievers Instruments, Inc., Boulder, CO). Nitric oxide concentrations were determined by injecting aliquots of 2 min old incubation mixtures directly into the analyzing cell containing phosphate buffer, pH 7.5. In the case of nitrite and nitrite plus nitrate, the reaction mixtures containing 1 mM peroxynitrite with or without 1 mM carbon dioxide and 10 μM tempol in 0.25 M phosphate buffer of various pHs were incubated for 15 min at room temperature and diluted 10 times before analysis. Samples for nitrite analysis were reduced in the analyzing cell containing saturated sodium iodide solutions in 50% acetic acid at room temperature. Samples for nitrite plus nitrate analysis were reduced in the analyzing cell containing a saturated solution of vanadium chloride in 1 M HCl at 90 °C. Authentic sodium nitrite and sodium nitrate were used as standards for quantification.

HPLC Assay of Phenol-Derived Products. Peroxynitrite (1 mM) was added to mixtures containing 5 mM phenol, 1 mM carbon dioxide, and 10 μM tempol in 0.25 M phosphate buffer. After 15 min incubation at room temperature, aliquots were analyzed by HPLC (*25*). The HPLC system consisted of a Waters Associates (Milford, MA) model 625LC instrument equipped with a rheodyne injector and a photodiode array detector (Waters 996). Chromatographic separation was carried out using two Nova-Pak C-18 (3.9 × 150 mm, 4 μm particle size) columns in series eluted with 10% (v/v) acetonitrile in 27 mM acetate/30 mM citrate buffer, pH 3.5, at a flow rate of 1 mL/min. Eluting compounds were monitored at 280 nm. The products were identified by comparison of their retention times and UV−visible spectra with those of authentic standards under identical chromatographic conditions. The products were quantified by integration of the corresponding chromatographic peaks and comparison with those of known concentrations of standards.

EPR Experiments. The EPR fast-flow spectra were recorded at room temperature (25 ± 2 °C) on a Bruker EMX spectrometer operating at 9.65 GHz and 100 kHz field modulation equipped with a Bruker ER4117 D-MTV dielectric mixing resonator with a 9 mm distance between the mixing cell and the resonator center (*19*). Tempol solutions were prepared in appropriate buffers with or without added sodium bicarbonate; in the former case, the solutions were left undisturbed for 5 min to permit bicarbonate/carbon dioxide equilibration. Peroxynitrite solutions were prepared with water. Tempol and peroxynitrite solutions were transferred to 60 mL plastic syringes mounted on a syringe infusion pump (Harvard apparatus pump 22). Spectra were recorded at about 8 ms after mixing at a continuous flow of 14 mL/min.

Results

Kinetic Studies. Stopped-flow experiments showed that 1−10 μM tempol did not significantly affect the rate of peroxynitrite decomposition (k_{obs} = 0.4 s^{-1}) (data not shown), confirming that the nitroxide does not react directly with the oxidant as previously proposed (*9*). However, in the presence of equimolar carbon dioxide (1.0 mM) that greatly accelerates peroxynitrite decomposition to produce a high flux of carbonate radical anion and

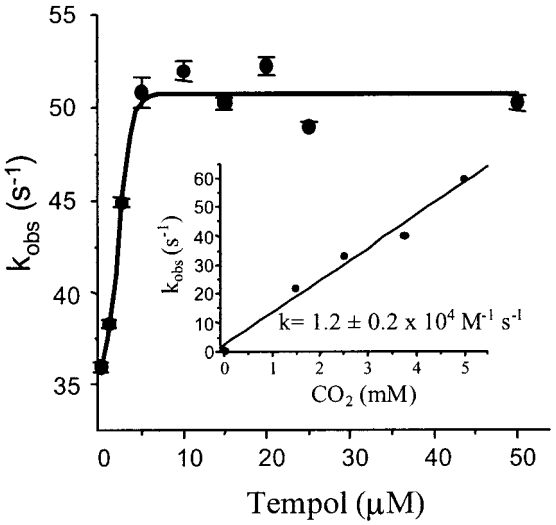

Figure 1. Effects of tempol on the apparent rate of peroxynitrite decomposition in the presence of carbon dioxide in 0.25 M phosphate buffer, pH 7.4 at 25 °C. In these experiments, 1 mM peroxynitrite was mixed with 2.5 mM carbon dioxide and the specified tempol concentrations. The k_{obs} obtained in the presence of 2.5 mM carbon dioxide and the absence of tempol (36 s^{-1}) was smaller than that expected from the literature value of the second-order rate constant of the reaction between peroxynitrite and carbon dioxide, pH 7.4 at 25 °C (2.6×10^4 $M^{-1}\cdot s^{-1}$) (*26*). The second-order rate constant determined under our experimental conditions (0.25 M phosphate buffer, pH 7.4, 25 °C) by following the rate of 200 μM peroxynitrite decay in the presence of excess carbon dioxide was (1.2 ± 0.2) $\times 10^4$ $M^{-1}\cdot s^{-1}$ (inset).

nitrogen dioxide (eq 3) [$k = (1.2-2.6) \times 10^4$ $M^{-1}\cdot s^{-1}$ at pH 7.4, 25 °C] (Figure 1 inset) (*26*), 10 μM tempol increased the rate of peroxynitrite decomposition by ca. 40% (data not shown). To confirm tempol's accelerating effects, the experiments were repeated in the presence of excess carbon dioxide (2.5 mM). Even under these conditions, the fast peroxynitrite decomposition (k_{obs} = 36 s^{-1}) was increased by low tempol concentrations, leveling off around 50 s^{-1} (Figure 1). Probably, in the presence of both carbon dioxide and tempol, an intermediate that reacts with peroxynitrite is produced fast enough to result in detectable changes of peroxynitrite decomposition rates. In the absence of carbon dioxide, the same intermediate is likely to be produced (see below). Its formation rate, however, depends on the slow spontaneous peroxynitrite decomposition precluding measurable changes in the overall peroxynitrite decay rates.

Assay of Evolved Oxygen. The amount of oxygen liberated during peroxynitrite decomposition under our experimental conditions followed the previously reported pH profile with about 20% of the initial oxidant decomposing to oxygen at pH 7.4 (Figure 2A) (*16*, *21*). In the presence of catalytic amounts of tempol (1−10 μM), oxygen evolution was constant from pH 5.5 to 8.1 and corresponded to about 30% of the initial peroxynitrite concentration (Figure 2A, Table 1). In the presence of carbon dioxide, oxygen evolution was barely detectable at pH 5.4, 6.4, and 7.4 (Figure 3, Table 1). This was expected because most of the peroxynitrite is rapidly decomposed to nitrogen dioxide and carbonate radical anion (eq 3). The latter oxidizes peroxynitrite comparatively slowly ($k = 7.7 \times 10^6$ $M^{-1}\cdot s^{-1}$) (*27*) as compared with the hydroxyl radical ($k = 4.8 \times 10^9$ $M^{-1}\cdot s^{-1}$), and its main decay route is likely to be reaction with contaminant nitrite (*16*). In the presence of catalytic

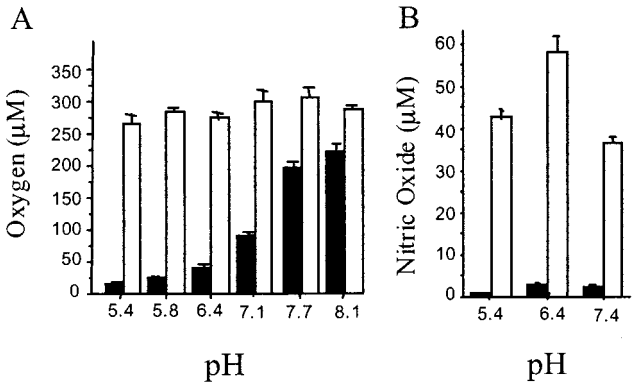

Figure 2. Effects of 10 μM tempol on the amounts of oxygen (A) and nitric oxide (B) produced during decomposition of 1 mM peroxynitrite at various pHs. The experiments were run in 0.25 M phosphate buffer at 25 °C and the assays performed as described under Experimental Procedures: in the absence (black bars) and presence (white bars) of tempol.

Table 1. Production of Oxygen, Nitrite, and Nitrate during the Decomposition of 1 mM Peroxynitrite under Various Experimental Conditions[a]

system	[oxygen] (mM)	[nitrite] (mM)	[nitrite] + [nitrate] (mM)
PN, pH 5.4	0.019 ± 0.005	0.060 ± 0.011	0.954 ± 0.062
PN and tempol, pH 5.4	0.266 ± 0.011	0.714 ± 0.037	0.960 ± 0.053
PN, pH 6.4	0.053 ± 0.007	0.145 ± 0.018	0.936 ± 0.054
PN and tempol, pH 6.4	0.277 ± 0.011	0.677 ± 0.032	0.958 ± 0.036
PN, pH 7.4	0.162 ± 0.011	0.255 ± 0.016	0.977 ± 0.056
PN and tempol, pH 7.4	0.349 ± 0.023	0.755 ± 0.029	0.938 ± 0.049
PN and CO_2, pH 5.4	0.020 ± 0.004	0.084 ± 0.014	0.891 ± 0.050
PN, CO_2, and tempol, pH 5.4	0.265 ± 0.007	0.722 ± 0.043	0.897 ± 0.059
PN and CO_2, pH 6.4	0.014 ± 0.005	0.118 ± 0.014	0.889 ± 0.064
PN, CO_2, and tempol, pH 6.4	0.282 ± 0.012	0.713 ± 0.039	0.955 ± 0.047
PN and CO_2, pH 7.4	0.019 ± 0.005	0.179 ± 0.012	0.956 ± 0.052
PN, CO_2, and tempol, pH 7.4	0.350 ± 0.024	0.762 ± 0.034	0.942 ± 0.044

[a] When present, the concentrations of carbon dioxide and tempol were 1 mM and 10 μM, respectively. The experiments were run in 0.25 M phosphate buffer at 25 °C and the assays performed as described under Experimental Procedures. The values correspond to the mean ± SD of the values obtained from three independent experiments. In the case of nitrite and nitrite plus nitrate, the values were corrected for the contaminant concentrations present in 1 mM peroxynitrite (0.205 ± 0.015 mM nitrite and 0.335 ± 0.023 mM nitrite plus nitrate) that were determined as described under Experimental Procedures.

amounts of tempol, however, oxygen evolution was again detectable (Figure 3) and in yields close to 30% of the initial peroxynitrite concentration (Table 1).

Assay of Nitrogen-Derived Products. In addition to oxygen, nitrite and nitrate are stable products of peroxynitrite decomposition (eqs 1−8). Nitrite and nitrite plus nitrate concentrations were measured by chemiluminescence under different experimental conditions. As shown in Table 1, nitrite yields increased in the presence of tempol, leveling off to ca. 70% of the initial peroxynitrite concentration both in the absence and in the presence of carbon dioxide. An increased yield of oxygen and nitrite in the presence of tempol (Table 1) is consistent with peroxynitrite oxidation to nitric oxide and

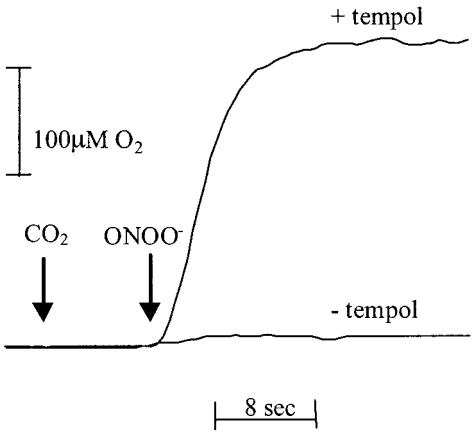

Figure 3. Oxygen evolution from peroxynitrite in the presence of carbon dioxide and tempol. At the time shown by the arrow, 1 mM peroxynitrite was added to equilibrated solutions of 2 mM carbon dioxide with or without 10 μM tempol as specified in the figure. The experiments were run in 0.25 M phosphate buffer, pH 7.4, at 25 °C.

Table 2. Production of 4-Nitrosophenol, 2-Nitrophenol, and 4-Nitrophenol during the Decomposition of 1 mM Peroxynitrite in the Presence of 1 mM Carbon Dioxide and 5 mM Phenol[a]

system	[4-nitrosoP][b] (mM)	[2-nitroP] (mM)	[4-nitroP] (mM)
PN, CO_2, pH 6.4	0.007 ± 0.003	0.068 ± 0.003	0.052 ± 0.003
PN, CO_2, and tempol, pH 6.4	0.124 ± 0.017	ND[c]	ND
PN, CO_2, pH 7.4	0.011 ± 0.003	0.106 ± 0.009	0.082 ± 0.008
PN, CO_2, and tempol, pH 7.4	0.268 ± 0.026	0.012 ± 0.004	0.014 ± 0.005

[a] When present, the concentration of tempol was 10 μM. The experiments were run in 0.25 M phosphate buffer at 25 °C and the assays performed as described under Experimental Procedures. Product analysis and quantification was performed by HPLC as described under Experimental Procedures. The values correspond to the mean ± SD of the values obtained from three independent experiments. [b] 4-Nitrosophenol, 2-nitrophenol, and 4-nitrophenol. [c] Not detectable.

oxygen (eqs 4−6). In agreement, tempol increased the amounts of nitric oxide that can be detected by chemiluminescence during peroxynitrite decay (Figure 2B). Detectable nitric oxide levels, however, were low and corresponded to about 5% of the initial peroxynitrite concentration in the absence (Figure 2B) and in the presence of carbon dioxide (data not shown). Such low values are probably due to the fast reaction between nitric oxide and nitrogen dioxide to produce dinitrogen trioxide ($k_5 = 1.1 \times 10^9\ M^{-1}\cdot s^{-1}$) (eq 5) (*22*). In agreement, addition of a dinitrogen trioxide trap, phenol, led to the detection of 4-nitrosophenol, whose yields attained ca. 30% of the initial peroxynitrite concentration at pH 7.4 and in the presence of carbon dioxide (Table 2). In parallel, the yield of phenol-nitrated products decreased as previously reported (*9*).

EPR Studies. EPR spectroscopy was used to follow a possible transient consumption of tempol during its interaction with peroxynitrite. Previously, LC/MS/MS was used to show that tempol acts as a true catalyst because it was recovered unaltered from peroxynitrite-containing incubations (*9*). Similarly, it was impossible to detect significant changes in the instantaneous concentrations of tempol by EPR of fast-flow mixtures of peroxynitrite (1 mM) and tempol (1−10 μM) (data not shown). In the presence of excess carbon dioxide (2.5 mM), however, a small (9−15%) but reproducible decrease in the intensity of the fast-flow EPR tempol spectrum was always detectable (see, for instance, Figure 4D). To better follow the small changes in the instantaneous tempol concentration, the spectra shown in Figure 4D were scanned with a modulation amplitude of 1 G, and the spectrum of the complete reaction mixture (broken lines) was displaced to the right. The intensity of the fast-flow EPR spectrum of the carbonate radical anion obtained in mixtures of 2.5 mM carbon dioxide and 1 mM peroxynitrite was also decreased by tempol (Figure 4A−C) in a concentration-dependent manner and became undetectable with 10 μM nitroxide (Figure 4, inset). It should be noted that the spectra shown in Figure 4A−C were scanned with 5 G of modulation amplitude to permit

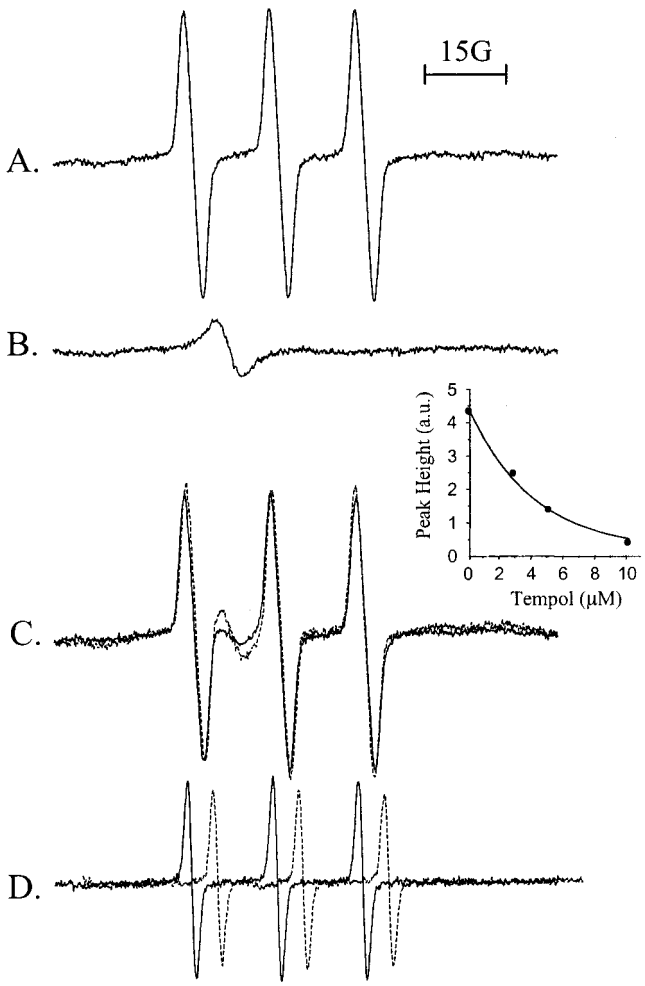

Figure 4. EPR continuous fast flow spectra of carbonate radical anion and tempol at pH 7.4. The spectra were obtained as described under Experimental Procedures: (A) tempol spectrum by mixing 5 μM tempol with buffer, (B) carbonate radical anion spectrum by mixing 1 mM peroxynitrite with 2.5 carbon dioxide, (C) tempol and carbonate radical anion spectrum by mixing 1 mM peroxynitrite with 2.5 mM carbon dioxide and 5 μM tempol; the superimposed spectrum in broken lines is the computer addition of (A) and (B); (D) the same as (A) but using 1 G modulation amplitude; the superimposed spectrum displaced to the left corresponds to (B) at 1 G modulation amplitude. The specified concentrations are those in the final reaction mixtures. The inset shows the concentration-dependent effect of tempol in inhibiting the peak height (a.u. = arbitrary units) of the carbonate radical anion signal obtained under the experimental conditions of (B). Instrumental conditions were as follows: microwave power, 2 mW; time constant, 163.84 ms; scan rate, 0.6 G/s; gain, 2.0×10^5; modulation amplitude, 5 G except for (D) where 1 G was used.

detection of the carbonate radical anion (*19*). These results indicate that extremely low levels of tempol-derived intermediates are present during catalysis. Also, they indicate that tempol reacts very rapidly with the carbonate radical anion.

Discussion

The results reported here confirm that tempol does not react directly with peroxynitrite but diverts its decomposition from nitrating to nitrosating species as previously reported (*9*). This latter work proposed a complex mechanism for explaining the effects of tempol that was based on the relative yields of phenol nitration and nitrosation obtained in the absence and presence of tempol. Here, we quantified the yield of most peroxynitrite-derived products (Figures 1−4, Tables 1 and 2). The main effect of tempol was to level off the amount of oxygen and nitrite produced from peroxynitrite to ca. 30% and 70% of the initial oxidant concentration, respectively (Figure 2A, Figure 3, Table 1). In addition, tempol increased production of a nitrosating species, probably dinitrogen trioxide, that attained yields close to 30% peroxynitrite at pH 7.4 as inferred from 4-nitrosophenol yield (Table 2). Thirty percent is roughly the yield of radicals produced from peroxynitrite in the absence (eq 2) and presence of carbon dioxide (eq 3) (*10*−*20*). Consequently, the results suggest that oxidation of tempol by peroxynitrite-derived radicals, hydroxyl radical (eq 9) or carbonate radical anion (eq 10), produces the corresponding oxoammonium cation which is reduced back to tempol by oxidizing peroxynitrite to oxygen and nitric oxide (eq 11). The latter reacts with peroxynitrite-derived nitrogen dioxide (eqs 2 and 3) to produce dinitrogen trioxide (eq 5).

$$\text{TP}-\text{NO}^{\bullet} + {}^{\bullet}\text{OH} \rightarrow \text{TP}-\text{N}{=}\text{O}^{+} + {}^{-}\text{OH} \qquad (9)$$

$$\text{TP}-\text{NO}^{\bullet} + \text{CO}_3^{\bullet -} + \text{H}^{+} \rightarrow \text{TP}-\text{N}{=}\text{O}^{+} + \text{HCO}_3^{-} \qquad (10)$$

$$\text{TP}-\text{N}{=}\text{O}^{+} + \text{ONOO}^{-} \rightarrow \text{TP}-\text{NO}^{\bullet} + {}^{\bullet}\text{NO} + \text{O}_2 \qquad (11)$$

This sequence of reactions is compatible with the known chemistry of tempol whose cycling between reduced and oxidized form has been previously shown to mediate several of its biochemical effects (*1*, *2*, *28*−*30*). The possibility of tempol being fast-oxidized to the oxoammoniun cation by peroxynitrite-derived nitrogen dioxide appears unlike due to its low redox potential (1. 0 V) as compared with those of the hydroxyl radical (2.3 V) and of the carbonate radical anion (1.8 V) (*31*). In addition, the pronounced increase of 4-nitrosophenol yield in the presence of tempol (Table 2) indicates that nitrogen dioxide is decaying mainly through reaction with produced nitric oxide (eq 5). Indeed, nitric oxide was hardly detectable under our experimental conditions (Figure 2B; see also Results). The above sequence of reactions also explains the low levels of 2- and 4-nitrophenol detected in the presence of tempol and carbon dioxide (Table 2). Tempol is oxidized by the carbonate radical anion that otherwise would oxidize phenol to the phenoxyl radical, an intermediate of phenol nitration (*32*−*34*).

It should be noted that both oxygen liberation and nitrite levels measured in the presence of tempol were higher than those predicted from 30% peroxynitrite being oxidized to oxygen and nitric oxide by the oxammonium cation. These yields can be estimated from eqs 4−8 because the stoichiometry of peroxynitrite oxidation does not depend on the oxidant species [hydroxyl radical (eq 4) or oxammonium cation (eq 11)]. Equation 8 predicts yields of 23% oxygen and 46% nitrite whereas we measured ca. 30% and 70%, respectively. However, eq 8 is a simplification that does not take into account the basal nitrite levels present in peroxynitrite preparations or the pH. Depending on the pH, spontaneous peroxynitrite decomposition yields from 0 to 50% oxygen and from 0 to 100% nitrite (*16*). This is because at slightly alkaline pHs, oxygen is also produced by routes other than peroxynitrite oxidation (see ref *16* for details). In the presence of tempol, the amount of liberated oxygen was pH- and carbon dioxide-independent (Figure 2A, Table 1), further indicating that oxygen is mainly produced from peroxynitrite oxidation by the oxoammonium cation (eq 11). Considering the complexities of the systems, the measured levels of nitrite and oxygen (Table 1) are pretty consistent with the mechanism proposed to explain tempol effects in the absence (eqs 1, 2, 9, and 11) and presence (eqs 3, 10, and 11) of carbon dioxide.

Unequivocal proof of the above mechanism would require the detection of the oxammonium cation. However, extremely low instantaneous concentrations of this species are produced because the changes detected in the intensity of the fast-flow EPR spectra of tempol under all tested experimental conditions varied from 0 to 15% (see, for instance, Figure 4D). This indicates that the rate constants of reactions 9−11 are extremely high. Indeed, the rate constant of the reaction between tempol and the hydroxyl radical is known to be close to the diffusion-controlled limit (*35*). The inhibitory effect of tempol on the instantaneous concentrations of the carbonate radical anion detected by continuous fast-flow EPR (Figure 4) indicates that the rate constant of their reaction is also extremely high, although the actual value has not been determined. The rate constant of peroxynitrite oxidation by the oxammonium cation should be close to the diffusion-controlled limit because the less reactive hydrogen peroxide is oxidized by the cation with a rate constant of 1.2×10^8 $M^{-1}{\cdot}s^{-1}$ (*36*). It is noteworthy that tempol accelerated to some extent the rate of peroxynitrite decomposition in the presence of carbon dioxide (Figure 1A). Such an accelerating effect was not observed in the absence of carbon dioxide because the rate of hydroxyl radical production, and consequently oxammonium cation formation, is too slow to significantly affect the rate of peroxynitrite decomposition. Likewise, the small decrease detected in the instantaneous tempol concentrations (maximum of 15%) was observed only in the presence of carbon dioxide (Figure 4; also see Results). Although a detailed kinetic analysis is precluded at this point, the present results and the known chemistry of tempol (*1*, *2*, *28*−*30*) are consistent with the proposed mechanism.

In conclusion, we propose that tempol diverts peroxynitrite decomposition from nitrating to nitrosating species by reacting with the most oxidizing peroxynitrite-derived radicals, the hydroxyl radical or the carbonate radical anion, to produce the oxammonium cation that oxidizes peroxynitrite to oxygen and nitric oxide. Thus, the protective effects of tempol against injury associated with an overproduction of nitric oxide (*3*−*6*) could be ascribed to its superoxide dismutase activity preventing peroxynitrite production (*2*), to catalysis of peroxynitrite decomposition to dinitrogen trioxide (this work), or to

both. Any of these possibilities supports the view that peroxynitrite and its derived radicals contribute to tissue pathology in inflammatory conditions.

Acknowledgment. We thank Dr. Dulcinéia S. P. Abdalla for providing access to the nitric oxide analyzer. This work was partially supported by grants to O.A. from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Financiadora de Estudos e Projetos (FINEP).

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TX015571Z