This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2005)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2005 Term

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Nitroxides as Antioxidants, beyond Spin Probes

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For 77:222, Spring 2005

24 Feb 2005

Abbreviations:
CHD, spiro [cyclohexane- 1,2'-doxyl] [spiro[cyclohexane- 1,2'-(4',4'-dimethyl-oxazolidine-3'-oxyl)]. **DMPO**, 5,5-dimethyl-1-pyrroline-N-oxide. **EPR**, electron paramagnetic resonance. **ESR**, electrons spin resonance. **MRI**, magnetic resonance imaging. **OXANO**: 2-ethyl-2,5,5-trimethyl-3-oxazolidinoxyl. $\mathbf{O_2^{\bullet-}}$, superoxide. **OXANOH**: 2-ethyl-2,5,5-trimethyl-3-oxazolidine. $\mathbf{^{\bullet}OOH}$, protonated superoxide **PNA**, polynitroxyl-albumin. **TPO**, 2,2,6,6-tetramethyl-piperidinoxyl. **TPL**, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl. **TPH**, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-hydroxyl.

Outline **Page**

Abstract

Stable nitroxide free radicals have been employed to probe various biophysical and biochemical processes involving oxidative stress. Previous studies demonstrated that nitroxides at non-toxic concentrations are effective as *in vitro* and *in vivo* antioxidants. Therefore, studying the mechanism underlying their antioxidant activity will help to improve therapeutic strategies by nitroxides. The capability of nitroxides to remove intracellular superoxides, to terminate radical-chain reactions, to oxidizes deleterious metal ions and prevent Fenton reaction may contribute their distinguished functions as antioxidants.

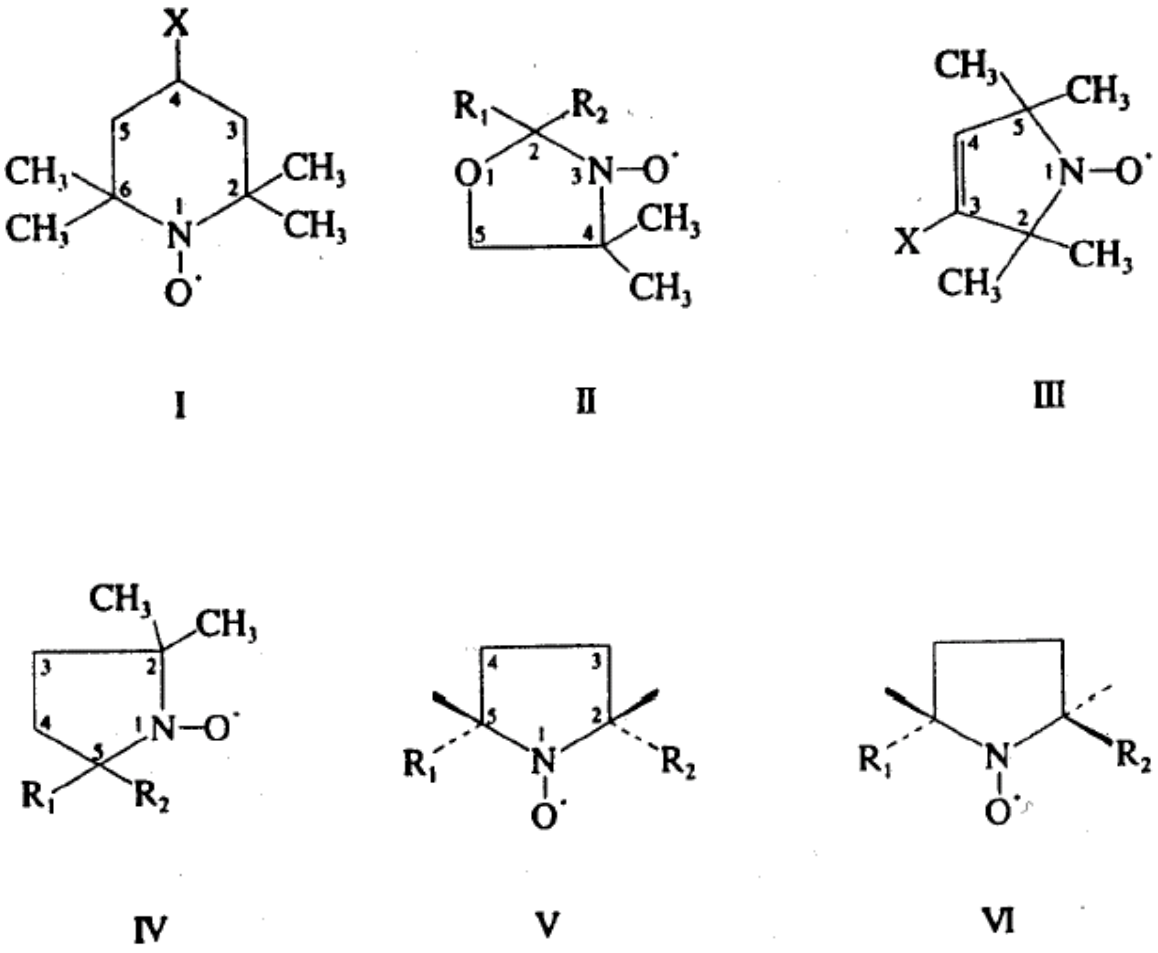
Introduction

Nitroxide stable free radicals or nitroxyl spin labels as they may be called, are chemically stable organic compounds that have an unpaired electron resulting in paramagnetic properties. Because of this, they have been used as imaging agents for electron spin resonance (ESR) studies and as contrast agents for magnetic resonance imaging (MRI) in animals [2]. An increasing number of studies demonstrated that nitroxides possess antioxidant functions, which suggested their important therapeutic potential in a wide range of diseases. In this paper, some chemical properties of nitroxides and their antioxidant functions and the mechanism behind that will be discussed.

The chemical properties of nitroxides

Nitroxides generally consist of a six-member ring piperidine derivative (such as TEMPO and TPO) or a five-member ring pyrroxamide derivative (such as ONANO) (Figure 1). The unpaired electron of the nitroxide moiety can be detected by EPR spectroscopy (Figure 2). Nitroxides are usually quite stable in aqueous solution, especially in alkali solutions [5]. The persistent nitroxide can still maintain signal intensity even at 250°C. Steric blocking methyl groups or alkyl groups may contribute to the kinetic stability [5]. Despite that rather immobilized electron in nitroxides, they can still undergo various reactions. Morris *et al* compared the reduction rates of five-membered pyrrolidine and pyrroline, and six-membered piperidine nitroxides. Their results showed an increased rate of reduction of six-membered nitroxides compared with those of the five-membered nitroxides [6]. The difference in the accessibility of their nitroxide group may contribute to this effect. A double bond in the five-membered nitroxyls increases the reduction rate

[6]. Results form Fuchs *et al* showed that the rate of reduction of nitroxides in shin varies considerably with nitroxide ring structure and substitution. The order of nitroxide stability in isolated human keratinocytes and human skin is imidazoline> pyrrolidine>piperidine>oxazolidine [7]. Cationic nitroxides are most unstable likely due to transmembrane electron shuttle or internalization [7].



I II III IV V VI

Figure. 1. **Structural classes of nitroxides**: (I) piperidine; (II) oxazolidein; (III) pyrroline; (IV-VI) pyrrolidine. From [1].

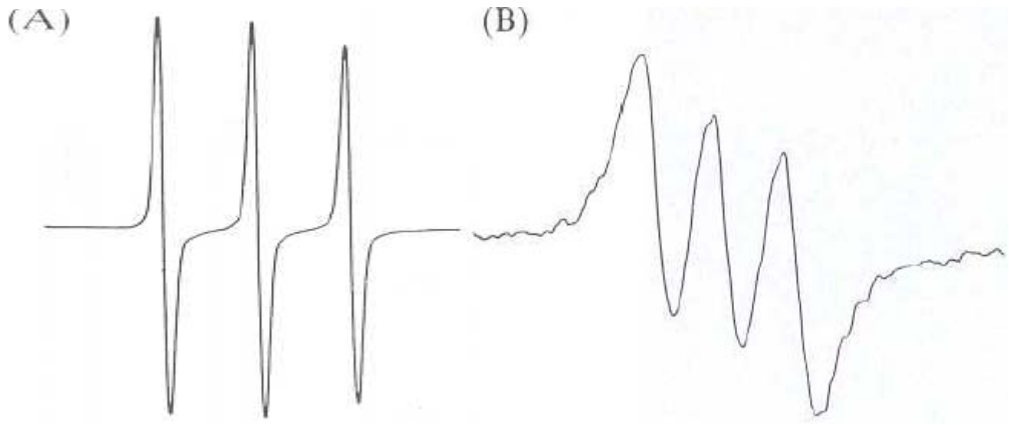
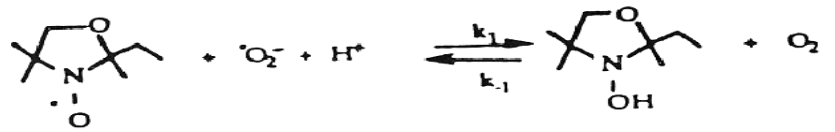


Figure 2: A, First derivative electron paramagnetic resonance spectrum of 5 mM TEMPOL in MEM in an EPR flat cell at ambient temperature. B, EPR spectrum of TEMPOL in the presence of broadening agent (100 mM $K_3Cr(C_2O_4)_3$) in MEM [4].

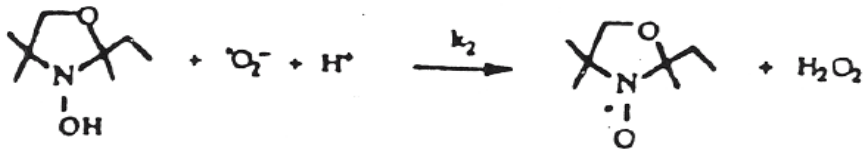
Nitroxides as antioxidants and mechanism

Remove $O_2^{\bullet-}$, nitroxides acts as scavengers of $O_2^{\bullet-}$ or as SOD mimics?

Nitroxides were found to be reduced to the corresponding hydroxylamine by $O_2^{\bullet-}$. This finding originally stemmed from the observation of $O_2^{\bullet-}$-induced spin-loss of OH spin-adduct of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The ESR signals of persistent nitroxide spin-adducts such as DMPO-OH rapidly decayed to a lower steady state when exposed to a continuous $O_2^{\bullet-}$-flux [8]. Oxazolidine nitroxides are supposed to dismutate $O_2^{\bullet-}$ through the following reaction pathways.



(1)

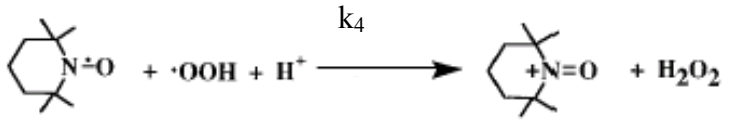


(2)

Superoxide reacts with OXANO through one electron reduction to yield diamagnetic ("ESR-silent") hydroxylamine OXANOH (Reaction 1). OXANOH and $O_2^{\bullet-}$ reacts to yield OXANO (Reaction 2) [9]. When OXANO reacts with $O_2^{\bullet-}$, rate constants k_1 and k_2 are 1.3 x 10^2 and 6.7 x 10^3 $M^{-1}s^{-1}$ respectively at pH 7.8 [9]. When TEMPO or TEMPOL react with $O_2^{\bullet-}$, rate constants at pH 7.8 range from 7 x 10^4 $M^{-1}s^{-1}$ to 1.2 x 10^5 $M^{-1}s^{-1}$ [8]. The overall reaction is the following:

$$2H^+ + 2O_2^{\bullet-} \longrightarrow H_2O_2 + O_2 \qquad (3)$$

Piperidine derivatives such as TPO and TPL are readily oxidized by $^{\bullet}OOH$ (protonated superoxide) to yield oxoammonium cation (Reaction 4), which in turn oxidizes another $O_2^{\bullet-}$ to molecular oxygen (Reaction 5) [10]. The reaction rate constants k_4 were 7.4 x 10^5 $M^{-1}s^{-1}$ and 2.0 x10^5 $M^{-1}s^{-1}$ for TPO and TPL, respectively [10]. Reaction 4 is the rate-limiting step in the catalytic dismutation of $O_2^{\bullet-}$ by nitroxides, because the second step (Reaction 5) is much faster having k_5 = 1.5 x 10^10 $M^{-1}s^{-1}$ [10].



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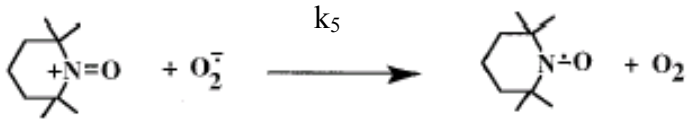
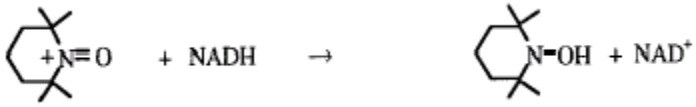


(5)

The net process of reaction 4 & 5 is reaction 3, in which $O_2^{\bullet-}$ induced depletion and regeneration of the nitroxides, and $O_2^{\bullet-}$ is removed without affecting nitroxides concentration. The above description supports the idea that nitroxides acts as SOD Mimics.

In the presence of reducing agents such as NADH and NADPH, transient oxoammonium cation was reduced through a two-electron reduction to its corresponsing hydroxylamin (Reaction 6) [10].



(6)

If only 1 mM NADH was added to the mix of $O_2^{\bullet-}$, and TPO, EPR signal decreased rapidly (Figure 3) [10]. From those results, nitroxides should be regarded as scavengers rather than SOD mimics.

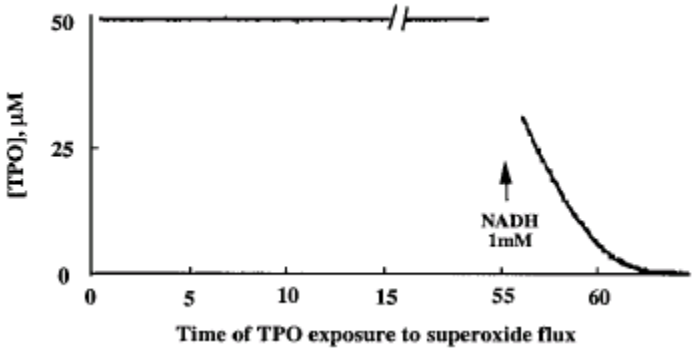
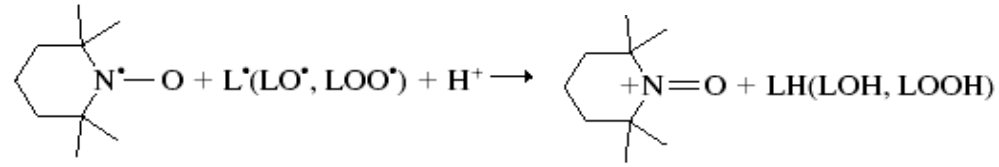


Figure. 3. **Invariance of the SOD mimic TPO subjected to superoxide flux over time.** A constant flux of ~16 mM/min O_2 was generated at 23 ± 2°C by 5 mM HX and 0.08 unit/mL XO in 50 mM phosphate buffer, pH 7, containing 50 mM TPO, 50 mM DTPA, and 260 units/mL catalase. The reaction mixture was inserted in a gas-permeable capillary, placed inside the EPR cavity, and flushed with air, whereas the intensity of TPO EPR signal was continuously monitored. Upon the addition of 1 mM NADH to the reaction mixture (*arrow*), the EPR signal of TPO decayed [10].

Terminating radical-chain reactions

Both nitroxide and hydroxylamine can reduce carbon-centered and oxygen-centered radicals and break the radical chain reaction (Reaction 7 & 8) [13].



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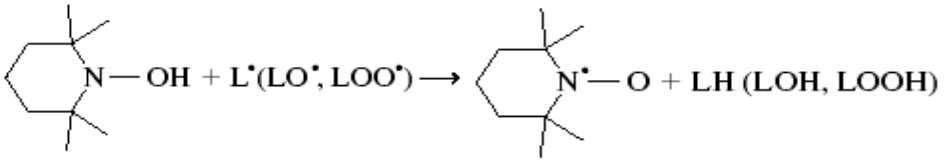
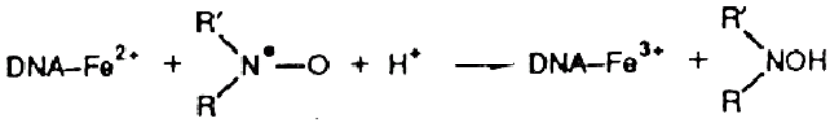


(8)

The high efficacy of both the nitroxide and its respective reduced form seem to result from the catalytic mode of their action. While switching back and forth between themselves (Reaction 1 & 2), stable nitroxides can terminate chain reaction of deleterious radicals with higher efficacy [13].

Preempt the Fenton reaction

Previous studies have shown that stable nitroxides are effective inhibitors of H_2O_2 - induced damage in mammalian cells [11]. Tissue injury can enhance iron mobilization and to increase the iron pool and free radical formation [13]. Mitchell found that, in the presence of CHD, DNA-Fe(II) reacted preferentially with CHD (reaction 9), which blocked the metal-catalyzed reduction of H_2O_2 to $^{\bullet}OH$ which is highly reactive and can cause damage to DNA [12].



(9)

Detoxification of hypervalent toxic metal species

Oxymyoglobin (oxyMbFe(II)), the major heme protein in muscle tissue, can undergo oxidation and yield $O_2^{\bullet-}$ and MbFe(III) [15]. H_2O_2 and MbFe(III) has been shown to produce a two-electron oxidation product of myoglobin by a heterolytic cleavage of the O-O bond of the peroxide coordinated to the heme (Reaction 10). The oxoferryl species are chemically reactive and can initiate free radical-mediated reactions that could result in biologic damage [14].

$$MbFe^{III} + H_2O_2 \longrightarrow {}^{\bullet}MbFe^{IV} + H_2O^{\bullet} \quad (10)$$

$$^{\bullet}MbFe^{IV} + H_2O_2 \longrightarrow MbFe^{IV} + O_2^{\bullet-} + 2H^+ \quad (11)$$

Besides this, the ferryl moiety can oxidize another H_2O_2 molecule to $O_2^{\bullet-}$ (Reaction 11). MbFe(IV) can oxidize critical targets of cells.

$$MbFe^{IV} + TPL \longrightarrow MbFe^{III} + TPL^{\cdot} \quad (12)$$

$$H_2O_2 + TPL^{+} \rightleftharpoons O_2^{\cdot-} + TPL + 2H^{+} \quad (13)$$

$$O_2^{\cdot-} + TPL^{+} \longrightarrow O_2 + TPL \quad (14)$$

Nitroxides can reduce and detoxify MbFe(IV), yielding the oxoammonium cation TPL^{+}, which can oxidize another H_2O_2 through Reaction 13 or generate O_2 by oxidizing $O_2^{\cdot-}$ through reaction 14 [14].

Summary

Nitroxides are chemically stable free radicals and can be detected by EPR spectra. The protection of oxidative damage by non-toxic levels of nitroxides have several plausible chemical explanations: 1) Remove superoxides. 2) Termination of free radical chain reactions. 3) Preempt the Fenton reaction. 4) Detoxification of hypervalent toxic metal species.

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