Volume 222, number 1, 75–78 FEB 05122 September 1987

A new approach for EPR detection of hydroxyl radicals by reaction with sterically hindered cyclic amines and oxygen

I. Rosenthal*, C. Murali Krishna, G.C. Yang*, T. Kondo and P. Riesz

**Division of Contaminant Chemistry, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, DC 20204* and *Radiation Oncology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Received 3 August 1987

Sterically hindered cyclic amines react with hydroxyl radicals in the presence of oxygen to yield stable nitroxide radicals which can be detected by EPR. This reaction provides a nonconventional spin-trapping tool for detection of hydroxyl radicals.

Hydroxyl radical; Cyclic amine; Spin trapping; EPR

1. INTRODUCTION

The possibility of identifying hydroxyl radicals in living cells during various natural or pathological metabolic processes by spin trapping has been frequently suggested [1–3]. However, the hydroxyl trapping by nitrones, specifically DMPO, which is most frequently used for this purpose, is plagued by chemical artifacts [4] and by the short lifetime of the spin adduct in the biological milieu due to the presence of a hydrogen atom at the α-carbon atom of the nitroxide [5].

The oxidation of sterically hindered, α,α'-tetra-substituted heterocyclic amines to nitroxide radical by singlet molecular oxygen [6] has been suggested as a detection method for the latter species [7–9], pending appropriate control tests. Many other strong oxidants may produce the same radical. Thus, oxidation of secondary amines by hydroperoxides, peroxides and peracids is one of the most important methods of preparing disubstituted nitroxides although the course of this reaction is not well known [10,11]. Nevertheless, these cyclic amines were reported not to react with hydroxyl radicals [12].

We found that α,α'-tetra-substituted cyclic secondary amines react with hydroxyl radicals in the presence of oxygen to yield stable nitroxide radicals.

2. EXPERIMENTAL

Four amines were tested: 2,2,6,6-tetramethylpiperidine (TMP), 2,2,6,6-tetramethyl-4-aminopiperidine (TMAP), 2,2,6,6-tetramethyl-4-piperidone (TMPone), and 2,2,6,6-tetramethyl-4-piperidol (TMPol) (Aldrich). Before use, the amines were purified by treatment with activated charcoal

Correspondence address: I. Rosenthal, Division of Contaminant Chemistry, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, DC 20204, USA; or P. Riesz, Radiation Oncology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)

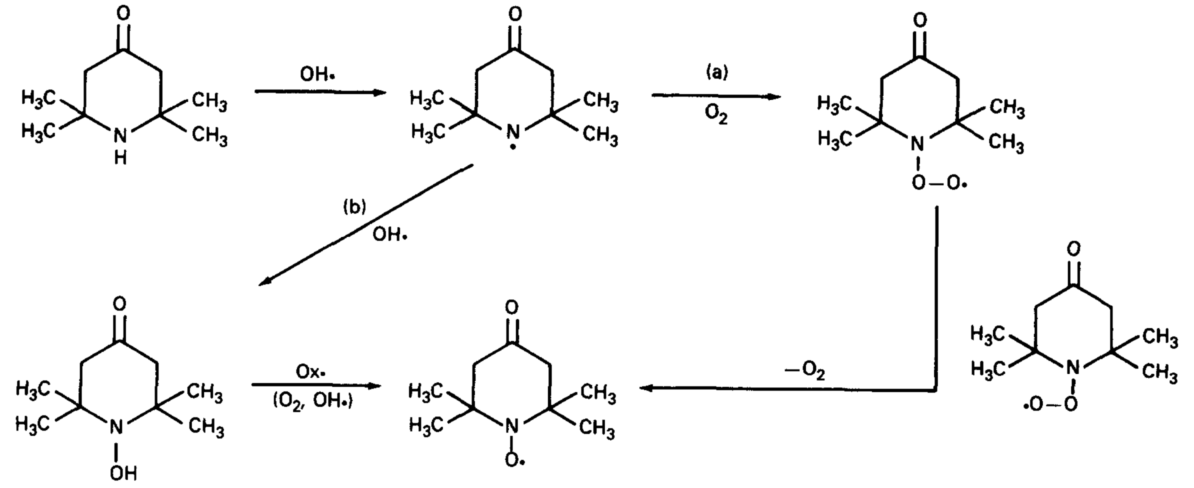

Fig.1. The chemical mechanism for generation of nitroxide radical by the reaction of TMPone with hydroxyl radical.

in aqueous solutions to remove the traces of nitroxides found in the commercial products. The γ-irradiations were performed in a ^{60}Co source (36 Gy/min). The EPR spectra were recorded with a Varian E-9, X-band spectrometer. The concentrations of the nitroxide radicals generated in each solution were determined by comparison with the standard curve of signals vs known concentrations of solutions of 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxyl [13].

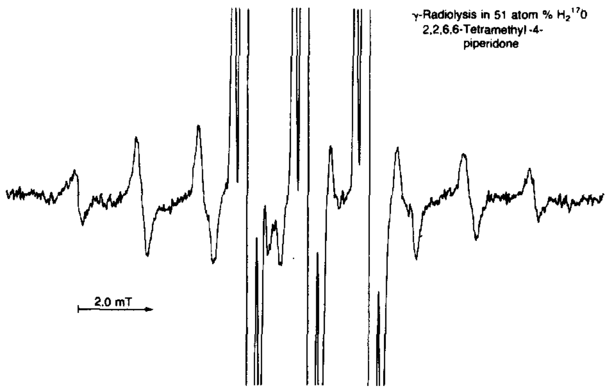

Fig.2. EPR spectrum of TMPone nitroxide generated in $H_2{}^{17}O$ (50%).

3. RESULTS AND DISCUSSION

The amines (10^{-3} M solutions in aerated water) were reacted with radiolytically generated hydroxyl radicals and the nitroxides formed were recorded by EPR spectroscopy as 1:1:1 triplets (TMP, TMAP and TMPol a_N = 1.7 mT, TMPone a_N = 1.6 mT). The participation of hydroxyl radicals in this oxidation was demonstrated by experiments performed in the presence of the competitive scavenger sodium formate (10^{-1} M, $k_{OH} = 2.9 \times 10^9\ M^{-1} \cdot s^{-1}$ [14]). No nitroxide could be detected in these experiments. Although cyclic amines are oxidized to nitroxides by H_2O_2 [9] and since the presence of sodium formate does not inhibit the formation of primary H_2O_2 ($G(H_2O_2) = 0.8$ [14]) this negative result also indicates that the H_2O_2 formed radiolytically (dose 540 Gy, i.e. at least 43 μmol·dm^{-3} H_2O_2) does not generate detectable radicals under our experimental conditions. Since sodium formate increases the formation of superoxide radical anions, this experiment also shows that this species does not react with the amine. This conclusion was further supported by reacting all these amines at room temperature with KO_2-saturated solutions in dry dimethyl sulfoxide. Again, no paramagnetic signals could be detected.

When radiolysis of the amines were carried out with oxygen-free, N_2O-saturated solutions, keeping all other conditions unchanged, no radicals could be detected either. Since the reaction of N_2O with e_{aq}^- doubles the number of hydroxyl radicals generated in solution, this result demonstrates the

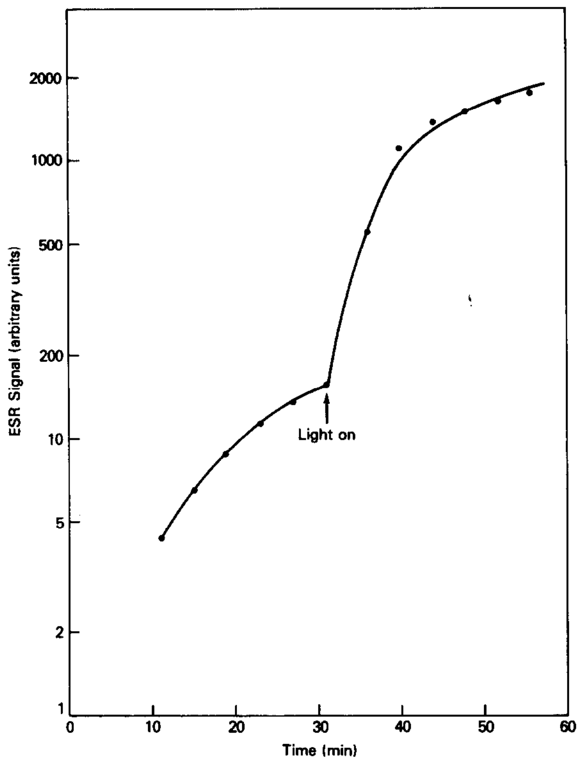

Fig.3. The formation of TMPone *N*-oxide vs reaction time. Initial amine concentration, 2×10^{-2} M; H_2O_2 concentration, 3×10^{-2} M; $\lambda_{exc} = 220$ nm (Schoeffel 1000 W high-pressure Hg-Xe lamp coupled with a Schoeffel grating monochromator); solution temperature, 21°C. The UV radiation was turned on 30 min after mixing the 2 reagents.

additional requirement for molecular oxygen in this oxidation. The chemical mechanism as exemplified by TMPone is depicted in fig.1. The relative contributions of the two pathways were determined by irradiating a solution of TMPone in $H_2{}^{17}O$ (51 atom% ^{17}O, Merck, Sharp & Dohme, Montreal). The resulting spectrum is a superimposition of two nitroxide radicals containing O^{16} and O^{17} (fig.2). The ^{16}O radical consists of 3 lines with $a_N = 1.6$ mT and the ^{17}O ($I = 5/2$) labeled radical of 8 lines ($a_N = 1.6$ mT, $a_O = 1.7$ mT). This 8 line spectrum can be explained as due to nearly equal values of a_N and a_O which reduce the expected 18 lines to 8 due to overlap. This experimental spectrum was in good agreement with the simulated spectrum using the above EPR parameters. The ratio of the [^{16}O]nitroxide and the [^{17}O]nitroxide was calculated from an EPR spectrum (not shown) in which the ^{16}O lines were recorded in scale and found to be 90:1, after correcting for the different multiplicities and for the 51% ^{17}O labeling of the water. This ratio is proportional to the occurrence of chemical routes (a) and (b).

Since the radiation dose, the G value for hydroxyl generation (G(OH) = 2.7 [14]), and the amount of nitroxide generated are known, the chemical yield for nitroxide formation [nitroxide detected (μmol) × 100/amount of OH$^{\cdot}$ generated (μmol)] could be calculated. Thus, for a content of 1 μmol amine, neutral pH, and 15×10^{-2} μmol OH$^{\cdot}$ generated, the chemical yields were less than 1% for TMP, TMPol, TMAP, and 19% for TMPone. The higher efficiency of TMPone in forming nitroxide points to amines substituted with electron-withdrawing groups as favorite candidates for use as hydroxyl radical 'spin traps'. As expected, higher chemical yields were obtained for a higher TMPone:OH$^{\cdot}$ ratio, i.e. for solutions exposed to lower radiation doses while all other parameters were kept unchanged. Thus, for an 8-times lower dose, a chemical yield of 26% was obtained. No pH effect in the range 6–7 was observed for the formation of TMPone nitroxide.

Finally, it was noted that the conversion of TMPone to nitroxide could also be carried out with hydroxyl radicals generated by photolysis of H_2O_2 (fig.3). The formation of the nitroxide radical prior to photolysis was not due to a Fenton reaction by involvement of traces of Fe^{2+}. This was shown in a separate experiment in which the rate of nitroxide formation in the dark was unaffected by the presence of the chelating agent diethylenetriaminepentaacetic acid (Aldrich) (TMPone, 2×10^{-2} M; H_2O_2, 3×10^{-2} M; DETAPAC, 2×10^{-4} M). However, the nitroxide can also be produced by the reaction with hydroxyl radicals resulting from Fenton reagent. Thus, a mixture of TMPone (2×10^{-2} M), H_2O_2 (6×10^{-2} M) and $FeSO_4$ (10^{-5} M) yielded, after a reaction time of 5 min, 4-times more nitroxide than in the absence of $FeSO_4$.

The chemistry for attaching sterically hindered cyclic amines to hydrophobic tails is known, as these compounds are the precursors of the corresponding spin labels. Thus, it seems likely that the present reaction can be used as a probe for simultaneous detection of hydroxyl radical and molecular oxygen during oxidative stress in cells or during lipid oxidation in biological membranes.

REFERENCES

[1] Janzen, E.G. (1980) in: Free Radicals in Biology (Pryor, W.A. ed.) vol.4, pp.115–154, Academic Press, New York.

[2] Kalyanaraman, B. (1983) in: Reviews in Biochemical Toxicology (Hodgson, E. et al. eds) vol.4, pp.73–139, Elsevier, Amsterdam, New York.

[3] Mason, R.P. (1984) in: Spin Labeling in Pharmacology (Holtz, J.L. ed.) pp.87–129, Academic Press, New York.

[4] Finkelstein, E., Rosen, G.M. and Rauckman, E.J. (1980) Arch. Biochem. Biophys. 200, 1–16.

[5] Samuni, A., Carmichael, A.J., Russo, A., Mitchell, J.B. and Riesz, P. (1986) Proc. Natl. Acad. Sci. USA 83, 7593–7597.

[6] Ivanov, V.B., Shlyapintokh, V.Y., Khvostach, O.M., Shapiro, A.B. and Rozantsev, E.G. (1975) J. Photochem. 4, 313–319.

[7] Lion, Y., Delmelle, M. and Van de Vorst, A. (1976) Nature 263, 442–443.

[8] Lion, Y., Gandin, E. and Van de Vorst, A. (1980) Photochem. Photobiol. 31, 305–309.

[9] Moan, J. and Wold, E. (1979) Nature 279, 450–451.

[10] Aurich, H.G. (1982) in: The Chemistry of Amino, Nitroso and Nitro Compounds and Their Derivatives (Patai, S. ed.) pt 1, suppl.F, Wiley, New York.

[11] Forrester, A.R., Hay, J.M. and Thomson, R.H. (1968) Organic Chemistry of Stable Radicals, Academic Press, London.

[12] Rigo, A., Argese, E., Stevanato, R., Orsega, E.F. and Viglino, P. (1977) Inorg. Chim. Acta 24, L71–L72.

[13] Wertz, J.E. and Bolton, J.R. (1972) Electron Spin Resonance, Elementary Theory and Practical Applications, McGraw-Hill, New York.

[14] Draganic, I.G. and Draganic, Z.D. (1971) The Radiation Chemistry of Water, Academic Press, New York.