MOLECULAR PHARMACOLOGY, 21:262–265

Production of Hydroxyl Radical by Decomposition of Superoxide Spin-Trapped Adducts

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Received October 2, 1980; Accepted November 16, 1981

SUMMARY

The spin trapping of superoxide by nitrone spin traps, in particular 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), was found to lead to the *de novo* production of hydroxyl radical and a nonradical species. Hydroxyl radical is then spin-trapped by DMPO, producing DMPO-OH. It was determined that a background level of approximately 3% DMPO-OH relative to DMPO-OOH is produced by this mechanism. Thus, the detection of hydroxyl radical by spin trapping with DMPO must be used with caution if the level of hydroxyl radical is less than 3% of the rate of superoxide generation. Several examples are cited in which investigators suggested that hydroxyl radical is produced when in fact the spin trapping of this free radical may have been an artifact of the system.

Spin trapping is a technique used to identify unstable free radicals, including superoxide and hydroxyl radicals (1–5). This method consists of allowing a nitrone or nitroso compound to react with a short-lived free radical and, in doing so, form a more stable nitroxide which can then be detected using conventional EPR spectrometry. Although this methodology is rather straightforward, there are a number of complications which are just now becoming apparent. For example, we have demonstrated that during the spin trapping of superoxide, using the cyclic nitrone DMPO,[1] the initial spin adduct, DMPO-OOH, is unstable and rapidly decomposes by almost exclusively a nonradical process into the more stable DMPO-OH and an EPR-invisible species (6).

During our investigation into the mechanism of oxygen radical production by human neutrophils, we observed that, no matter how diligent we were in removing metal ion impurities from our reaction mixtures, we invariably spin-trapped a small but quantifiable amount of hydroxyl radical. Attempts to identify the metalloenzyme responsible for hydroxyl radical generation were unsuccessful. We also determined that small quantities of hydroxyl radical were spin-trapped in all other aqueous systems where DMPO and superoxide were present. In this communication, we present evidence to demonstrate that, during the spin trapping of superoxide by either cyclic or acyclic nitrones, the superoxide spin adduct is initially produced which rapidly decomposes into a variety of products, including hydroxyl radical. This decomposition and the concomitant production of hydroxyl radical does not require the presence of metal ions.

The spin traps α-[4-pyridyl-1-oxide]-N-tert-butyl nitrone (4-POBN) and phenyl-N-tert-butyl nitrone (PBN) were obtained from Aldrich Chemical Co. (Milwaukee, Wisc.) and Eastman (Rochester, N. Y.), respectively, whereas DMPO was synthesized according to the method of Bonnett *et al.* (7). TMAS was prepared as described previously (8). All buffers were passed through a Chelex-100 ion exchange column (Bio-Rad, Inc., Richmond, Calif.) to remove trace metal impurities such as iron (9, 10). DETAPAC (0.1 mM) was added to sequester further any residual trace metal ions. Although the superoxide adduct of DMPO, DMPO-OOH, decomposes at a faster rate in the presence of iron, this decomposition is still rapid in the absence of a catalyst. An aqueous solution of TMAS was prepared immediately prior to its use by dissolving TMAS (1 mg/ml) in a solution containing 10 mM NAOH and 0.1 mM DETAPAC.

DMPO-OOH was prepared by adding 0.050 ml of the TMAS solution to 0.50 ml of a 0.1 M potassium phosphate buffer (pH 7.4) containing 0.18 M DMPO and 0.1 mM DETAPAC. To determine whether or not hydroxyl radical was being produced, 0.34 M ethanol (95%) was added. If hydroxyl radical were present it would react with ethanol to generate α-hydroxyethyl radical (11), whereas superoxide does not undergo such an electron abstraction (11). Thus, ethanol should both inhibit the trapping of hydroxyl radical and result in the formation of a new species due to the spin trapping of α-hydroxyethyl radical (Fig. 1). This phenomenon was, in fact, observed, as illustrated in Table 1.

At pH 7.4, the dismutation of superoxide is essentially

This investigation was supported in part by Grant DAAG 29-80-K-0075 from the Department of Defense.

[1] The abbreviations used are: DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; 4-POBN, α-[4-pyridyl-1-oxide]-*N*-*tert*-butyl nitrone; PBN, phenyl-*N*-*tert*-butyl nitrone; TMAS, tetramethyl ammonium superoxide; DETAPAC, diethylenetriaminepentaacetic acid.

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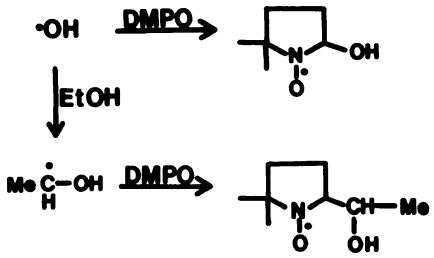


FIG. 1. *Reaction of hydroxyl radical with either DMPO to form DMPO-OH, or ethanol to produce α-hydroxyethyl radical which then reacts with DMPO*

complete within 1 sec, whereas DMPO-OOH decomposes with a half-life of approximately 2 min. Therefore, by varying the order of addition of DMPO, superoxide, and ethanol, it is possible to verify that hydroxyl radical is produced by the decomposition of DMPO-OOH and to rule out other mechanisms for the generation of this free radical. The results of these experiments are shown in Table 1. Even if ethanol were added after the DMPO-OOH was produced (and therefore after the superoxide had dismutated), the DMPO-α-hydroxyethyl adduct was still formed. Thus, the DMPO-OOH adduct decomposes to a powerful oxidant which can oxidize ethanol yielding α-hydroxyethyl radical, and which reacts with DMPO to produce a species identical with the hydroxyl radical spin-trapped adduct, DMPO-OH.

The data in Table 2 further verify this observation. When DMSO, formate, or ethanol was added to preformed DMPO-OOOH, the radicals produced were the same as those generated by the photolysis of hydrogen peroxide in the presence of these chemicals and DMPO (Fig. 2). We estimate that the yield of DMPO-OH from DMPO-OOH decomposition is approximately 3%.

TABLE 1

Production of hydroxyl radical via decomposition of DMPO-OOH

The numbers refer to the relative EPR peak heights of the superoxide, hydroxyl, and α-hydroxyethyl radical adducts of DMPO, measured 8 min after addition of 50 μl of TMAS in a 10 mM NAOH solution to 500 μl of a 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM DETAPAC. The concentrations of DMPO and ethanol were 0.18 M and 0.34 M, respectively. The data indicate that DMPO-OOH decomposes to produce a powerful oxidant which can oxidize ethanol into the α-hydroxyethyl radical. This is the same radical formed by reaction of hydroxyl radical with DMPO, and is consistent with the notion that DMPO-OOH decomposes to produce hydroxyl radical.

Order of addition[a]	DMPO-OOH	DMPO-OH	DMPO-EtOH
EtOH + TMAS, then DMPO	0	0	0
ETOH + DMPO, then TMAS	9	16	11
DMPO + TMAS, then EtOH	9	17	11
DMPO + TMAS	10	29	0

[a] The order of addition refers to the fact that two components were mixed together, 10 seconds allowed to elapse, then the third reactant was added.

The data presented in Table 2 demonstrate that the superoxide adducts of 4-POBN and PBN decompose to give hydroxyl radicals. The yields of hydroxyl radical (as measured by spin trapping) produced via decomposition of 4-POBN-OOH and PBN-OOH were considerably less than that observed from DMPO-OOH decomposition, and, in the case of 4-POBN, hydroxyl radical was barely detectable (Fig. 3).

There exist several possible mechanisms for homolytic decomposition of hydroperoxides (13); however, we believe that the probable mechanism for the decomposition of DMPO-OOOH into hydroxyl radical and a nonradical

TABLE 2

Spin trapping of hydroxyl radical in the presence of inhibitors

In each case, Adduct 1 is the hydroxyl radical trapped, whereas Adduct 2 is a secondary radical due to reaction of hydroxyl radical with the inhibitor. DMSO, Dimethyl sulfoxide.

Spin trap	System	Inhibitor	Adduct 1		Adduct 2	
			A_N	A_H^β	A_N	A_H^β
DMPO	Photolysis of H_2O_2	None	14.9[a]	14.9[a]		
DMPO	Decomposition of DMPO-OOH	None	14.9	14.9		
DMPO	Photolysis of H_2O_2	Ethanol	14.9	14.9	15.8	22.8
DMPO	Decomposition of DMPO-OOH	Ethanol	14.9	14.9	15.8	22.8
DMPO	Photolysis of H_2O_2	DMSO	14.9	14.9	16.4	23.4
DMPO	Decomposition of DMPO-OOH	DMSO	14.9	14.9	16.4	23.4
DMPO	Photolysis of H_2O_2	$NaHCO_2$	14.9	14.9	15.6	18.7
DMPO	Decomposition of DMPO-OOH	$NaHCO_2$	14.9	14.9	15.6	18.7
PBN	Photolysis of H_2O_2	None	15.3[a]	2.75[a]		
PBN	Decomposition of PBN-OOH	None	15.3	2.75		
PBN	Photolysis of H_2O_2	Ethanol	15.3	2.75	16.2	3.34
PBN	Decomposition of PBN-OOH	Ethanol	15.3	2.75	16.2	3.34
4-POBN	Photolysis of H_2O_2	None	14.93[a]	1.69[a]		
4-POBN	Decomposition of 4-POBN-OOH	None	—	—		
4-POBN	Photolysis of H_2O_2	Ethanol	14.93	1.69	15.56	2.59
4-POBN	Decomposition of 4-POBN-OOH	Ethanol	—	—	15.60	2.65

[a] The literature values for the hyperfine splitting constants are as follows: DMPO-OH, $A_H = A_N = 14.9$ G (5); PBN-OH, $A_N = 15.3$ G, $A_H^\beta = 2.75$ (1); 4-POBN-OH, $A_N = 14.95$, $A_H^\beta = 1.68$ (12).

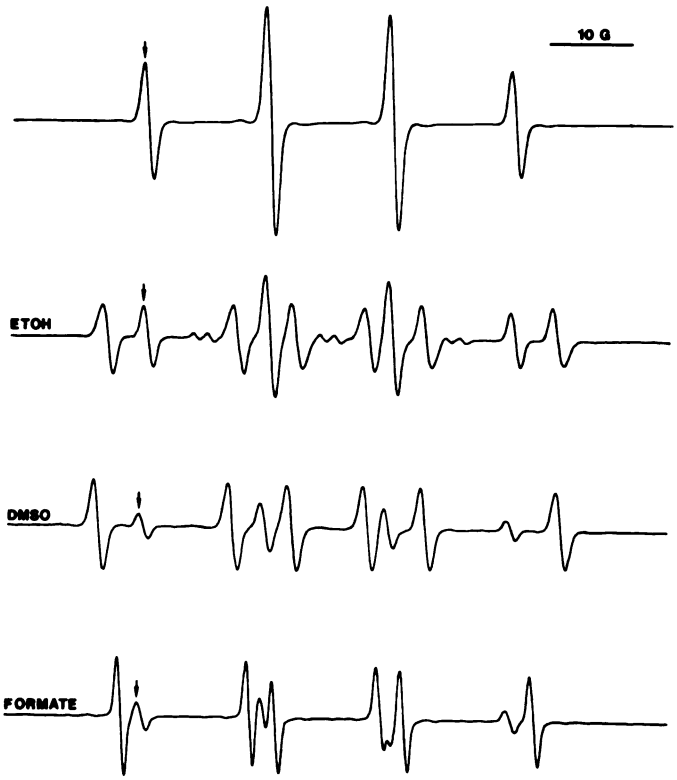
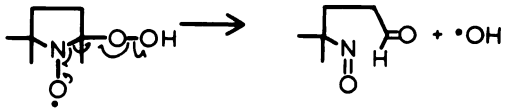


FIG. 2. *Effect of hydroxyl radical scavengers on the spin trapping of hydroxyl radical*

Hydroxyl radical was generated by the UV photolysis of 0.6% hydrogen peroxide in a 0.1 M chelexed potassium phosphate buffer (pH 7.4) containing 0.18 M DMPO and 0.1 mM DETAPAC. Where indicated the the following scavengers were added: 4% EtOH, 4% dimethyl sulfoxide (*DMSO*), and 2% formic acid. The *arrow* indicates the location of hydroxyl radical spin-trapped by DMPO for a reference point. The microwave power was 10 mW and the modulation frequency was 100 kHz with an amplitude of 0.5 G. The sweep time was 25 G/min and the response time was 0.3 sec.

species is unique. In the case of alkyl hydroperoxides, theoretical values for the activation energy of unimolecular homolysis are in the range of 42–43 Kcal/mole and experimental values are typically much lower (13). However, for DMPO-OOH, the number of unpaired electrons remains constant (Scheme 1), and one would therefore predict a more favorable activation energy.



SCHEME 1

The introduction of nitrone spin traps into a superoxides-generating system can lead to the *de novo* production of hydroxyl radical. This is an important consideration to bear in mind when spin-trapping studies are undertaken. There will be a background level of approximately 3% DMPO-OH relative to DMPO-OOH. Thus, detection of hydroxyl radical by spin trapping with DMPO must be interpreted with caution if the level of hydroxyl radical production in a system under study is less than 3% of the rate of superoxide generation. An example of this situation occurs with stimulated neutrophils (14). Despite this pitfall, two groups have detected DMPO-OH in spin-trapping experiments with stimulated neutrophils and reported that this was proof of hydroxyl radical formation by the neutrophils (15, 16). Finally, it should be pointed out that thiols can carry out the direct reduction of DMPO-OOH to DMPO-OH by a hydroxyl radical-independent mechanism (13, 17). This is an important consideration to keep in mind when conducting spin-trapping experiments in biological systems.

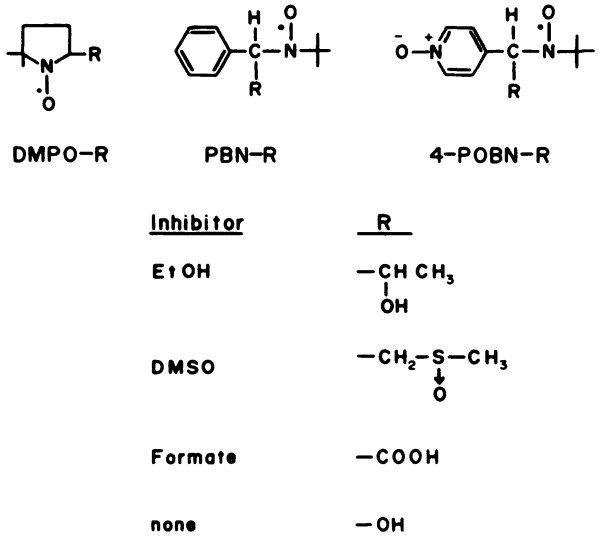


FIG. 3. *Structure of spin-trapped adducts noted in Table 2*

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