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Original Contribution

Metabolic activation of sulfur mustard leads to oxygen free radical formation

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ABSTRACT

We recently published electron paramagnetic resonance (EPR) spin trapping results that demonstrated the enzymatic reduction of sulfur mustard sulfonium ions to carbon-based free radicals using an in vitro system containing sulfur mustard, cytochrome P450 reductase, NADPH, and the spin trap α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) in buffer (A.A. Brimfield et al., 2009, *Toxicol. Appl. Pharmacol.* 234:128–134). Carbon-based radicals have been shown to reduce molecular oxygen to form superoxide and, subsequently, peroxyl and hydroxyl radicals. In some cases, such as with the herbicide paraquat, a cyclic redox system results, leading to magnified oxygen free radical concentration and sustained tissue damage. Low mustard carbon radical concentrations recorded by EPR in our in vitro system, despite a robust (4.0 mM) sulfur mustard starting concentration, led us to believe a similar oxygen reduction and redox cycling process might be involved with sulfur mustard. A comparison of the rate of mustard radical–POBN adduct formation in our in vitro system by EPR at atmospheric and reduced oxygen levels indicated a sixfold increase in 4-POBN adduct formation (0.5 to 3.0 μM) at the reduced oxygen concentration. That result suggested competition between oxygen and POBN for the available carbon-based mustard radicals. In parallel experiments we found that the oxygen radical-specific spin trap 5-*tert*-butoxycarbonyl-5-methylpyrroline-*N*-oxide (BMPO) detected peroxyl and hydroxyl radicals directly when it was used in place of POBN in the in vitro system. Presumably these radicals originated from O_2 reduced by carbon-based mustard radicals. We also showed that reactive oxygen species (ROS)–BMPO EPR signals were reduced or eliminated when mustard carbon radical production was impeded by systematically removing system components, indicating that carbon radicals were a necessary precursor to ROS production. ROS EPR signals were completely eliminated when superoxide dismutase and catalase were included in the complete in vitro enzymatic system, providing additional proof of oxygen radical participation. The redox cycling hypothesis was supported by density functional theory calculations and frontier molecular orbital analysis.

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Toxic organic compounds such as iodonium ions [1,2], pyridylium ions [3–5], quinones, and others [6–8] can be activated to carbon-based free radicals by enzymatic reduction. In some cases these radicals can reoxidize by donating an electron to other molecules in their environment and then be enzymatically rereduced, setting up a redox cycling system [4–6]. As long as the resulting carbon-based radicals fall in the proper position in the pecking order of relative reduction potentials [9], one of the sites to which they can donate an electron directly is molecular oxygen, which results in the formation of superoxide radicals. Alternatively, the donation of an electron to oxygen can occur indirectly via transition metal intermediates. Either route initiates a secondary nonenzymatic reactive oxygen cascade, yielding peroxyl and hydroxyl radicals, magnifying the final concentration of radicals produced from the original carbon-based species and increasing tissue damage [4,6].

We recently reported that cyclic sulfonium ions spontaneously arising from chloroethyl vesicants such as sulfur mustard, chloroethyl ethyl sulfide, chloroethyl methyl sulfide, and nitrogen mustards [10,11] can be converted to carbon-based free radicals in vitro when they undergo one-electron reduction by pyridine nucleotide-driven flavoenzymes [12]. In follow-up work we became interested in using electron paramagnetic resonance (EPR) to measure the concentration of mustard radical–spin trap adducts formed under the influence of NADPH-cytochrome P450 reductase. However, our results indicated such a low level of mustard radical–spin trap adduct formation compared to what was expected with our starting mustard concentration (4.0 mM) that we began to question our understanding of mustard–spin trap adduct formation. There seemed to be competition for the available carbon-based mustard radical between our spin trap, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN), and an entity that was either very short lived or that generated no EPR signal under our analysis conditions. The work cited above [4–7] suggested that our unknown reactant might be oxygen. The experimental results upheld

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that point of view. Additional analysis of the data using density functional theory and frontier molecular orbital analysis [13] reinforced the feasibility of a redox cycling mechanism.

Materials and methods

Reagents

Superoxide dismutase from human erythrocytes, diethylenetriaminepentaacetic acid (DTPA), β-NADPH, mono- and dibasic-potassium phosphate, and Chelex-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The stable free radical Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), used to generate the standard curve enabling the measurement of free radical adduct concentration [14], was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Catalase was obtained from Worthington Biochemicals (Lakewood, NJ, USA). The spin trap 4-POBN in high purity form came from Enzo Life Sciences (Plymouth Meeting, PA, USA). The oxygen radical-specific spin trap 5-*tert*-butoxycarbonyl-5-methylpyrroline-*N*-oxide (BMPO) was from Applied Bioanalytical Laboratories (Sarasota, FL, USA). Sulfur mustard (98% purity) was produced by the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD, USA) and stored at −70°C until needed. Recombinant porcine NADPH-cytochrome P450 oxidoreductase was a kind gift from Professor Bettie Sue Masters of the Department of Biochemistry, University of Texas Health Sciences Center (San Antonio, TX, USA).

Vesicant safety

The chloroethyl thioethers and amines are potent, volatile vesicating agents that cause delayed production of erythema, massive water-filled blisters, and necrosis when they come into contact with human skin. They are active in either vapor or liquid form. Exposure of the eyes can cause conjunctivitis, temporary blindness, and corneal opacity. Inhalation can lead to pneumonia, chronic bronchitis, and asthma [15]. Care must be exercised to prevent skin contact and inhalation of vapors when working with these compounds. Therefore, all work was carried out in a fume hood while wearing long sleeves, eye protection, and rubber gloves (preferably nitrile).

Enzymatic carbon or oxygen radical generation

Carbon or oxygen free radical production was followed in an incubation mixture consisting of 4.8 mM NADPH, 8 nM NADPH-cytochrome P450 reductase in Chelex-100-treated 0.1 M potassium phosphate buffer, pH 7.5, containing 0.25 M NaCl, 1.0 mM DTPA, and 25 mM spin trap. Vesicant concentration was nominally 4.0 mM. This is referred to as complete incubation mixture.

EPR spectrometry

Mustard-related carbon or oxygen free radical production was recorded using a Bruker EMX Plus EPR spectrometer equipped with a 4119 HS cavity (Bruker Biospin Corp., Billerica, MA, USA). Unless indicated otherwise in the figure legends, the instrument was operated at room temperature at a microwave frequency of 9.77 GHz and 100 kHz modulation field at a power setting of 12.62 mW, a scan rate of 0.61 G/s, modulation amplitude set at 100 G, and receiver gain of 1.26×10^4. Samples were introduced into the EPR in 10-mm flat cells. Each spectrum was the accumulation of 64 scans (time constant 5.12 ms, conversion time 5 ms). Experimental spectra were integrated individually to determine the area under the curve using WinEPR version 2.22 rev. 12 (Bruker Biospin Corp.). Area was converted to concentration using the equation for a one-electron free radical standard curve produced using Tempol according to the method of Barr et al. [14]. The results were incorporated into kinetic plots. When necessary, mixed oxygen radical spectra formed using BMPO [15] were simulated and deconvoluted using Winsim software version 0.98 developed at the National Institute of Environmental Health Science (Research Triangle Park, NC, USA).

Buffer deoxygenation

In preparation for EPR experiments requiring reduced oxygen levels, Chelex-treated phosphate buffer containing DTPA was degassed in 10-ml volumes in the lyophilizer jar of a Freezone 6 lyophilizer (Labconco Corp., Kansas City, MO, USA). Buffer was held in a 20-ml glass scintillation vial sealed with a serum vial stopper pierced by an 18-gauge hypodermic needle and placed in the freeze-drying bottle. The vacuum over the buffer was increased slowly until bubbling ceased. At that point, the vacuum was released and the container repressurized by bleeding N_2 into the evacuated lyophilizer jar from a nitrogen source. The process was repeated several times to ensure O_2 removal. The remaining sample preparation consisted of adding the degassed buffer to measured quantities of the enzyme, the substrates, and the spin trap inside a glove bag under a nitrogen atmosphere.

Documentation of oxygen reduction

Partitioning of carbon-based mustard radicals between POBN–adduct formation and O_2 reduction was evaluated by two processes. In one set of experiments we compared the EPR signal produced by POBN adduct formation in complete incubation mixture made with deoxygenated buffer with the signal produced in complete incubation mixture made with buffer containing ambient dissolved oxygen. This allowed us to test the effect of oxygen level on mustard radical–POBN adduct production. The incubation mixture in this case contained 25 mM 4-POBN.

For comparison we used the spin trap BMPO in place of 4-POBN to detect oxygen radicals directly [16,17] and to demonstrate the dependence of oxygen radical signal production on mustard reduction to carbon free radicals. In this case we measured oxygen free radical formation in complete incubation mixture containing atmospheric dissolved oxygen and 25 mM BMPO. We evaluated the contribution of each component of the complete mixture to oxygen radical generation by replacing each, one at a time, with buffer and observing the relative magnitude of the oxygen radical–BMPO adduct signal on the EPR. Finally, we included superoxide dismutase (500 IU/ml) and catalase (500 IU/ml) in the complete incubation mixture containing BMPO, to document the elimination of the reactive oxygen species (ROS) signals, demonstrating that ROS were the source of the EPR signal. Final sample volume was 0.5 ml in each case.

Quantum mechanics calculations

To help characterize the chemistry that drives the production of superoxide by sulfur mustard, geometry optimizations in the gas phase were performed for molecular oxygen, superoxide radical anion, the carbon-based mustard radical, and the cyclic mustard sulfonium ion at the B3LYP/6-31 + g (d, p) level of theory using the Quantum Mechanics Package G03 revision C.02 (Gaussian, Inc., Wallingford, CT, USA) [18]. Each energy minimum was verified by performing a vibrational analysis. The existence of a redox cycle involving mustard radical and molecular oxygen was rationalized using postcalculation frontier molecular orbital analysis [13]. This case being that of a radical species, we used the energies corresponding to the β-electron as a matter of convention.

Frontier molecular orbital analysis

For a bimolecular, open-shell system consisting of two chemical species A and B, there are two directions for electron flow. Either A is oxidized by the transfer of an electron from A to B or it is reduced

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by the transfer of an electron to B from A. Frontier molecular orbital analysis [13] can be used to discriminate between the two cases. In this situation we used the technique to validate the direction of electron flow in our cyclic redox model resulting from the enzymatic reduction of the cyclic mustard sulfonium ion. According to frontier electron molecular orbital theory the energetic preference for the direction of electron flow from species A to species B is predicted by Eq. (1),

$$\Delta E_{A-B} = k/\left(E_{HOMO_A} - E_{LUMO_B}\right), \quad (1)$$

where k is a proportionality constant and E_{HOMO_A} and E_{LUMO_B} are the respective highest occupied molecular orbital and lowest unoccupied molecular orbital energies for interacting species A and B. Because these studies involved open-shell systems, HOMO and LUMO energies for the β electron were used as a matter of convention in this report. Because we were interested in qualitatively assessing this interaction, k was assumed to have a value of 1. As is obvious from the equation, electron transfer from A to B is favored when the HOMO–LUMO energy difference is small and negative.

Results

The presence of dissolved oxygen in our in vitro incubation mixture had a distinct effect on the rate of formation of the mustard–POBN adduct. When dissolved oxygen concentration was reduced by subjecting the reaction buffer to high vacuum and then backflushing with nitrogen, the concentration of mustard–POBN adduct produced increased sixfold compared to adduct formed at ambient dissolved oxygen levels (from 0.5 to 3.0 μM) (Fig. 1). A comparable outcome occurred when sulfur mustard was replaced by chloroethylethyl sulfide (CEES; results not shown), frequently used as a model compound for sulfur mustard. This was not a surprising result, considering the apparent mechanistic similarity between sulfur mustard and CEES [12,19]. This outcome strongly suggested competition between O_2 and POBN for the available carbon-based mustard free radical.

It became obvious that the direct detection of oxygen radical formation would help to prove that ROS were involved. Direct probing for reactive oxygen species by EPR is possible using the spin trap BMPO. Following the production of radical adducts over time using an EPR lag-time plot (Fig. 2) indicated a rapid buildup of BMPO adduct to 4.8 μM during the first 75 min of data collection followed by a period of decay that tailed out asymptotically until the analysis was ended. Simulation (Fig. 3) and deconvolution (Fig. 4) of individual spectra taken at the peak of the curve confirmed the presence of both peroxyl radical and hydroxyl radical adduct signals as described in [16].

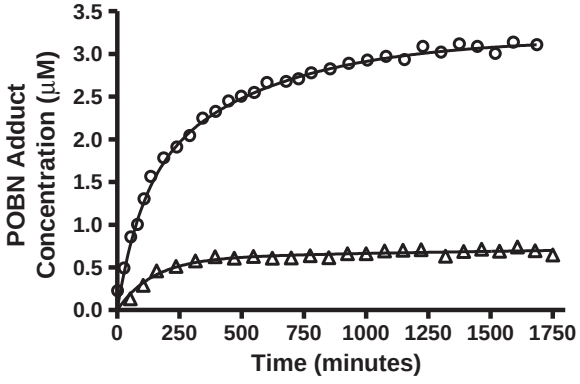

Fig. 1. Representative comparison of mustard–POBN adduct formation at atmospheric (△) and reduced (○) oxygen concentrations with all other components of the incubation mixture held constant.

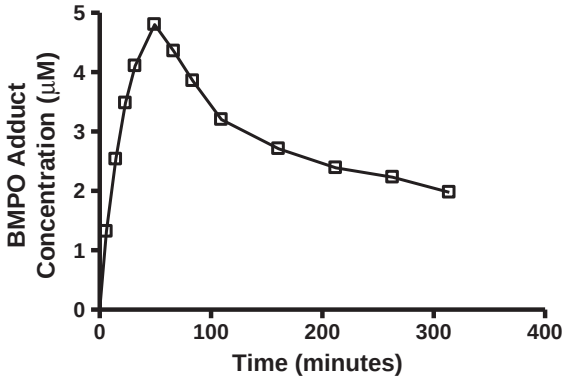

Fig. 2. A representative graph illustrating the concentration of oxygen free radicals measured by EPR after reduction of sulfur mustard by cytochrome P450 reductase. The spin trap BMPO (specific for reactive oxygen species) was substituted for POBN. A superoxide–BMPO radical adduct rapidly accepts a proton to become a peroxyl radical adduct. In enzymatic systems, this adduct is likely to be enzymatically reduced to a BMPO hydroxyl radical [15].

Success in detecting oxygen radicals directly with BMPO led us to test our understanding of the reaction sequence producing ROS by replacing the components of the in vitro spin trapping mixture one at a time with buffer and rerunning the EPR spectra. In earlier work [12] we showed that removal of any component of the complete in vitro mixture should reduce or eliminate the EPR signal from POBN adducts. Fig. 5 illustrates an effort to apply the same principle to the generation of oxygen radicals using the oxygen-specific spin trap BMPO. A reduction in oxygen radical production by inhibiting mustard (carbon) radical formation would tie the production of oxygen radicals

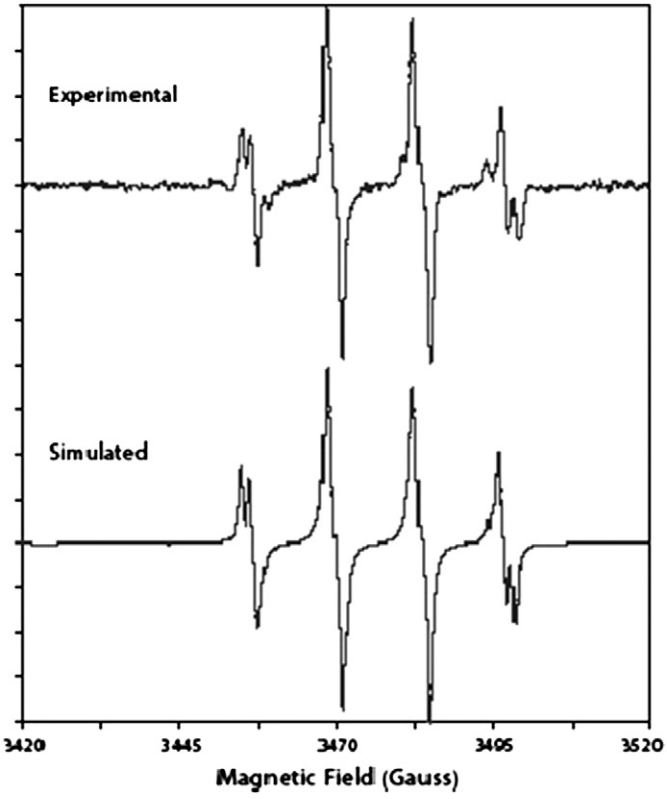

Fig. 3. (Top) The EPR spectrum of the experimental BMPO–oxygen radical adduct mixture taken at the peak of the curve shown in Fig. 2 after approximately 75 min of data collection. (Bottom) The computer simulation of the same experimental curve. The area under the curve is made up of contributions from two species of reactive oxygen–BMPO adduct, each having two isomeric forms [15]. The major contribution is from peroxyl radical with a small contribution by the hydroxyl radical species.

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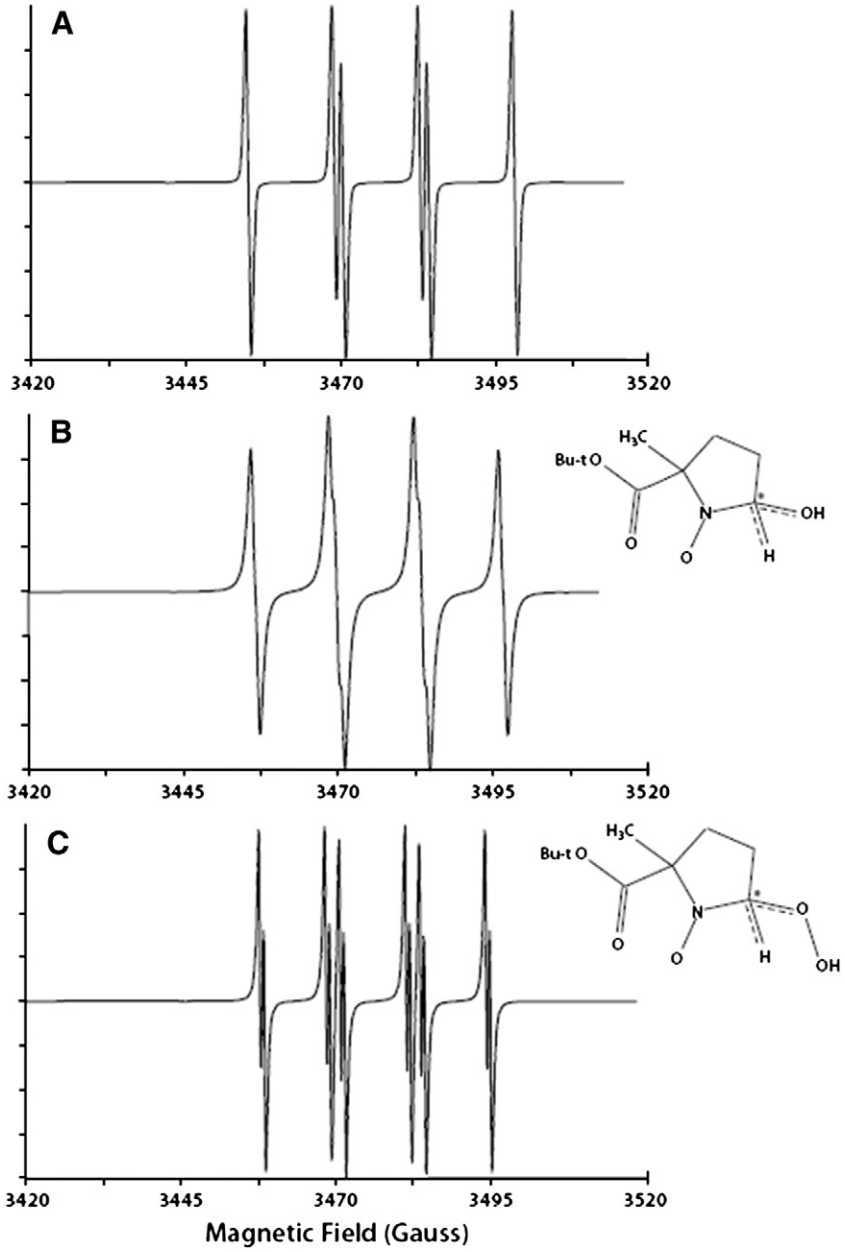

Fig. 4. The deconvolution of the simulated experimental BMPO–ROS adduct mixture (Fig. 3, simulated). The original simulation contained four components representing the spectra resulting from (A and B) two stereoisomeric forms of BMPO–hydroxyl radical adduct and (C) two stereoisomeric forms of BMPO–peroxyl radical adduct. The peroxyl adduct (structure shown to the right of spectrum (C)) simulation was not separated into its components because of space limitations and very small hyperfine splitting constants. The structure between the two upper spectra is a generalized BMPO–hydroxyl radical adduct. The dotted lines indicate bonds that vary with the isomeric structure.

directly to carbon radical generation and reinforce our attempts to incorporate the redox cycling concept.

Spectrum A in Fig. 5 shows the EPR signal produced using the complete in vitro mustard radical-generating system containing NADPH, cytochrome P450 reductase, sulfur mustard, and the chelating agent DTPA in phosphate buffer with the O_2-specific spin trap BMPO in place of POBN. The BMPO spectrum with four main peaks in a 1:2:2:1 arrangement and their associated fine structure are characteristic of oxygen radical signals detected using this spin trap [16,17]. The spectrum is made up of signals originating with adducts of peroxyl and hydroxyl radicals. The fine structure reflects the superposition of the BMPO–peroxyl radical adduct on top of the BMPO–hydroxyl radical adduct (see Figs. 3 and 4) [16].

Spectrum B in Fig. 5 illustrates the effect of removing sulfur mustard from the mixture. In essence we have eliminated the source of the cyclic sulfonium ions that are enzymatically reduced to yield carbon-based free radicals. In so doing, we have eliminated the source of carbon radicals, reducing O_2 to superoxide. The magnitude of the signal is greatly diminished, indicating the formation of a much lower concentration of oxygen radicals, although the basic four peak structure is still evident. Clejan and Cederbaum [4] attributed this effect to the reduction of oxygen to superoxide by reduced cytochrome P450 reductase so long as an intermediate electron acceptor was present, in this case probably a complex of Fe^{3+} with DTPA. Although the phosphate buffer used to prepare our system was passed through Chelex-100, trace amounts of di- or trivalent metal cations originating with the phosphate salts may be carried into the in vitro mixture.

Spectrum C in Fig. 5 reflects the removal of both mustard and DTPA from the complete mixture. Its signal is weaker still than that of spectrum B, but continues to show the presence of a BMPO–oxygen signal. The reduced signal strength reflects the outcome of removing both components because DTPA-chelated Fe^{3+} can serve as an electron acceptor as well [4].

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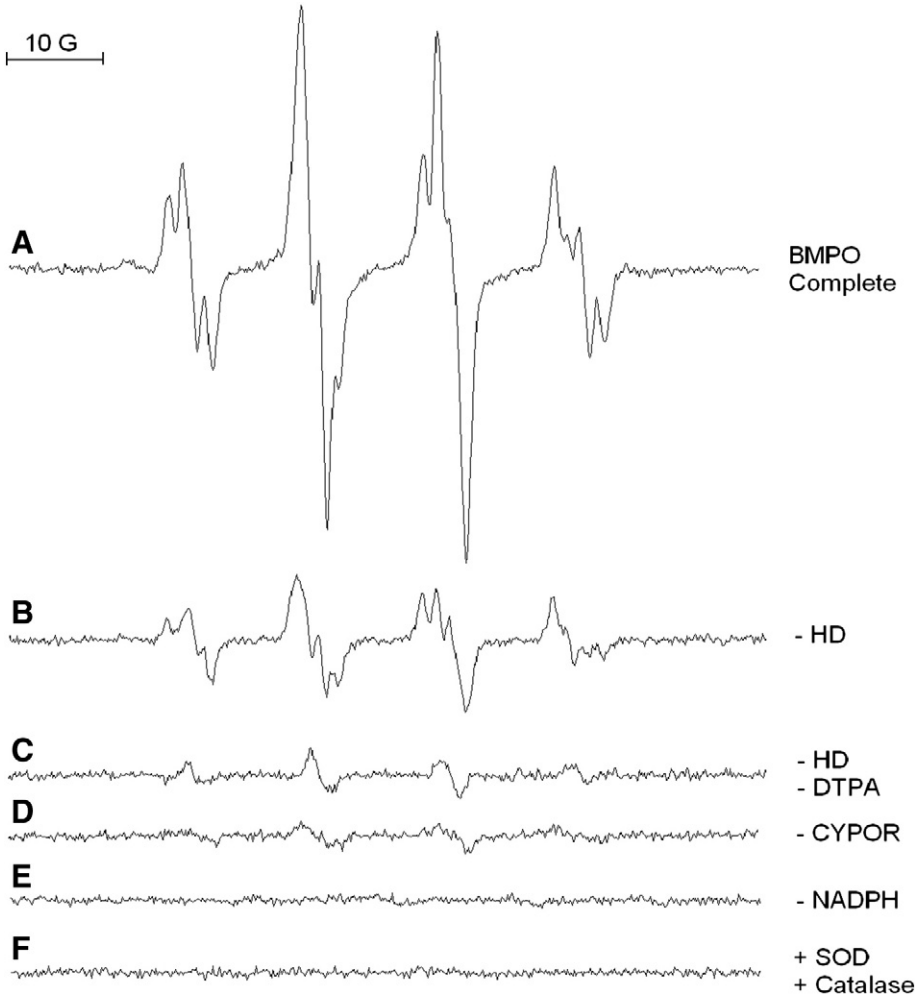

Fig. 5. Experimental spectra comparing the EPR signal from BMPO–oxygen adducts arising from the in vitro incubation mixture as individual components of the mixture are removed and replaced with an equal volume of buffer. The rest of the mixture is left intact. These results tie ROS production to mustard carbon-based radical production, providing evidence for redox cycling and oxygen radical cascade formation. The 10 G (Gauss) line defines the width of the scans.

Under these circumstances the number of species capable of accepting electrons from NADPH and donating electrons to O_2 is further reduced to just the reductase alone. Clejan and Cederbaum [4] attributed this effect to the direct reduction of oxygen by cytochrome P450 reductase, a process that has been documented by others as well [20,21].

Spectrum D in Fig. 5 is the signal that results when the reductase is removed from the otherwise complete incubation mixture. Removal of the reductase resulted in a much reduced efficiency of electron transfer to oxygen as is reflected in the signal intensity. When NADPH, the ultimate electron source, was removed, the BMPO–adduct signal disappeared (spectrum E). Further indication of ROS involvement (Fig. 5, spectrum F) occurred when the formation of BMPO adduct signals was eliminated by the addition of superoxide dismutase and catalase to the otherwise complete incubation mixture. These enzymes destroy any superoxide radicals by dismutation to peroxide and oxygen followed by conversion of the peroxide to oxygen and water.

We carried out quantum mechanics calculations to determine the HOMO and LUMO energies (in E_h, or hartrees) for the mustard sulfonium ion, mustard radical, molecular oxygen, and superoxide radical (Table 1). With these values in hand we were able to use frontier molecular orbital analysis to predict the direction of electron transfer.

Analysis of the reduction of molecular oxygen by mustard radical (Eq. (1), Table 2A) yielded an energy change of −9.22 for transfer of an electron from the mustard radical (A) to molecular oxygen (B). A smaller energy change of −2.64 resulted for the transfer of an electron from molecular oxygen to the mustard radical. Therefore, there is a strong thermodynamic preference for the passage of an electron from mustard radical to molecular oxygen to produce superoxide. The effect of this reaction is the simultaneous regeneration of mustard sulfonium ion that again becomes available for enzymatic reduction. Therefore, the reduction of molecular oxygen seems to drive a redox cycle that amplifies the tissue damage resulting from mustard exposure.

There remains a possibility that because the sulfonium is also a product of O_2 reduction by mustard radical and because sulfonium ion is readily reduced, superoxide could reduce the sulfonium ion again, setting up the equilibrium in Eq. (2):

$$\text{mustard radical} + O_2 \leftrightarrow \text{mustard sulfonium ion} + \text{superoxide radical}. \quad (2)$$

Table 1
HOMO and LUMO energies in hartrees resulting from the density functional theory calculations for the molecules under consideration.

Compound	E_{HOMO}	E_{LUMO}
Mustard radical	−0.2403	−0.0926
Molecular oxygen	−0.4719	−0.1318
Superoxide	0.0793	0.1871
Sulfonium ion	−0.4422	−0.2303

For singly occupied HOMOs, as in singly reduced radicals, the β spin orbital energies were used as a matter of convention.

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Table 2A
The results of frontier molecular orbital theory analysis applying Eq. (1) to the density functional theory results for mustard radical and molecular oxygen given in Table 1.

Compound	E_{SHOMO}	E_{LUMO}	$1/(A_{SHOMO}-B_{LUMO})$ (ΔE_{A-B})	$1/(B_{SHOMO}-A_{LUMO})$ (ΔE_{B-A})
Mustard radical (A)	−0.2403	−0.0926	−9.22	
Molecular oxygen (B)	−0.4719	−0.1318		−2.64

S in SHOMO designates a singly occupied molecular orbital as HOMO.

Frontier molecular orbital analysis was also used to assess the viability of this idea. The results in Table 2B show that the reduction of sulfonium ion by superoxide is strongly energetically disfavored, with an energy change of +3.23. Therefore, there is no expectation of an equilibrium. The cyclic redox mechanism proposed can be said to be driven solely via the reduction of molecular oxygen by enzymatically produced mustard radical.

Discussion

We were able to summarize our results from the manipulation of the oxygen concentration (Fig. 1), from the direct measurement of oxygen radicals (Figs. 2–4), and from the manipulation of the components of our in vitro system (Fig. 5) in the form of a pathway outlining a proposed mechanism for the initiation and maintenance of mustard free radical production by redox cycling of reduced chloroethyl sulfide sulfonium cations (Fig. 6).

Step 1 shows loss of chloride and the spontaneous formation of the strained, three-membered cyclic sulfonium ion of a chloroethyl mustard. This is a first-order intramolecular rearrangement with assistance from the neighboring sulfur [10]. We established in our previous work [12] that the formation of this strained ring structure was required for enzymatic reduction and free radical formation. Only cyclic ethyl sulfonium ions were reduced.

Step 2 represents the enzymatic reduction step in which one of a number of pyridine nucleotide-driven flavin reductases can donate a single electron to the cyclic sulfonium ion to form the carbon-based mustard free radical shown at step 3. The structure of the radical itself was worked out in our earlier work using EPR [12].

At step 4, based on our experiments in which we reduced oxygen concentration during spin trapping analysis, we propose that the carbon-based radical might interact by either of two competing routes in vitro. It could form an EPR-detectable POBN adduct by alkylating the spin trap, or it could donate an electron to molecular oxygen causing the formation of a superoxide anion radical and a new sulfonium ion, neither of which would produce an EPR signal in our system. Because there would be no spin trap available in an in vivo situation, the main route during actual exposure would be oxygen radical formation. This, no doubt, is what formed the basis for our observation of reduced POBN adduct formation in the presence of increased levels of oxygen (Fig. 1); oxygen was in competition with POBN for the available mustard radical. This point of view is supported by the

Table 2B
The results of frontier molecular orbital theory analysis applying Eq. (1) to the density functional theory results for superoxide radical and sulfonium ion given in Table 1.

Compound	E_{SHOMO}	E_{LUMO}	$1/(A_{SHOMO}-B_{LUMO})$ (ΔE_{A-B})	$1/(B_{SHOMO}-A_{LUMO})$ (ΔE_{B-A})
Superoxide (A)	0.0793	0.1871	+3.23	
Sulfonium ion (B)	−0.4422	−0.2303		−1.59

S in SHOMO designates a singly occupied molecular orbital as HOMO.

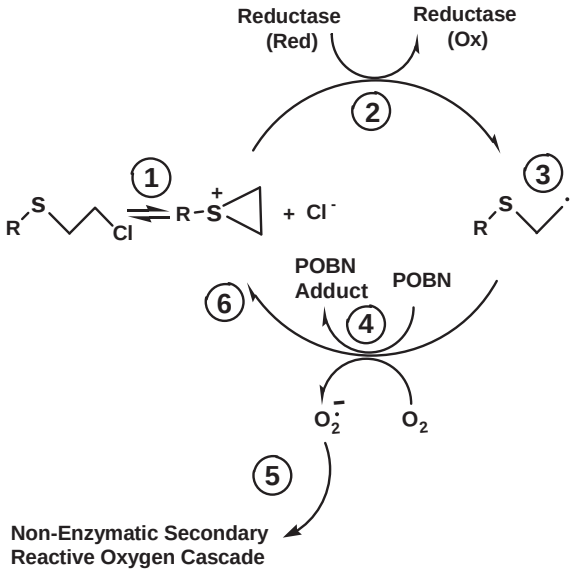

Fig. 6. Proposed mechanism for the initiation and maintenance of free radical production by redox cycling of reduced chloroethyl sulfide sulfonium cations. (Step 1) Spontaneous first-order formation of sulfonium ions by intramolecular cyclization with loss of chloride. (Step 2) Pyridine nucleotide reductase-driven enzymatic reduction of the cyclic sulfonium to give the (step 3) mustard carbon free radical. (Step 4) Competitive reaction of the mustard free radical with the spin trap to give the POBN adduct or, via reduction of molecular oxygen, to yield the superoxide radical initiating the nonenzymatic secondary reactive oxygen cascade (step 5). (Step 6) Recyclization caused by neighboring group participation of the sulfur atom to form a carbocation (sulfonium ion) ready for enzymatic rereduction.

frontier molecular orbital calculations, which show that the formation of superoxide anion is energetically favorable (Table 2A).

In step 5, superoxide radicals formed by the reduction of molecular oxygen are converted to peroxyl and hydroxyl radicals via the nonenzymatic secondary reactive oxygen cascade [6]. It is at this stage that the concentration of oxygen free radicals would be magnified. In step 6 the cyclic sulfonium ion is re-formed via neighboring group participation, with the sulfur atom making it ready for enzymatic rereduction and reinitiation of the redox cycle.

The sequence depicted in step 6 is supported by the results from frontier molecular orbital analysis. This analysis confirmed that oxidation of mustard radical by molecular oxygen is very energetically favorable. This oxidation results in the formation of sulfonium ion and superoxide radical. At this point, the redox cycle can operate only if there is a substantial concentration of mustard sulfonium ion available for enzymatic rereduction. The results from Table 2B predict that reduction of sulfonium ion by superoxide radical is an energetically disfavored process. Therefore, there is no equilibrium between superoxide and mustard radical. The absence of this equilibrium predicts that our cyclic redox model is driven solely by formation of superoxide.

There are three significant consequences of that result. The first is that the redox cycle does not depend on the nature of the reductant that transforms mustard sulfonium ion to mustard radical [3,12]. As long as there are cellular reducing agents available to convert mustard ion to mustard radical and a supply of reducing equivalents the redox cycle will continue to operate.

The second consequence is a corollary of the first. Inhibition of any specific mustard ion reductant will not have a significant effect on the redox cycle. As long as there are reducing agents available to reduce mustard ion, the redox cycle will continue. Although the overall production of superoxide may be slightly decreased because of a lower concentration of mustard radical, kinetically slower reducing agents will still provide a concentration of mustard radical to feed the redox cycle.

The third consequence is that compounds or conditions capable of interrupting the supply of molecular oxygen would disrupt the redox

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cycle. As was stated previously, it is formation of superoxide that drives the redox cycle. Therefore, if the supply of oxygen were disrupted, the concentration of mustard radical would increase. Although carbon radicals are extremely reactive, once reacted, they would not be able to participate in the redox cycle. Therefore the cycle would be broken.

The knowledge that carbon-based radicals and ROS play a role in mustard toxicity leads one to speculate about the therapeutic possibilities inherent in excluding oxygen from sites of skin exposure. Graham et al. [22] showed that vacuum dressings reduced the healing time for mustard injuries on the skin. Additionally, early researchers looking at radiation damage observed that during the free radical-producing process of X-irradiation, increasing oxygen concentration enhanced toxicity and anoxia reduced it [23]. The complete exclusion of oxygen by physically isolating a site of mustard exposure and bathing it with an inert gas may reduce ROS formation and also lead to reduced tissue damage and more rapid healing.

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