The iron chelator deferoxamine (Desferal; DSFL) reacts with peroxidases and $\text{H}_2\text{O}_2$ to form the DSFL radical (DSFL$^-$), which can be detected by EPR spectroscopy. We have found that DSFL$^-$ formation resulting from exposure to $\text{H}_2\text{O}_2$ and any of a number of different peroxidases is greatly enhanced in the presence of the nitro compound pyridine N-oxide. 2-Methyl-2-nitrosopropane and pyridine N-oxide were additionally capable of increasing enzymatic peroxidase activity as measured by o-dianisidine and/or tetramethylenediamine oxidation. Furthermore, at high concentrations of 4-POBN (50 mM) in the absence of DSFL, we detected a peroxidase/$\text{H}_2\text{O}_2$-dependent 12-line EPR spectrum that likely represents a 4-POBN/4-POBN nitrogen-centered spin adduct. In the presence of both 4-POBN (10 mM) and DMPO (100 mM), an 18-line EPR spectrum was observed consistent with formation of a DMPO/4-POBN nitrogen-centered spin adduct. Thus, the nitro spin trap 4-POBN can enhance the peroxidase-mediated formation of DSFL$^-$, possibly via the formation of a transient 4-POBN radical species. These data suggest the importance of assessing the potential for nitro spin traps to both inhibit and enhance biological oxidation prior to their use as potential pharmacological agents.

Deferoxamine (Desferal; DSFL)$^1$ is a potent iron chelator used clinically to treat iron overload (1). By binding iron in such a way that it greatly hinders redox cycling, DSFL can reduce the potential for damage to normal tissue from iron-catalyzed HO$^\cdot$ formation (2). Reaction of various peroxidases (e.g. HRP, LPO, MPO, and eosinophil peroxidase) with $\text{H}_2\text{O}_2$ in the presence of DSFL leads to the formation of the DSFL radical (DSFL$^-$), a nitroxide radical that can be detected by EPR spectroscopy (3). DSFL$^-$ is capable of damaging vitamins and enzymes (4), and its formation is thought to be dependent on formation of Compound II of the peroxidase (5), although the exact reaction process remains ill defined.

We recently demonstrated that human neutrophils, monocytes, and eosinophils generate HO$^\cdot$ via a peroxidase-dependent mechanism involving MPO or eosinophil peroxidase (6, 7). In the course of work examining the ability of DSFL to modulate this HO$^\cdot$ formation in its role as an iron chelator, we observed that MPO-dependent formation of DSFL$^-$ increased dramatically in the presence of the nitro spin trap 4-POBN. Due to their oxidant-scavenging properties, nitro spin traps have been studied as potential therapeutic modalities for a variety of pathological processes believed to be mediated by reactive oxidant species (8–11). Our observations with 4-POBN and DSFL raised the possibility that some nitro spin traps could enhance rather than inhibit peroxidase-mediated oxidant injury under some circumstances. Accordingly, we examined 1) whether 4-POBN enhances the ability of other peroxidases to generate DSFL$^-$, 2) the extent to which spin traps other than 4-POBN enhance peroxidase-mediated DSFL$^-$ formation, and 3) the mechanism(s) involved.

**EXPERIMENTAL PROCEDURES**

**Materials**

HRP and bovine LPO were obtained from Sigma. Human MPO was provided by Dr. William Nauseef (University of Iowa, Iowa City, IA). Human eosinophils were isolated as described previously (7, 12) and lysed by repeated freeze-thaw cycles in order to obtain crude lysates with eosinophil peroxidase activity. DSFL, L-tryptophan, 3-hydroxybenzaldehyde, 4-POBN, 5,5-dimethyl-1-pyrroline 1-oxide (DMPO), 2-methyl-2-nitrosopropane (MNP) dimer, and phenyl-tert-butyl nitroxide (PBN) were also obtained from Sigma. Salicylic acid was obtained from Fisher. Pyridine N-oxide (PNO), 4-methyl-PNO, pyridine, and diethylentriaminepentaacetic acid (DTPA) were obtained from Aldrich.

**EPR Spectroscopy**

For the generation of DSFL$^-$, 100 $\mu$M $\text{H}_2\text{O}_2$ was added to a solution containing 1–100 $\mu$M DSFL and one of the following: HRP (2–20 $\mu$M), LPO (2–20 $\mu$M), MPO (40–400 units/mI), or lysed eosinophils. After 10 min, samples were transferred to an EPR flat cell, and the resulting EPR spectra were obtained at room temperature using a Bruker Model ESP 300 spectrometer. Unless otherwise stated, instrumental settings were as follows: microwave power, 20 milliwatts; modulation frequency, 100 kHz; modulation amplitude, 0.9 G; sweep width, 0.4 G; and response time, 0.6 s.

All EPR experiments were performed in 20 mM phosphate buffer, pH 7.4.
4-POBN Enhances DSFL Peroxidation

RESULTS AND DISCUSSION

Formation of the DSFL Radical by the Peroxidase H$_2$O$_2$ System—DSFL, an iron chelator used clinically in cases of iron overload, is susceptible to enzymatic oxidation by various peroxidases (3, 5). Consistent with the results of others (3), addition of DSFL to a HRP/H$_2$O$_2$ system yielded the characteristic DSFL$^\cdot$ as detected by EPR (Fig. 1). The steady-state concentration of DSFL$^\cdot$ increased as the level of DSFL added to the system increased, but it was not linear over the 100-fold range of DSFL concentrations used. Under identical experimental conditions, we observed DSFL$^\cdot$ formation by MPO, LPO, and lysed eosinophils (i.e. eosinophil peroxidase) in the presence of H$_2$O$_2$ (data not shown). In each case, both H$_2$O$_2$ and peroxidase were required for the formation of DSFL$^\cdot$ (data not shown). Formation of this radical by the HRP/H$_2$O$_2$ system was clearly observed at DSFL concentrations as low as 10 $\mu$M. When administered to humans, stable plasma concentrations of DSFL as high as 20 $\mu$M have been reported (16). Thus, at in vivo sites of inflammation in such treated individuals, neutrophil-derived MPO and eosinophil-derived eosinophil peroxidase could lead to the formation of DSFL$^\cdot$.

DSFL$^\cdot$ seen in the above experiments is likely a direct product of the action of the peroxidase/H$_2$O$_2$ system on DSFL (3). It has been reported that DSFL$^\cdot$ can be formed directly by a scavenging reaction between DSFL and HO$^\cdot$ (3). This suggested that the DSFL$^\cdot$ we observed could be a result of DSFL reacting with HO$^\cdot$ generated by the peroxidase/H$_2$O$_2$ system, forming DSFL$^\cdot$ as a consequence. However, using the highly sensitive 4-POBN/ethanol spin-trapping system (6, 17, 18), we were unable to detect significant production of HO$^\cdot$ by the combination of H$_2$O$_2$ and HRP, LPO, or MPO (data not shown). These data suggest that peroxidase enzymes form DSFL$^\cdot$ predominantly via their oxidizing intermediates, i.e. Compounds I and/or II, and not via formation of a HO$^\cdot$ intermediate.

Enhanced DSFL Radical Formation in the Presence of 4-POBN—In the course of the above experiments, we surprisingly observed a 2–5-fold enhancement of DSFL$^\cdot$ formation in the presence of the nitrotrone spin trap 4-POBN at the concentration (10 mM) typically employed in our spin-trapping studies (Fig. 2). Results were qualitatively similar regardless of the peroxidase employed, implying a common mechanism of action in the enhancement of DSFL$^\cdot$ formation. However, the magnitude of increase varied with the peroxidase employed. In the presence of 20 $\mu$g/ml HRP, this enhancement was typically 4-fold, whereas with 20 $\mu$g/ml LPO, this enhancement was as high as 20-fold. Enhanced DSFL$^\cdot$ formation could be observed at 4-POBN concentrations as low as 200 $\mu$M and increased linearly over the range of 200 $\mu$M to 10 mM 4-POBN. Furthermore, by reducing the levels of peroxidase 10-fold, the effects of 4-POBN became more dramatic. Thus, in the presence of 2 $\mu$g/ml LPO and 200 $\mu$M 4-POBN, DSFL$^\cdot$ was easily detected (Fig. 3B), whereas in the absence of 4-POBN (Fig. 3A), it was not seen.

Effect of Other Compounds on DSFL Radical Formation—In an effort to better understand the mechanism(s) responsible for the enhancement of DSFL$^\cdot$ formation, we tested the ability of various compounds representative of the structural moieties of 4-POBN to enhance the formation of DSFL$^\cdot$ by the various peroxidase/H$_2$O$_2$ systems. Initially, we examined the two main structural components of 4-POBN. The nitroso spin trap MNP is contained in the structure of 4-POBN, but has no aromatic ring structure (Fig. 4). In contrast, the compound PNO, while not a spin trap, has a pyridine ring structure that is identical to that found in 4-POBN (Fig. 4), i.e. it includes a nitrogen oxide...
group, but lacks the MNP-like side chain. Surprisingly, at a concentration of 10 mM, both MNP and PNO demonstrated a significant enhancement of DSFL\(^z\) formation, but to a lesser extent than 4-POBN (Fig. 3, compare A with E and A with F). Qualitatively similar increases in DSFL\(^z\) formation in the presence of 4-POBN and MNP, but not PNO, have been observed with MPO and isolated eosinophils (data not shown). This raised the possibility that the nitrone moiety and the aromatic ring structure are at least partially responsible for the enhancement of DSFL\(^z\) formation.

One of the possible explanations for the partial activity of PNO was its lack of a side chain at the 4-position of the aromatic ring. We thus tested 4-picoline N-oxide (4-methyl-PNO) (Fig. 4) in the peroxidase/H\(_2\)O\(_2\) system. Surprisingly, no enhancement of DSFL\(^z\) formation was seen in the presence of this compound (data not shown). Another related compound of interest was pyridine, representing the aromatic ring of PNO without the oxide group (Fig. 4). Addition of 10 mM pyridine to the peroxidase/H\(_2\)O\(_2\) system also demonstrated no increase in DSFL\(^z\) formation (data not shown). Thus, two compounds, MNP and PNO, which share structural features with 4-POBN, were able to enhance DSFL\(^z\) formation, implying a role for the nitrone moiety as well as the nitrogen oxide moiety in the aromatic ring.

We were also interested to see if this enhancement was seen with other nitrene spin traps. The spin trap PBN is structurally similar to 4-POBN, differing only at the 4-position of the aromatic ring (Fig. 4). This feature makes PBN far more lipophilic than 4-POBN. At a concentration of 10 mM, the highest concentration we were able to effectively utilize due to solubility constraints, PBN demonstrated no enhancement of DSFL\(^z\) formation (Fig. 3, A and D). This implies that the spin-trapping nitrene moiety is not involved, but rather it is the nitrogen oxide group in the aromatic ring (Fig. 4) that is important for the enhanced radical formation in the presence of 4-POBN.

Another commonly used nitrene spin trap is DMPO. Aside from the shared nitrene group, DMPO is structurally dissimilar to 4-POBN (Fig. 4). Addition of up to 100 mM DMPO to the peroxidase/H\(_2\)O\(_2\) systems did not lead to enhancement of peroxidase-mediated DSFL\(^z\) formation under our experimental conditions (Fig. 3, A and C). The lack of effect with DMPO suggests that the enhancement of peroxidase-mediated DSFL\(^z\) formation is not a general property of nitrene spin traps, but that features unique to the 4-POBN molecule are responsible for the effect.

Effect of Spin Traps and Related Compounds on Peroxidase Activity—We next wanted to determine whether the enhancement of DSFL\(^z\) formation was the result of a direct interaction between the nitrene compound and DSFL or simply the result of a generalized increase in peroxidase activity. We chose to measure enzymatic peroxidase activity using two distinct assay systems, the oxidation of tetramethylbenzidine and the oxidation of o-dianisidine.

Addition of 4-POBN to either the HRP or LPO system failed to demonstrate any significant increase in peroxidase activity (Table I). DMPO, PBN, and 4-methyl-PNO, all of which failed to demonstrate an increase in DSFL\(^z\) formation, also failed to demonstrate increased peroxidase activity (Table I). MNP and PNO, however, did demonstrate an enhancement of peroxidase activity (Table I). The increases in enzymatic peroxidase activity seen with MNP and PNO were of the same magnitude as those seen in the spin-trapping experiments (Table I and Fig. 3). Pyridine also demonstrated a large increase in enzymatic activity.

![Fig. 3. DSFL\(^z\) formation in the presence of various spin traps.](http://www.jbc.org/)

![Fig. 4. Chemical structures of 4-POBN, PBN, MNP, PNO, 4-methyl-PNO, DMPO, and pyridine.](http://www.jbc.org/)

**Table I**

|          | HRP | o-Dianisidine | LPO | o-Dianisidine |
|----------|-----|---------------|-----|---------------|
| 4-POBN   | 1.01 ± 0.07 (5) | 1.04 ± 0.05 (5) | 1.09 ± 0.14 (7) | 0.94 ± 0.11 (5) |
| MNP      | 2.80 ± 0.50 (3)\(^a\) | 2.43 ± 0.21 (7)\(^b\) | 2.41 ± 0.45 (4)\(^b\) | 4.72 ± 0.88 (5)\(^b\) |
| PNO      | 1.59 ± 0.20 (3)\(^a\) | 1.22 ± 0.06 (5)\(^b\) | 1.28 ± 0.21 (5) | 1.34 ± 0.09 (5)\(^b\) |
| 4-Methyl-PNO | 0.98 ± 0.06 (3) | 0.98 ± 0.03 (4) | 0.92 ± 0.11 (3) | 1.18 ± 0.16 (4) |
| Pyridine | 4.21 ± 0.54 (3) \(^b\) | 6.75 ± 1.08 (4)\(^b\) | 0.91 ± 0.06 (3) | 0.97 ± 0.12 (3) |
| PBN      | 0.78 ± 0.09 (4) | 1.02 ± 0.05 (5) | 0.83 ± 0.05 (5) | 0.93 ± 0.13 (4) |
| DMPO     | 0.86 ± 0.08 (3) | 1.15 ± 0.05 (4) | 1.01 ± 0.06 (4) | 0.97 ± 0.09 (3) |

\(^a\) TMB, tetramethylbenzidine.

\(^b\) Statistically significant compared with control (no spin trap) at p < 0.05.
HPR activity, which was not seen with LPO (Table I). The large increase in enzymatic HPR activity observed in the presence of pyridine was surprising given the lack of enhancement of DSFL\(^*\) formation seen with this compound in the spin-trapping studies.

We can thus conclude that the enhanced DSFL\(^*\) formation seen with the peroxidase/H\(_2\)O\(_2\) system in the presence of 4-POBN is not attributable to its ability to increase peroxidase activity. It is possible that the ability of both MNP and PNO to enhance DSFL\(^*\) formation is explainable on the basis of an increase in enzymatic activity, but this is in contrast to the pyridine data showing an increase in enzymatic activity with no concomitant increase in DSFL\(^*\) formation. These data suggest that it may be the ring structure, which 4-POBN and PNO share, that is of critical importance to the ability of 4-POBN to enhance peroxidase-dependent DSFL\(^*\) formation.

Soriani et al. (5) have proposed that peroxidase-mediated DSFL\(^*\) formation requires the formation of Compound II of the peroxidase. Inclusion of the Compound II-promoting agents I-tryptophan, 3-hydroxybenzaldehyde, or salicylate (all at 10 mM) in the DSFL/H\(_2\)O\(_2\)/peroxidase system yielded no observable increase in DSFL\(^*\) formation under our experimental conditions (data not shown). Thus, the enhancement of DSFL\(^*\) formation seen with 4-POBN is likely not due to an increase in peroxidase Compound II formation.

Spin Trapping a Radical from 4-POBN in the Peroxidase/H\(_2\)O\(_2\) System—Rather than directly increasing the enzymatic activity of the peroxidase, it is possible that 4-POBN is an oxidizable substrate for the peroxidase, forming a radical species that in turn abstracts an electron (hydrogen atom) from DSFL to form DSFL\(^*\). This would have the net effect of increasing the steady-state level of DSFL\(^*\). If this transient 4-POBN species were a free radical (4-POBN\(^{\cdot}\)), we hypothesized that it might be possible to detect its presence directly using EPR. However, previous experiments described above showed that the reaction of either HPR or LPO with H\(_2\)O\(_2\) (without DSFL) in the presence of 10 mM 4-POBN yields no detectable spin-active species. Since this could be related to a very short half-life of such a 4-POBN radical, we explored the possibility that spin trapping could be used to detect such a species.

When 10 mM 4-POBN was added to either the HPR/H\(_2\)O\(_2\) or LPO/H\(_2\)O\(_2\) system in the presence of 100 mM DMPO, the EPR spectrum shown in Fig. 5A was generated. No spectrum above background was observed in the absence of 4-POBN. We propose that this spectrum represents a DMPO/4-POBN spin adduct (a\(_{1N}\) = 15.9 G, a\(_{2N}\) = 13.85 G, and a\(_{3N}\) = 4.64 G). A computer-simulated spectrum with these parameters is shown in Fig. 5B.

Further evidence for the formation of a 4-POBN radical during the reaction of the peroxidases, H\(_2\)O\(_2\), and 4-POBN was obtained by increasing the concentration of 4-POBN to 50 mM. Under such conditions, we were able to routinely observe a 12-line EPR spectrum consistent with the formation of a 4-POBN/4-POBN spin adduct, where a\(_{1N}\) = 1.8 G, a\(_{2N}\) = 14.9 G, and a\(_{3N}\) = 1.85 G (Fig. 6A). A computer-simulated spectrum with these parameters is shown in Fig. 6B.

The detection of the above spin adducts strengthens the possibility that the enhancement of DSFL\(^*\) formation by 4-POBN is due to the generation of an oxidizing 4-POBN radical species. This radical in turn enhances the effectiveness of peroxidase-mediated oxidation of DSFL. We assume that the 4-POBN radical species being trapped is derived from the pyridyl-associated nitroso moiety, but cannot rule out completely an association with the tert-butyl-associated nitroso moiety.

In summary, we have shown that under certain conditions, the nitroso spin trap 4-POBN, the nitroso compound MNP, and the nitrogen oxide compound PNO can enhance DSFL radical formation by several peroxidases. It is likely, however, that these compounds work in different ways to achieve the same end. The biological implications of these observations remain unclear. Nevertheless, nitrones and other related compounds have been investigated as pharmacological agents for the treatment of various pathological states (8–11).

Our data suggest the need for caution in the use of such agents in conditions in which peroxidase-derived oxidants may be involved. Peroxidase-mediated oxidation of compounds other than DSFL could potentially be enhanced in the presence of compounds such as 4-POBN. 4-POBN has been observed to have toxic effects. Normally perfused rat hearts demonstrated a 40% loss of cardiac function following a 15-min exposure to...
4-POBN. In addition, rat hearts reperfused following 30 min of ischemia, which typically recover 40–55% of their preischemic function, demonstrated no recovery if they received prior exposure to 4-POBN. These observations may relate to 4-POBN’s susceptibility to oxidation by peroxidase-like processes. In light of evidence demonstrating a role for peroxidase-mediated oxidation products in human pathology and given the increasing use of nitrone spin traps as pharmacological agents, it is important to evaluate the potential for these nitrone spin traps to both abrogate and enhance free radical-mediated oxidation.

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The Spin Trap α-(4-Pyridyl-1-oxide)-N-tert-butyl nitronitrone Stimulates Peroxidase-mediated Oxidation of Deferoxamine: IMPLICATIONS FOR PHARMACOLOGICAL USE OF SPIN-TRAPPING AGENTS

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