Highlight Review

Proposed Mechanisms for HOOOH Formation in Two Typical Enzyme Reactions Responsible for Superoxide Anion Production in Biological Systems

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Abstract

We investigated the hypoxanthine (HPX)–xanthine oxidase (XOD) reaction by examining the chemiluminescence (CL) response mediated by a luminol analog, 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)-dione sodium salt (L-012). It was found that addition of a high concentration of dimethyl sulfoxide (DMSO), a potent $^{\bullet}OH$ scavenger, could not completely reduce the CL response. This result suggests the existence of an unknown reactive oxygen intermediate other than $O_2^{-\bullet}$ and $^{\bullet}OH$. We further examined the HPX–XOD reaction and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidation reaction by applying an electron spin resonance (ESR) spin-trapping method. In both reaction systems, similar responses were observed. That is, addition of DMSO increased the formation of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) –OOH in a concentration-dependent manner. This indicates that scavenging of $^{\bullet}OH$ increases the detected $O_2^{-\bullet}$ level, further suggesting the existence of an intermediate oxygen species derived from $O_2^{-\bullet}$ and $^{\bullet}OH$. One candidate for this species is HOOOH, presumably formed in the following way.

$$O_2^{-\bullet} + H^+ + {}^{\bullet}OH \rightarrow HOOOH$$

♦ Introduction

Oxygen is one of the most important biomediators for aerobic organisms, since these organisms take oxygen into the body during respiration, and the consumed oxygen is used for oxidation–reduction reactions. These reactions generate four kinds of active intermediates called strict reactive oxygen species (ROSs): superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). A dismutation reaction of $O_2^{-\bullet}$ with water results in the formation of H_2O_2 and $^1O_2$.[1] H_2O_2 is then converted to $^{\bullet}OH$ by a Fenton-like reaction or a metal-ion-catalyzed Haber–Weiss reaction.[2,3] $^1O_2$ is also generated by a peroxidase, H_2O_2, and halide system, such as myeloperoxidase-catalyzed bactericidal action.[4–7]

Beside these ROSs, attention has gradually been paid to hydrogen trioxide (HOOOH) in the past decade. Several reports have suggested that RO_3H (R = H and alkyl) species are key intermediates in both natural and polluted atmospheric environments[8,9] and in low-temperature ozonization of various organic substances.[10] In the case of biological systems, it has been reported that all antibodies have the ability to catalyze the oxidation of water by $^1O_2$ to generate H_2O_2 and probably O_3 as well. The authors of that study postulated that antibodies carry the reaction through HOOOH as a key intermediate, as in the following reaction 1.[11]

$$2{}^1O_2 + 2H_2O \rightarrow 2HOOOH \rightarrow H_2O_4 + H_2O_2 \rightarrow {}^3O_2 + 2H_2O_2 \quad (1)$$

Although HOOOH has not yet been detected in biological systems in vivo, its in vitro production has been confirmed experimentally.[12,13] For instance, it has been shown that HOOOH generated reductantly from ozone decomposes into H_2O and $^1O_2$ in a process catalyzed by a water molecule.[14] The reverse reaction is surmised to be catalyzed by one or more molecules of water, as described above.[11]

Recently, we postulated the existence of HOOOH as a new reactive intermediate oxygen species found in hypoxanthine (HPX) and xanthine oxidase (XOD) reactions[15] and in nicotinamide adenine dinucleotide phosphate (NADPH) oxidation reactions (unpublished data). Our interest is in whether HOOOH plays an important role in biological systems. Therefore, this Highlight Review focuses on the possible mechanisms for HOOOH formation in these two typical enzyme reactions of XOD and NADPH oxidase.

♦ Xanthine Oxidase Reaction

XOD is known to generate $O_2^{-\bullet}$ when acting on its substrates in the presence of oxygen.[16–18] The consumption of oxygen by the XOD system has been postulated as the following pathway.[17]

$$HPX + XOD + 2O_2 \rightarrow 2H^+ + 2O_2^{-\bullet} \quad (2)$$

$$2O_2^{-\bullet} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

Furthermore, it has been reported that superoxide dismutase (SOD) inhibits the lipid peroxidation caused by XOD, as does catalase, indicating that both $O_2^{-\bullet}$ and H_2O_2 are essential intermediates.[18]

Electron spin resonance (ESR) spectrometry using the spin-trapping agent 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has been applied to characterize the different radical species generated by XOD, along with the mechanisms of their generation.[19] In those authors' study, the reaction of xanthine with XOD equilibrated with air resulted in DMPO–OOH (an adduct from DMPO and $O_2^{-\bullet}$) and DMPO–OH (an adduct from DMPO and $^{\bullet}OH$). Since α-hydroxylethyl or methyl radicals are generated in the presence of ethanol or dimethyl sulfoxide (DMSO),

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respectively, both of which are potent scavengers of $^{\bullet}OH$, it has been shown that DMPO–OH is generated directly from $^{\bullet}OH$ rather than from the breakdown of DMPO–OOH.

Taking the reported results together, the following reactions 2–4 are proposed for the HPX–XOD system.

$$HPX + XOD + 2O_2 \rightarrow 2H^+ + 2O_2^{-\bullet} \quad (2)$$

$$2O_2^{-\bullet} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH \quad (4)$$

As shown by these reactions 2–4, the enzyme system generates $^{\bullet}OH$ as well as $O_2^{-\bullet}$, so it has been widely used for the screening and development of novel antioxidants as functional active ingredients for neutraceuticals, cosmeceuticals, and pharmaceuticals.[20–26]

ESR spin-trapping and chemiluminescence (CL) methods are widely used for analyses of ROSs such as $O_2^{-\bullet}$ and $^{\bullet}OH$. The ROS selectivity of CL methods, however, is poor. In addition, the luminol CL reaction is well documented, whereas the reaction processes in ROS generation systems, such as the HPX–XOD system, are not clear. It has been reported that a luminol-mediated CL response occurs in the HPX–XOD system, according to the following reactions 2–7.[27]

$$HPX + XOD + 2O_2 \rightarrow 2H^+ + 2O_2^{-\bullet} \quad (2)$$

$$2O_2^{-\bullet} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH \quad (4)$$

$$^{\bullet}OH + LH^- \rightarrow OH^- + H^+ + L^{-\bullet} \quad (5)$$

$$L^{-\bullet} + O_2^{-\bullet} \rightarrow LO_2^{2-} \quad (6)$$

$$LO_2^{2-} \rightarrow N_2 + AP^{*2-} \rightarrow AP^{2-} + h\nu \quad (7)$$

LH^-: luminol monoanion, $L^{-\bullet}$: luminol radical, AP^{2-}: aminophthalate dianion, AP^{*2-}: electronically excited state of AP^{2-}.

The reactions show that LH^- reacts with $^{\bullet}OH$ to form $L^{-\bullet}$, and the resultant $L^{-\bullet}$ reacts with $O_2^{-\bullet}$. According to the reactions, if $^{\bullet}OH$ is completely scavenged, no CL response is assumed to be observed. Thus, we previously examined the effect of DMSO, a potent $^{\bullet}OH$ scavenger, on the CL response in the HPX–XOD system.[15] In that study, we used a luminol analog, 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)-dione sodium salt (L-012), as a CL probe. Figure 1a shows the effect of DMSO on the L-012-mediated CL response in the HPX–XOD system. When HPX was added to the mixture, a rapid CL response was observed, and the CL response was reduced by the presence of DMSO in a concentration-dependent manner. Figure 1b, which shows the DMSO-induced inhibition rate, demonstrates that DMSO at a concentration of 240 mM maximized the reduction of the CL response, and an even higher concentration of DMSO reduced the CL response by up to 90%. In contrast, the CL response was reduced by 99% or more in the presence of either SOD or DMPO (data not shown).[15] As described above, LH^- reacts with $^{\bullet}OH$ to form $L^{-\bullet}$, and then the resultant $L^{-\bullet}$ reacts with $O_2^{-\bullet}$.[22] Thus, maximal scavenging of $^{\bullet}OH$ is assumed to cause the complete lack of CL response. The addition of DMSO, however, could not reach a level near 100% reduction of the CL response, so we speculate the existence of an intermediate, other than $^{\bullet}OH$, that is reactive to LH^-. Since one candidate intermediate is $O_2^{-\bullet}$, we examined whether L-012 reacts with $O_2^{-\bullet}$ by applying an ESR spin-trapping method. In either the presence or absence of DMSO, different concentrations of L-012 were added to the HPX–XOD system, and the DMPO–OOH adduct was monitored by ESR. As shown in Figure 2, even in the presence of DMSO, the DMPO–OOH formation was inhibited by L-012 in a concentration-dependent manner, although the inhibition was weaker than in the absence of DMSO. Our preliminary study showed that the rate constant for LH^- with $^{\bullet}OH$ is smaller than that

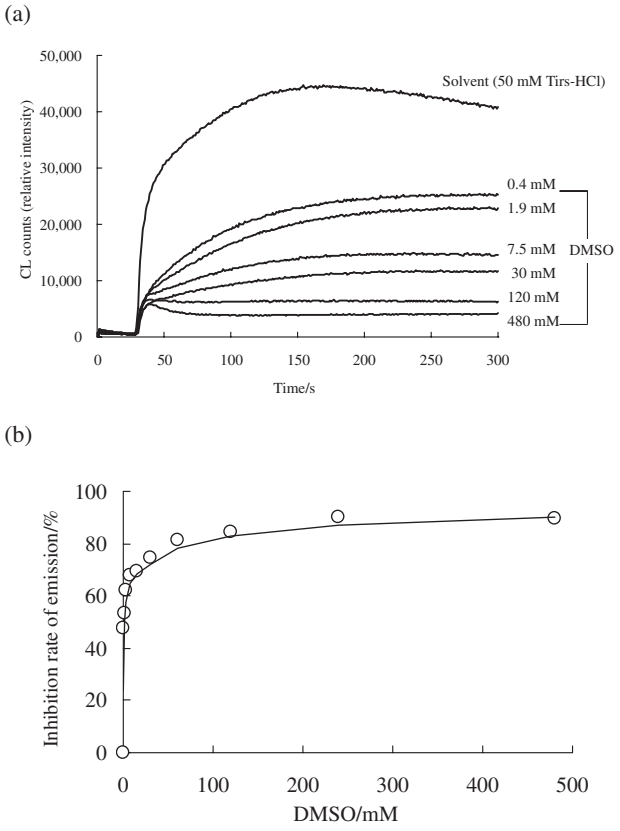

Figure 1. Effect of DMSO, a potent $^{\bullet}OH$ scavenger, on the L-012-mediated CL response in the HPX–XOD system. (a) Representative CL responses, and (b) inhibition rate of the CL response through the addition of different concentrations of DMSO. Each CL response curve or inhibition rate value represents the mean of duplicate trials.

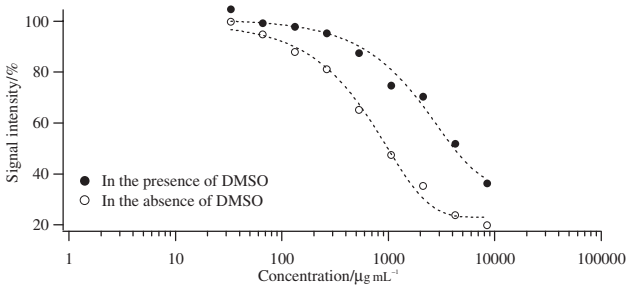

Figure 2. Inhibition curves for DMPO–OOH formation, obtained by the addition of different concentrations of L-012 in the presence or absence of DMSO in the HPX–XOD system. Each value represents the mean of duplicate trials.

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for mannitol with $^{\bullet}OH$ (order of 10^8),[28,29] and the rate constant for DMSO with $^{\bullet}OH$ has been reported to be on the order of 10^9.[27] In other words, the rate constant for DMSO with $^{\bullet}OH$ is much higher than that for LH^- with $^{\bullet}OH$, so that the high concentration of DMSO used in the study was sufficient to completely scavenge $^{\bullet}OH$. Thus, under the condition of a lack of $^{\bullet}OH$, L-012 likely competes with DMPO to react with $O_2^{-\bullet}$. From these considerations, the reactions 2–7 given above should likely be amended as follows, in which LH^- reacts directly with $O_2^{-\bullet}$ to form $L^{-\bullet}$, as reported in a previous paper.[30]

$$HPX + XOD + 2O_2 \rightarrow 2H^+ + 2O_2^{-\bullet} \quad (2)$$

$$2O_2^{-\bullet} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH \quad (4)$$

$${}^{\bullet}OH + LH^- \rightarrow OH^- + H^+ + L^{-\bullet} \quad (5)$$

$$LH^- + O_2^{-\bullet} + H^+ \rightarrow L^- + H_2O_2 \quad (8)$$

$$L^{-\bullet} + O_2^{-\bullet} \rightarrow LO_2^{2-} \quad (6)$$

We found an interesting phenomenon in another experiment, in which the effect of different concentrations of DMSO on DMPO–OOH and DMPO–OH formation was examined by the ESR spin-trapping method, as shown in Figure 3. DMPO–OOH formation was increased two- to threefold by the addition of DMSO, whereas DMPO–OH formation was almost completely reduced. These results suggest an interaction between $O_2^{-\bullet}$ and $^{\bullet}OH$. We postulate a possible interaction via the following reaction.

$$O_2^{-\bullet} + H^+ + {}^{\bullet}OH \rightarrow HOOOH \quad (9)$$

Although this should be confirmed in a future study, HOOOH may contribute to the luminol-mediated CL response in the following way.

$$HOOOH + {}^{\bullet}OH \rightarrow HOOO^{\bullet} + H_2O \quad (10)$$

$$HOOO^{\bullet} + LH^- \rightarrow L^{-\bullet} + HOOOH \quad (11)$$

$$L^{-\bullet} + O_2^{-\bullet} \rightarrow LO_2^{2-} \quad (6)$$

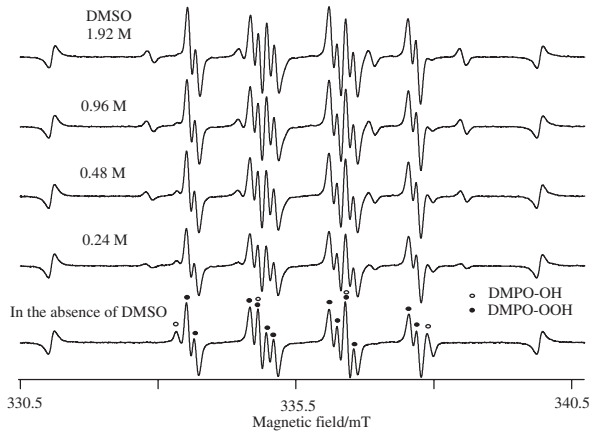

Figure 3. Representative ESR spectra showing the effect of DMSO, a potent $^{\bullet}OH$ scavenger, on DMPO–OOH and DMPO–OH formation in the HPX–XOD system.

Together with the possibilities of direct reaction of LH^- with $O_2^{-\bullet}$ to form $L^{-\bullet}$ and interaction of $O_2^{-\bullet}$ and $^{\bullet}OH$ to generate HOOOH, we finally postulate the following reactions 2–11 for the HPX–XOD system, as summarized schematically in Figure 4.

$$HPX + XOD + 2O_2 \rightarrow 2H^+ + 2O_2^{-\bullet} \quad (2)$$

$$2O_2^{-\bullet} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH \quad (4)$$

$${}^{\bullet}OH + LH^- \rightarrow OH^- + H^+ + L^{-\bullet} \quad (5)$$

$$LH^- + O_2^{-\bullet} + H^+ \rightarrow L^- + H_2O_2 \quad (8)$$

$$O_2^{-\bullet} + H^+ + {}^{\bullet}OH \rightarrow HOOOH \quad (9)$$

$$HOOOH + {}^{\bullet}OH \rightarrow HOOO^{\bullet} + H_2O \quad (10)$$

$$HOOO^{\bullet} + LH^- \rightarrow L^{-\bullet} + HOOOH \quad (11)$$

$$L^{-\bullet} + O_2^{-\bullet} \rightarrow LO_2^{2-} \quad (6)$$

$$LO_2^{2-} \rightarrow N_2 + AP^{*2-} \rightarrow AP^{2-} + h\nu \quad (7)$$

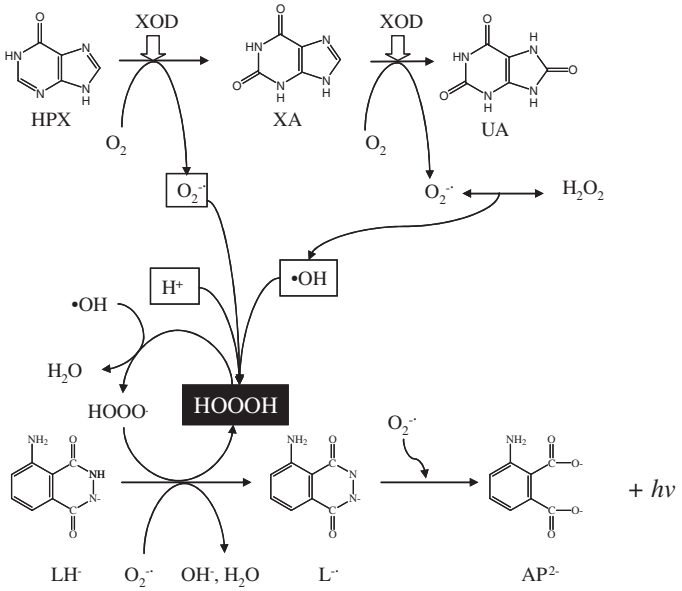

Figure 4. Schematic illustration of the proposed mechanism inducing the luminol-mediated CL response.

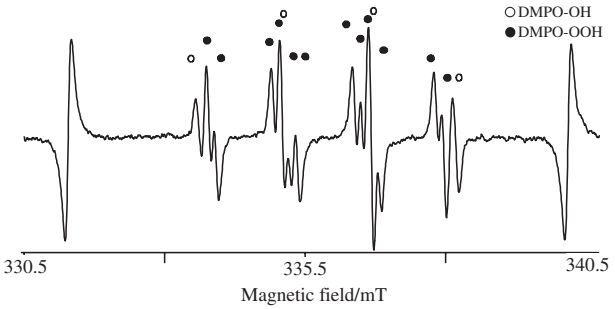

Figure 5. Representative ESR spectrum obtained from the NADPH–NADH oxidase system.

♦ *NADPH Oxidase Reaction*

Phagocytic cells such as neutrophils and macrophages play an important role in host defense against microbial infections. The microbicidal mechanism consists of phagocytosis of pathogens and production and subsequent release of ROSs and bactericidal proteins to phagosomes. During the microbicidal response, professional phagocytic cells produce $O_2^{-\bullet}$, a precursor of microbicidal oxidants.[31–33] The process involves activation of NADPH oxidase, which catalyzes the reduction of molecular oxygen to $O_2^{-\bullet}$ at the expense of NADPH in the phagocytes.

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{-\bullet} + H^+ \qquad (12)$$

The necessity of NADPH oxidase in host defense is demonstrated by the recurrent, life-threatening infections occurring in patients with chronic granulomatous disease, a disorder in which NADPH oxidase is nonfunctional because of a deficiency in its oxidase components.[34–36] Besides the essential role of NADPH oxidase in the innate immune system, it is also a prominent contributor to a variety of inflammatory disorders. A vast amount of circumstantial evidence implicates ROSs produced uncontrollably by phagocytic cells as mediators of inflammation, shock, and ischemia/reperfusion injury.[37]

The NADPH oxidases are a group of plasma-membrane-associated multicomponent enzymes consisting of at least two components bound to the plasma membrane ($gp91^{phox}$ and $p22^{phox}$, which together form the flavocytochrome b_{558}), three cytosolic components ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$), and a small GTPase Rac.[38] In resting phagocytic cells, NADPH oxidases are dormant, and their components exist separately in the membrane and in the cytosol. Once phagocytic cells are primed by appropriate stimuli, NADPH oxidases are activated to produce $O_2^{-\bullet}$ by association of these cytosolic components with the plasma or phagosome membrane components.[39–42]

In our study using the cell-free NADH oxidase system with the ESR spin-trapping method, $^{\bullet}OH$ was detected as shown in Figure 5. It was presumably formed according to the following reaction 4 given by Hodgson and Fridovich.[27]

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH \qquad (4)$$

Figure 6 shows representative ESR spectra demonstrating the effect of DMSO on DMPO–OOH and DMPO–OH formation in the NADPH oxidation reaction, and Figure 7 summarizes the effect of DMSO on the signal intensities of DMPO–OOH and DMPO–OH. The inset figure in Figure 7 shows the negative correlation between DMPO–OOH and DMPO–OH formation.

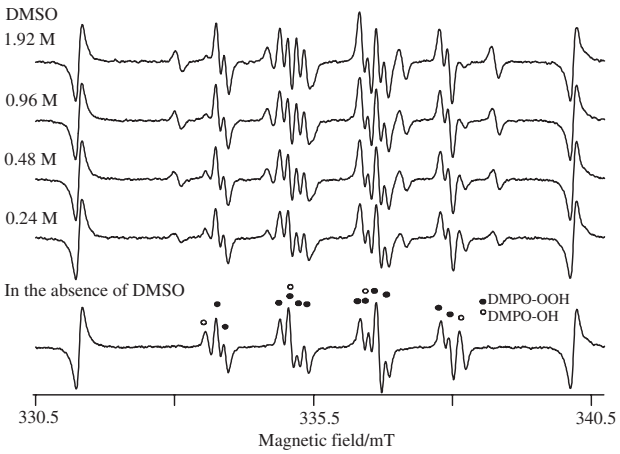

Figure 6. Representative ESR spectra demonstrating the effect of DMSO, a potent $^{\bullet}OH$ scavenger, on DMPO–OOH and DMPO–OH formation in the NADPH–NADH oxidase system.

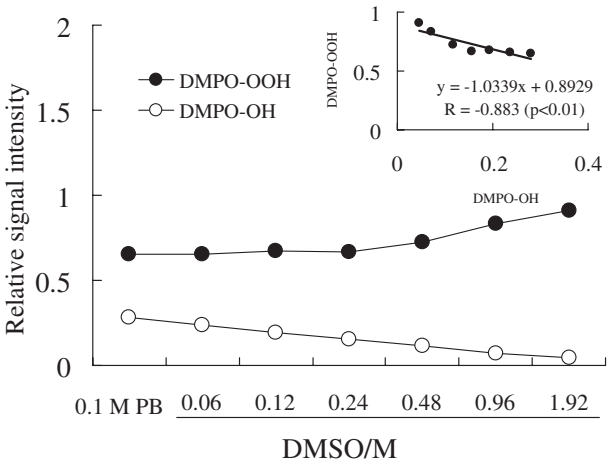

Figure 7. Effect of different concentrations of DMSO on the relative signal intensities of DMPO–OOH and DMPO–OH. The relative signal intensities of DMPO–OOH and DMPO–OH were obtained by dividing each by the signal intensity of Mn^{2+} as an internal standard. Each value represents the mean of duplicate trials.

Addition of different concentrations of DMSO augmented the DMPO–OOH level and reduced the DMPO–OH level in a concentration-dependent manner. In other words, scavenging of $^{\bullet}OH$ increases the detected $O_2^{-\bullet}$ level, suggesting the existence of an intermediate oxygen species derived from $O_2^{-\bullet}$ and $^{\bullet}OH$. We thus postulate that this species might be HOOOH, as reported in a previous study on the HPX–XOD system.[14]

$$O_2^{-\bullet} + H^+ + {}^{\bullet}OH \rightarrow HOOOH \qquad (9)$$

As with Figure 4 summarizing the proposed mechanism for HOOOH formation in the HPX–XOD system, Figure 8 schematically illustrates the proposed mechanism for HOOOH formation in the NADPH–NADH oxidase system.

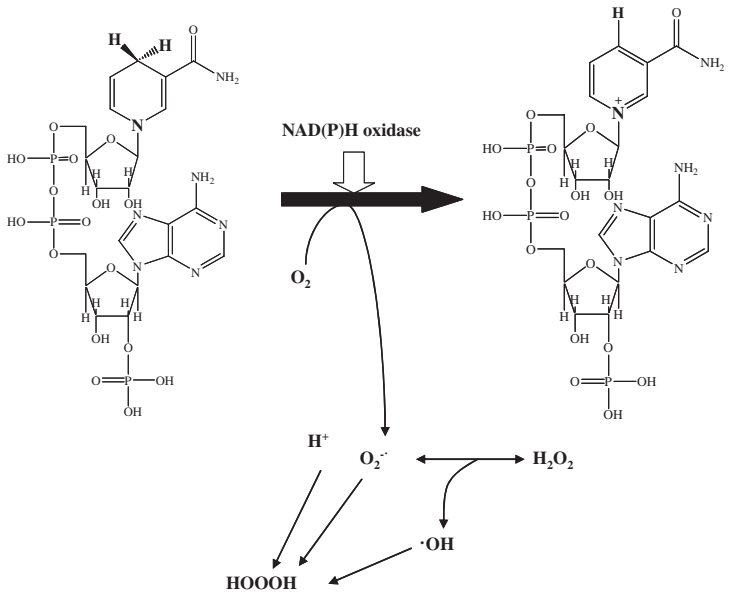

Figure 8. Schematic illustration of the proposed mechanism by which HOOOH is formed in the NADPH oxidation reaction.

♦ *Conclusion*

XOD, which catalyzes the oxidation of HPX to xanthine and further catalyzes the oxidation of xanthine to uric acid, plays an important role in catabolism of purines. In addition, XOD is thought to be a primary source of ROSs that mediate ischemic-reperfusion-related injuries.[43] NADPH oxidase is essential in host defense through ROS production of primed phagocytic cells, while ROSs produced uncontrollably by phagocytic cells act as mediators of inflammation. In these important biological systems, $O_2^{-\bullet}$, $^{\bullet}OH$, and H_2O_2 have been considered the major ROSs. Our recent studies propose both the existence of HOOOH as an additional ROS and mechanisms for its formation. As a next step, detection of HOOOH and the biological implications of HOOOH should be addressed.

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