What Is Responsible for the Initiating Chemistry of Iron-Mediated Lipid Peroxidation: An Update

Zhiyong Cheng[†] and Yuanzong Li*

The Key Laboratory of Bioorganic & Molecular Engineering, College of Chemistry & Molecular Engineering, Peking University, Beijing, China 100871

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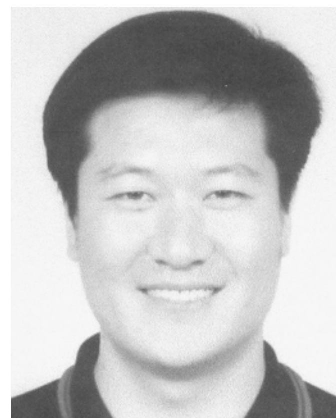
1. Introduction

In the past few decades, extensive studies have been performed and have shown the causative role of iron-mediated lipid peroxidation (LPO) in human diseases and in the deterioration of the quality of food products.[1,2] Because LPO-induced changes in the permeability and fluidity of membranes compromise the functions of receptors, ion channels, and other proteins on biomembranes,[3] LPO is associated with various pathological events, such as inflammation,[4] postischemic reperfusion injury,[5] atherosclerosis,[6] ethanol toxicity,[7] and cancer.[8] Moreover, there have been reports on the possible involvement of LPO in the toxicities of drugs[9] and environmental pollutants[10] and in the acute or chronic consequences of trauma.[11−13]

Chemical research[1,2,14,15] indicates that LPO is a set of radical-mediated chain reactions. The overall process consists of three stages: initiation, propagation, and termination. Such a chain reaction is initiated by the abstraction of a hydrogen atom from the reactive methylene group (LH) of lipids, and the process of H-abstraction leaves behind one unpaired electron on the carbon atom of the methylene group, which promotes the rearrangement of the double bonds adjacent to the methylene group and produces alkyl radicals ($L^•$). In the presence of oxygen, alkyl radicals react to form peroxyl radicals ($LOO^•$) that can abstract a hydrogen atom to produce lipid peroxide (LOOH) and propagate the reactions.

* To whom correspondence should be addressed. Telephone: +86-10-6275 7954. Fax: +86-10-6275 1708. E-mail: yzli@pku.edu.cn.
† Present address: Howard Hughes Medical Institute, Department of Biological Chemistry and Molecular Pharmacology, Harvard University Medical School, 300 Longwood Ave, Boston, MA 02115.



Zhiyong Cheng received his Ph.D. degree from Peking University (China) in 2003, with Dr. Prof. Yuanzong Li as his adviser. His thesis work was focused on the mechanisms of the antioxidant functions of phenolic compounds against iron-mediated oxidative damage to biomolecules. After postdoctoral research at the University of Michigan (Ann Arbor), where he studied the redox properties of the thioredoxin system in *Drosophila Melanogaster* under the supervision of Drs. Profs. Charles H. Williams and David P. Ballou, Dr. Cheng joined Harvard University Medical School in 2006. He is currently working with Dr. Prof. Morris F. White (Howard Hughes Medical Institute, Department of Biological Chemistry and Molecular Pharmacology) on the redox signaling and mitochondrial function in diabetic pathogenesis.

Yuanzong Li received his Ph.D. in Chemistry at Lanzhou University of P. R. China (with Professor Yaozu Chen) and competed postdoctoral research at Peking University (with Prof. Yunxiang Ci). He has been active in research at Hull University, U.K. (1995), Tokushima University, Japan (1999), and Harvard University, U.S.A. (2002–2003). Currently he is a professor of Chemistry at Peking University (Beijing, P. R. China) with his research focusing on molecular recognition and biochemical analysis along with QSAR study of phenolic antioxidants against oxidative stress.

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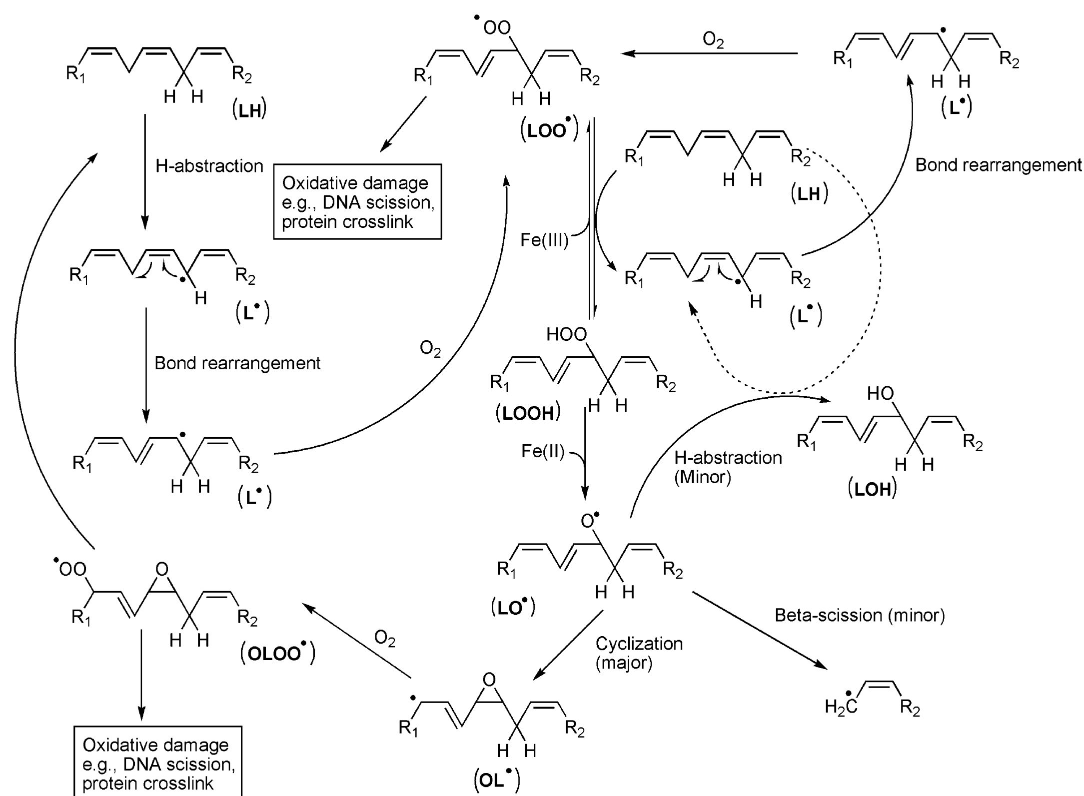


Figure 1. Overview of iron-catalyzed LPO and formation of lipid-derived radicals.

The resultant LOOH is cleaved in the presence of active iron ions, forming more reactive species such as LOO•, alkoxyl (LO•), or epoxyperoxyl radical (OLOO•) (Figure 1). These species can abstract H-atoms from lipid to propagate LPO or terminate the chain reaction by dimerization or by attacking proteins and DNA to cause oxidative damage and protein/DNA cross-links.[16−17]

Currently there is no doubt that iron (often as a complex) is an effective catalyst in the process of LPO,[18−23] but the mechanism by which iron initiates LPO is still under open debate. The Fenton reaction has long been considered as the likely mechanism for producing highly oxidizing hydroxyl radicals (•OH) to abstract hydrogen atoms from polyunsaturated fatty acids (PUFAs),[24,25] but H_2O_2-degrading enzymes or hydroxyl radical scavengers rarely inhibit the iron-dependent peroxidation.[26−28] Similarly, superoxide anion radical ($O_2^{\bullet-}$) as an LPO initiator[29,30] via the Haber−Weiss reaction or direct H-abstraction reactions was doubted because superoxide dismutase (SOD) had no effect on the superoxide-present LPO systems.[31,32] Given that iron-dependent LPO in systems comprised initially of Fe(II) and phospholipid liposomes requires some Fe(II) oxidation and that LPO in systems containing Fe(III) and liposomes requires some Fe(III) reduction, the hypothesis involving an Fe(II)−O_2−Fe(III) complex was developed by Aust et al., who suggested that a critical ratio of Fe(II)/Fe(III) (1:1) should be achieved for the maximal rate of LPO.[26,33,34] Nevertheless, some doubts have arisen concerning the Fe(II)−O_2−Fe(III) complex as the initiating species. For example, attempts to isolate such a complex have failed,[33,35] and the Fe(II)/Fe(III) ratio of 1:1 was not necessary for maximal stimulation of LPO in some systems,[36,37] arguing against the proposal of a specific stoichiometric complex for LPO initiation. The substitution of Pb(II) or Al(III) for Fe(III) in stimulating Fe(II)-mediated LPO of liposomes and microsomes[38−40] suggested that Fe(III) is not imperative for the LPO initiation in those systems. In accordance with the oxidizing intermediates formed in peroxidase-catalyzed reactions, some groups suggested that the iron-related peroxidation might be initiated by ferryl ion (FeO^{2+})[41,42] or perferryl ion ($Fe^{2+}O_2$).[43,44] These conflicting reports have confused our understanding of the initiating mechanisms of LPO, and they may even mislead mechanistic investigations on LPO-mediated cell injuries or antioxidant activities of certain compounds if not resolved.[18,45,46]

Fortunately, two essential points shown in the literature have clearly suggested some competent LPO initiators and led to an elegant explanation of these discrepancies. First, the LPO products were found in enantiomeric excess from natural sources, which cannot be achieved by achiral species at all (e.g., •OH or $O_2^{\bullet-}$). In this respect, enzymes (such as lipoxygenases) using PUFAs as reductant substrates should be the prime candidates because they catalyze LPO in an enantiospecific way.[47−49] In addition, the hypervalent iron species generated by heme proteins or simple iron complexes showed selective reactivity toward lipids and should also be candidates as LPO initiators according to their oxidizing ability. Second, the widely occurring contamination of lipid hydroperoxide (LOOH) in lipid samples potentiates LPO initiation in these systems (in the sense of abstracting the first hydrogen atom)[26,50] and is at least partially responsible for the controversy. Iron may stimulate peroxidation by decomposing peroxides to lipid radicals, which then abstract hydrogen and propagate the chain reaction.[51,52] But the detection of superoxide or hydroxyl radicals is not necessarily an indicator of their function in LPO initiation. In this review, we provide a comprehensive discussion on proposed LPO initiators in the literature of the past few decades, focusing on the most effective species, namely, LOOH-derived lipid radicals (secondary initiation), hypervalent iron complexes,

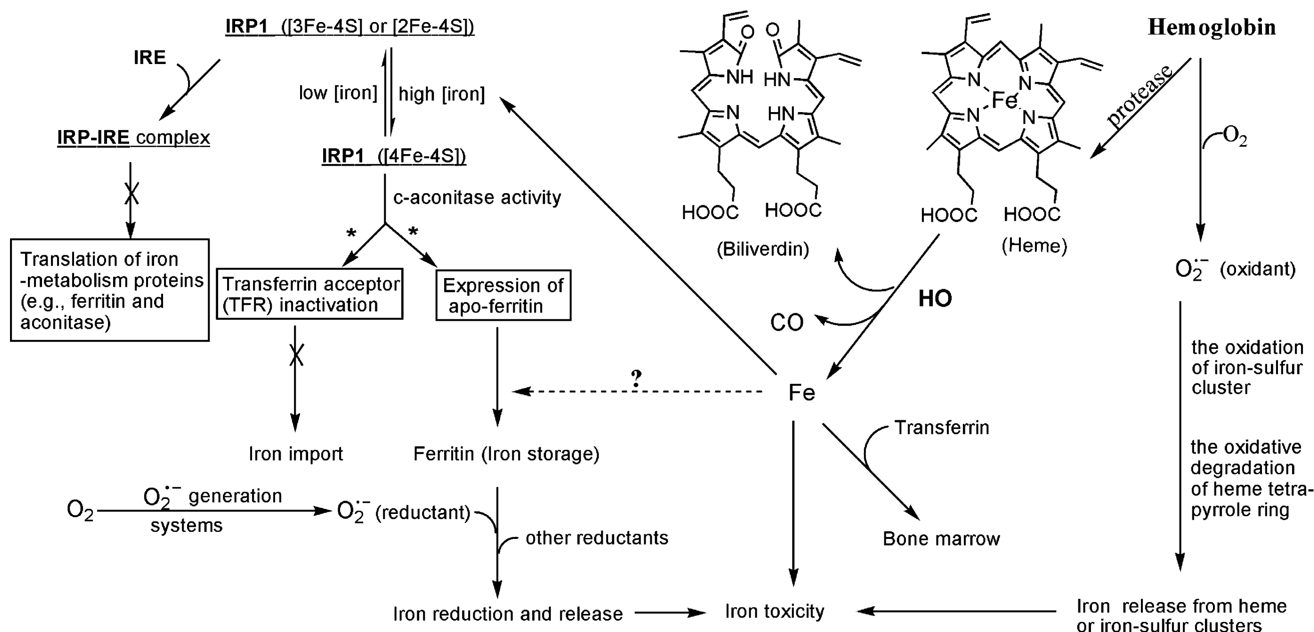


Figure 2. Overview of iron regulation and iron release under stress conditions. Note that IRP2 cannot assemble the [4Fe−4S] cluster or exhibit aconitase activity at high iron concentration but is degraded by proteasomes. At low iron concentration, IRP2 will bind to the IRE of mRNA in a similar way to IRP1. IRP, iron regulatory protein; IRE, iron regulatory element; HO, heme oxygenase; Hb, hemoglobin. The symbol ∗ represents excitation/stimulation, and × means inhibition or reduction.

and enzymatic oxidation. The role of Fe(II), Fe(III), chelators, and other metal cations in LPO initiation and inhibition is discussed, providing an overall picture of the initiating chemistry for LPO.

2. Sources of Iron in LPO

Iron is an essential element required for growth and survival of almost every organism. It is reported that about 65% of iron exists in hemoglobin-bound forms, 10% is present in myoglobin, cytochromes, and iron-containing enzymes, and 25% is bound to the iron storage proteins, ferritin and haemosiderin.[1] The liganding of iron enables it to vary the oxidation state, reduction potential, and electronic spin configuration and thus to play a variety of roles in biology: on one hand, iron is the essential cofactor for a variety of proteins, e.g., cytochromes and other heme-containing proteins and iron−sulfur proteins;[19] on the other hand, it is also a potential catalyst for chemical reactions involving free radical formation and subsequent oxidative stress and cell damage.[42,53] For example, there is considerable evidence that reaction of active forms of oxygen with PUFAs in biomembranes can result in cell damage in the presence of iron.[47,54−60] In some cases, even iron-containing proteins were reported to induce LPO.[19,61] The reaction of hydrogen peroxide with Fe(II) hemoglobin (oxyHb and deoxyHb) and Fe(III) hemoglobin (metHb) produces ferrylhemoglobin (ferrylHb) and oxoferrylhemoglobin (oxoferrylHb), respectively,[62,63] and both of them are implicated in cellular and tissue damage.[64,65] The direct cytotoxic effects associated with hemoglobin (Hb) or myoglobin (Mb) have been ascribed to their redox reactions with peroxides that form the ferryl oxidation state of the proteins, analogous to compounds I and II formed in the catalytic cycle of many peroxidase enzymes.[66] This higher oxidation state of the protein is a potent oxidant capable of promoting oxidative damage to most classes of biological molecules including LPO.

Cellular iron uptake, storage, and utilization are strictly controlled in organisms in order to minimize the pool of potentially toxic “free iron”; this is the function of iron regulatory proteins (IRPs) and iron storage proteins (Figure 2). This tight control prevents potential iron-mediated health disturbances.[1,67,68] IRPs are cytosolic trans regulators able to bind to specific RNA stem−loop structures called iron-responsive elements (IREs), located on the mRNA of several proteins relevant to cellular iron metabolism.[69−71] The IRP−IRE complex formation leads to decreased translation of genes containing an IRE in their 5′ untranslated regions. Proteins regulated in this way include ferritin, mitochondrial aconitase, and erythroid 5-aminolevulinic acid synthetase (eALAS). At high levels of intracellular iron, IRP2 is degraded but IRP1 assembles a [4Fe−4S] cluster, which switches the function of IRP1 from IRE binding to acting as a cytoplasmic isoform of aconitase (c-aconitase). This leads to the high expression of apo-ferritin for greater iron storage and destabilizes the transferrin receptor for reduced iron import. When intracellular iron levels decrease, the [4Fe−4S] cluster is disassembled, resulting in a loss of the c-aconitase activity and the restoration of IRE binding activity.[70] Such a coordinated regulation of gene expression maintains a balance between iron storage and uptake; iron release from this type of iron−sulfur protein will not be a toxicological problem because it is under strict regulation.

Under physiological conditions, however, the release of free iron due to nonenzymatic degradation of heme proteins has been implicated in toxicity.[66,72,73] The iron contained in ferritin can be released by reducing agents such as superoxide ion, dihydroflavins, and paraquat.[74] Under oxidative stress conditions, superoxide is generated in a short burst, which is effective in reducing the ferric (the stored form in ferritin) to ferrous and causing iron release (Figure 2).[75,76] A variety of enzyme systems, such as the xanthine/xanthine oxidase (XO) system, the nicotinamide adenine dinucleotide (NADH)/nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, and the hypoxanthine (HX)/XO system, participate in generation of $O_2^{\bullet-}$.[77,78] Also, some biochemical autoxidation process may produce superoxide and contribute

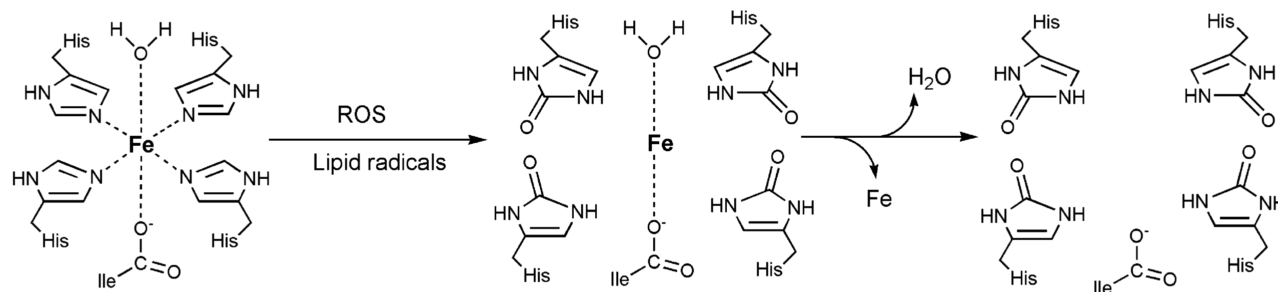
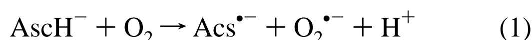
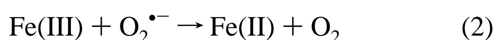


Figure 3. Schematic for the oxidation of the labile histidine residues and iron release at the active site during lipoxygenase suicide. Rabbit reticulocyte 15-lipoxygenase, which has four conserved histidines (His361, His366, His541, and His545) and a C-terminal isoleucine in its active site, is taken as an example. The attack of these labile histidines by ROS or lipid-derived radicals produces 2-oxo-histidine, which has no iron-ligating capacity, thus leading to iron release.

to iron release, such as the autoxidation of ascorbate[27,79] and hemoglobin.[80] Ascorbate autoxidation (reaction 1) is subject to kinetic and thermodynamic restraints, but the reaction can be greatly favored in the presence of Fe(III) or Fe(III) ligand (such as ferritin) as a direct one-electron acceptor (reaction 2), concomitant with increasing generation of superoxide.[81,82] In the case of hemoglobin, its reaction with H_2O_2 can also produce $O_2^{\bullet-}$ radical anion[83,84] in addition to its autoxidation.[80] The superoxide generated in the heme pocket was ideally located to oxidize the tetrapyrrole rings, leading to heme degradation, iron release, and formation of two fluorescent products.[84] It was also found that H_2O_2 promotes more iron release from glycated hemoglobin than that from nonglycated hemoglobin.[85] The iron released from heme degradation is a potential source of oxidative damage in red cells and endothelial cells.[83,84,86]


$$AscH^- + O_2 \rightarrow Acs^{\bullet-} + O_2^{\bullet-} + H^+ \qquad (1)$$


$$Fe(III) + O_2^{\bullet-} \rightarrow Fe(II) + O_2 \qquad (2)$$

Cell stimulation can activate a broad spectrum of enzymes.[87] In addition to those participating in superoxide generation from phagocyte attack (e.g., XO), lipoxygenases (LOXs) and proteases are two important classes involved in iron release under stress conditions. The active site of LOX comprises a non-heme iron cofactor and conserved labile histidines to ligate the iron,[88−90] and the LOX pathway may stimulate the oxidation of the histidines and switch on iron release because activation of LOXs by cell injury elicits oxidation of PUFAs and produces oxidizing intermediates, such as hydroperoxide, alkoxyl, and peroxyl radicals.[47] Hydroxyl radicals derived from the Fenton reaction or Habor−Weiss reaction were also implicated in the oxidant attack on histidines.[91] When oxidizing intermediates are generated in the neighborhood of the labile histidine residues at the active center, they can oxidize histidines into 2-oxo-histidines that lack iron-chelating ability (Figure 3).[47,91,92] As a result, LOXs can be deactivated in a suicide way and switch on iron release during massive cell injury/stimulation. Another class of activated enzyme is protease, which can remove the protein cover/exterior from iron-containing proteins and contribute to iron release. A case of considerable interest is hemoglobin proteases, which in malaria parasites were found to ingest host hemoglobins to supply amino acids for the growth and maturation of the parasites.[93−95] The release of heme after hemoglobin degradation can be a powerful prooxidant in LPO,[96] and the resultant radical intermediates are likely to cause further damage to host cells by creating oxidative stress.[97] In this respect, heme oxygenase (HO) may exert a coupled cellular protection with ferritin that sequesters the free iron released by HO-catalyzed breakdown of heme.[98,99] Enzymatic heme degradation by the HO produces biliverdin, carbon monoxide, and iron.[100,101] Before being sequestered by ferritin or assembling the [4Fe−4S] cluster in the aconitase pathway, the released iron may be available for the catalysis of deleterious oxidation like LPO and membrane damage.[102] The toxicity of HO-liberated iron can be prevented somewhat by biliverdin or its further reduction product (bulirubin) because both of them are antioxidants.[101,103] In addition, (apo)ferritin and transferrin may exert a primary defense because they can effectively chelate/store iron in a form of ferritin[104−108] or chelate/transport iron to the bone marrow for recycling.[109,110] Considerable evidence has been reported that release of iron via HO-mediated catabolism of heme is a trigger for *de novo* synthesis of ferritin or ferritin apoprotein,[98,99,104,111,112] and the extent of induction ferritin synthesis is directly related to the size of the chelatable iron pool.[99] This induction protected cells from subsequent exposure to toxic concentrations of hemin, and pretreatment with apoferritin protected astrocytes from hemin toxicity in a concentration-dependent fashion.[104,111] In a study on the adaptive response to oxidative stress in human skin fibroblasts, supplement of deferrioxamine could substitute for ferritin synthesis to protect against ultraviolet A radiation where an increased level occurs to the heme catabolizing enzyme HO-1.[98] Thus, the combined action of heme oxygenase-1 and ferritin appears to act as a primary defense against heme-mediated injury: HO converts heme iron from a lipid-soluble to a water-soluble form; thus, the need for iron sequestration is rapidly met by induction of ferritin synthesis. It must be noted that, however, the direct evidence is still lacking about whether apo-ferritin uptakes/sequesters heme-released iron directly and how this occurs. Studies using stable iron isotopes[113,114] could be good choices to pursue such a purpose.

As a practical matter, agents that induce abnormal iron release will be harmful, while those with an inhibiting effect will protect the lipids from peroxidation. A recent report by Yanagida et al. showed that fosfomycin inhibited the gentamicin-induced LPO by depressing the iron release from mitochondria.[115] Chlorhexidine (CHX), an anphipatic and antiseptic agent used in dentistry, increased iron release from ferritin by approximately 13-fold when compared to control values and significantly enhanced the iron-dependent LPO.[116] Similar effects were also reported for naproxen and salicylic acid,[117] dopamine and DOPA,[118] and the metabolites resulting from the reactions of benzene with superoxide radical generating agents.[119] Note that the reactivity of iron varies greatly depending upon its liganding environment. Oxygen

Table 1. Effects of Hydroxyl Radical Scavengers and Spin-Trapping Compounds on H_2O_2−Fe(II)- and LOOH−Fe(II)-Induced LPO in Micelles[a]

inhibitor	H_2O_2−Fe(II) system (TTAB)		LOOH−Fe(II) system (SDS)	
	O_2 consumption (μM)	inhibition (%)	O_2 consumption (μM)	inhibition (%)
control[b]	42.6	0	25.3	0
formate (50 mM)[c]	8.9	79	26.0	0
mannitol (50 mM)[c]	29.8	30	24.1	0
tert-BuOH (50 mM)[c]	37.0	13	26.2	0
DMPO (1 mM)[c]	36.6	14	25.6	0
PBN (1 mM)[c]	22.6	47		

[a] Reprinted with permission from ref 55. Copyright 1988 Elsevier Inc. [b] Reaction mixture of the control system in tetradecyltrimethylammonium bromide (TTAB) micelles contained 50 mM TTAB, 30 μM $FeSO_4$, 5 mM linoleic acid, and 0.1 mM H_2O_2; the control system for sodium dodecyl sulfate (SDS) contained 50 mM SDS, 30 μM $FeSO_4$, 5 mM linoleic acid, and 68 μM LOOH. [c] All the experimental conditions are the same as those in the control experiment except for the presence of the corresponding scavengers or spin-trapping compounds. Incubation was at 20 °C for 15 s (pH 6.0).

ligands prefer Fe(III); thus, the reduction potential of the iron is decreased. Conversely, nitrogen and sulfur ligands stabilize Fe(II); thus, the reduction potential of the iron is increased.[81] Therefore, chelators with oxygen ligands, such as citrate, promote the oxidation of Fe(II) to Fe(III), while chelators that contain nitrogen ligands, such as phenanthroline, inhibit the oxidation of Fe(II). Many chelators, such as EDTA and Desferal (DFO), will bind both Fe(II) and Fe(III); however, the stability constants are much greater for the Fe(III)−chelator complexes. Therefore, these chelators will bind Fe(II) and subsequently promote the oxidation of the Fe(II) to Fe(III) with the concomitant reduction of molecular oxygen to partially reduced oxygen species.[19] Since the maximal coordination number of iron is six, the hexadentate chelators can provide more consistently inert complexes due to their ability to completely saturate the coordination sphere of the iron atom and, consequently, deactivate the "free iron" completely. For example, DFO is a very effective antioxidant in clinical application because of its potential to markedly decrease the redox activity of iron.[1]

3. Proposed Initiators of LPO

3.1. Reactive Oxygen Species (ROS)

Theoretically, molecular oxygen cannot react with PUFAs because of kinetic constraints, but ground state oxygen can be transformed into more reactive forms called reactive oxygen species (ROS). The toxicity of iron has often been attributed to its role in the generation of ROS, such as the Fenton reaction for hydroxyl radical formation, reduction of molecular oxygen to produce superoxide and superoxide-derived oxidants,[21,120,121] and decomposition of LOOH to generate alkoxyl and peroxyl radicals.[51,52]

3.1.1. Hydroxyl Radical

Fenton-type reactions, which produce reactive hydroxyl radicals ($^{\bullet}OH$; see reaction 4), have been implicated in such oxidative processes as the reperfusion period[122−124] and the injury of mouse-derived C2C12 myotubes treated with iodoacetate and sodium cyanide.[125] Normally, the Haber−Weiss reaction occurs at a negligible rate constant (1 M^{-1} s^{-1} or less),[126] but it can be an important source of hydroxyl radicals in the presence of Fe^{III} or Fe^{III} ligand (reactions 2−4), and it can possibly induce LPO under conditions of oxidative stress.[127,128] For example, initiation of LPO *in vitro* or *in vivo* by Fe(III) and O_2 has been hypothesized to occur via production of hydroxyl radicals arising from the iron-catalyzed Haber−Weiss reaction.[129]

$$\mathrm{Fe(III)} + O_2^{\bullet -} \rightarrow \mathrm{Fe(II)} + O_2 \quad (2)$$

$$O_2^{\bullet -} + HO_2^{\bullet} + H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$H_2O_2 + \mathrm{Fe(II)} \rightarrow \mathrm{Fe(III)} + {}^{\bullet}OH + OH^- \quad (4)$$

From a theoretical point of view, the ability of hydroxyl radical to behave as a powerful oxidant of unsaturated lipids is unquestionable because it is the most potent oxidant formed from oxygen ($E_{m,7}$ = +2.31 V).[2,130] Indeed, the Fenton reagent was found to induce LPO; some "$^{\bullet}OH$ scavengers" or enzymes such as catalase and superoxide dismutase (SOD) could effectively inhibit those processes (Table 1).[33,55,125,131] In some research, hydroxyl radicals were directly detected by spin trapping agents (5,5-dimethyl-1-pyroline-*N*-oxide, DMPO) in systems where LPO was occurring (Table 1).[55,125,132] However, these findings are not convincing enough to establish the role of hydroxyl radicals in LPO initiation, and some of the conclusions are highly conflicting and doubtful.[27,133,134]

First, it is known that Fenton chemistry proceeds via formation of an oxo−iron(IV) intermediate to generate hydroxyl radical.[135−138] The hypervalent oxo−iron species is also highly reactive and can undergo H-abstraction reactions to initiate LPO (see sections 3.4 and 4.1 for details). Thus, Fenton-type reactions are certain to initiate LPO, but such a phenomenon is far from corroborating the real role of hydroxyl radicals in LPO initiation.

Second, the term "hydroxyl radical scavenger" is a misnomer because the hydroxyl radical is very oxidizing and will react with most organic chemicals at near diffusion-limited rates. Accordingly, the inhibiting effects of these "scavengers" on the LPO induced by the Fenton reagent have led to contradictory conclusions. As indicated in Tables 1 and 2, mannitol showed little inhibitory function on a LPO system where $^{\bullet}OH$ was involved,[27] but it acted as a powerful antioxidant in a Fe(II)−H_2O_2 stimulated LPO system.[55] The major reasons for this controversy can be divided into two aspects: (a) Hydroxyl may not participate in LPO initiation at all. The generation of hydroxyl radicals via Fenton reaction (the H_2O_2−Fe^{II} system) was found at a higher level in sodium dodecyl sulfate (SDS) micelles than in tetradecyltrimethylammonium bromide (TTAB) micelles; however, LPO was only observed in TTAB micelles (Table 1, column 2) instead of in SDS micelles.[55] In contrast, Fe(II)−LOOH could readily

Table 2. Effects of Oxygen Radical Scavengers and Iron Chelators on Ascorbate (0.1 mM)-Stimulated LPO in Several Regions of the Human Brain (% of the LPO That Was Measured in the Control Experiment with Ascorbate Alone)[a]

agents	regions[b]			
	caudate	PFC	temporal	cerebellum
mannitol (10 μM)[c]	100 ± 17	93 ± 9	89 ± 5	92 ± 5
indomethacin (10 μM)	94 ± 6	94 ± 6	110 ± 9	97 ± 6
Trolox (50 μM)	8.8 ± 2.7[d]	35 ± 13[d,e]	40 ± 5[d,e]	45 ± 9[d,e]
deferroxamine (10 μM)	1.0 ± 1.7[d]	−0.7 ± 2.4[d]	1.3 ± 1.5[d]	1.4 ± 1.4[d]
SOD (50 μg/mL)	13 ± 2[d]	8.8 ± 1.9[d]	22 ± 6.8[d]	29 ± 12[d]
catalase (50 μg/mL)	170 ± 60	320 ± 44[d,e]	390 ± 47[d,e]	340 ± 47[d,e]

[a] The effects of the agents (at the final concentration shown) were demonstrated on ascorbate-stimulated LPO in membranes from the brain regions shown. Results are expressed as percentages of the LPO observed in the presence of ascorbate alone (corrected for effects on basal LPO). The mean ± SEM values are shown. Reprinted with permission from ref 27. Copyright 1996 Blackwell Synergy. [b] Minced tissue was placed in ice-cold HEPES buffer (20 mM, pH 7.4 at 4 °C) plus 2 mM $MgSO_4$ and frozen quickly in a dry ice and acetone bath. Frozen aliquots were thawed rapidly at 37 °C, diluted 1:10 (v/v) in the same buffer, and homogenized. The homogenate was diluted to a final 50-fold dilution of the original mince (v/v) in buffer and centrifuged at 39,000*g* for 20 min at 4 °C. The pellets were rinsed in fresh buffer and resuspended to the 50-fold dilution (repeated four times). The final rinsed pellets were resuspended in HEPES buffer (pH 7.4) for a final concentration of ~20 mg of protein/mL of suspension, frozen rapidly, and stored at −70 °C until the time of assay. LPO was assessed by measuring thiobarbituric acid reactive species (TBARS, λ_{max} = 532 nm) of the samples where 0.1 ± 0.03 mg of protein from prepared particulate membrane fragments was diluted in a final volume of 500 μL of HEPES buffer (pH 7.4, 34.4 mM, 37 °C). PFC denotes prefrontal cortex. The ascorbate concentrations used in this experiment and control are both 0.1 mM. [c] Mannitol had no inhibiting effect on the LPO even at a high concentration of 1 mM. [d] $p < 0.05$ vs ascorbate alone in the same region. [e] $p < 0.05$ vs the effects of the same agent in the caudate.

stimulate significant LPO in SDS micelles (Table 1, column 4) presumably via a secondary-initiation mechanism (see section 3.3). Inhibition of •OH formation by either SOD or catalase has been demonstrated by ESR techniques or chemical methods,[34,139] but the effects of these two enzymes on the LPO where •OH was present varied dramatically from inhibition to no effect to enhancement.[33] The same phenomenon was observed from Table 2: SOD caused inhibition by more than 70%, but catalase showed significant enhancement of the LPO in all cases. Such conflicting functions seriously questioned the role of •OH in LPO initiation but will direct the discussion to a very interesting topic: "secondary initiation" and the dual functions of Fe(II), where the conflicting roles of SOD and catalase in LPO can be well explained (see section 3.3). (b) These compounds are actually not as "specific" as expected for scavenging hydroxyl radicals. Many of the commonly used "hydroxyl radical scavengers" can also affect the reactivity of iron with other constituents via the chelation of iron as DFO always does (Table 2). Mannitol inhibits the oxidation of Fe(II) by H_2O_2, whereas benzoate stimulates the oxidation of Fe(II) by H_2O_2. Similarly, the initial rate of LPO by Fe(II) is inhibited by mannitol but stimulated by benzoate.[33,34] Due to their very high reactivity, hydroxyl radicals have been shown to attack many of the commonly used buffers, including phosphate, Tris-HCl, HEPES, and MOPS;[140,141] thus, these buffers are also hydroxyl radical scavengers in some sense. Care must be exercised in this respect during experimental design and data analysis, especially when hydroxyl radical-quenching activity is evaluated for some compounds (like mannitol) with which the buffers would be competing for the hydroxyl radical. In Table 2, mannitol of high concentration (1 mM) along with 34.4 mM HEPES buffer did not show a significant effect in LPO inhibition. The original authors thus concluded that hydroxyl radical was not involved in the LPO system. This is a relatively safe conclusion. However, the contribution of HEPES buffer must be taken into consideration to avoid an artifact if the case of mannitol did show significant LPO-inhibiting effects.

Third, the detection of hydroxyl radicals as DMPO−OH may be an artifact because hypervalent-iron species were found to give the same products by oxidizing DMPO (see section 4.1 for details). Thus, the DMPO−OH product may not be indicative of the direct role of hydroxyl radicals in LPO initiation. That is why electron paramagnetic resonance (EPR) detected the DMPO−OH product in a LPO model system composed of Fe(II)/H_2O_2/phospholipid liposomes, but catalase and "hydroxyl radical scavengers" (i.e., mannitol and benzoate) did not show a LPO-inhibiting effect as expected (either stimulating or inhibiting).[34] Similar findings were also reported for the oxidation of egg yolk phosphatidylcholine liposome[142] and the LPO induced by the XO system in the presence of Fe−ADP.[143] Finally, the very high reactivity of the hydroxyl radical leads to several intrinsic restraints in LPO initiation: (a) Due to their very limited lifetime (about 1 ns), it is difficult to conceive that hydroxyl radicals can migrate from generation sites in the aqueous regions to hydrophobic membrane compartments where LPO is triggered. They may react with other biomolecules first and disappear before arriving inside the lipid micelle because of their very high reactivity and random (nonspecific) property of attack.[26,144] (b) The indiscriminate and near diffusion-limited reactivity of hydroxyl radicals makes it possible for them to attack any part of the lipid molecule to produce a variety of products, not just to attack the bis-allylic double bond to initiate LPO chain reactions. Thus, this is per se a severe limitation to the idea regarding hydroxyl radical as a LPO initiator.[33,145] Most recently, a series of studies by theoretical methods or in the gas phase have been reported on the reactivity of hydroxyl radicals with short-chain unsaturated organic compounds.[146−154] Hydroxyl radical was shown to undergo electrophilic addition reaction at low temperature (≤450 K) and H-abstraction reaction at high temperature (≥1000 K).[146−149] Hashimoto and Iwata even found that H-abstraction mainly occurs to saturated organic compounds,[150] whereas addition usually occurs to molecules containing a double bond (π electron) and results in hydroxylation products.[151−154] Although such evidence has not been obtained under aqueous/physiological conditions, the overwhelmingly competing fashion of the electrophilic addition mechanism versus the H-abstraction reaction should not be overlooked in the mechanistic studies concerning LPO.

Direct evidence to dismiss hydroxyl radical as LPO initiator was also obtained by different groups independently. As long as the conditions were kept anaerobic,[26] H_2O_2 and Fe(II) were unable to induce the production of diene

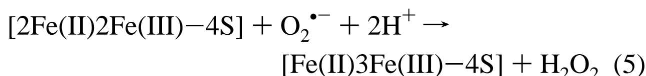
conjugation even in hours. In addition, Braughler et al. did not detect hydroxyl radical in a LPO system induced by the combinations of Fe(III)−Fe(II).[134] A seemingly better explanation of the role of hydroxyl radicals in LPO initiation is the site-specific mechanism,[155] where the binding of a transition metal ion to the biological target is a prerequisite for the production of hydroxyl radical-mediated cell damage.[156] The hydroxyl radicals produced at the membrane surface, probably near the phosphate moiety of the lipid where iron is trapped by ionic binding, will not be able to penetrate to the unsaturated fatty acid region and thus will fail to promote LPO; hydroxyl radical can only initiate LPO provided it is formed in hydrophobic regions of membranes. In the latter case, a critical amount of hydroxyl radical is formed directly at the site of oxidative attack.[156] Accordingly, Fukuzawa et al. reported that the presence of iron in the hydrophobic phase of membranes was required for H_2O_2-dependent LPO,[55] and the solubility of the iron complex in the lipid phase of membranes was a critical determinant of its catalytic effectiveness in initiating LPO.[157] Unfortunately, the site-specific mechanism is seriously questioned by the H_2O_2-induced inhibition against iron-mediated LPO.[158−160] Further study also indicated that the addition of H_2O_2 did not enhance but strongly suppressed the Fe^{II}−DTPA-induced diene conjugation. These findings argue strongly against the opinion that hydroxyl radicals resulted in hydrogen abstraction and initiated LPO.[26,161] Assuming that the site-specific mechanism is sound, LPO will be inhibited by hydroxyl radical traps only in the presence of detergent dispersed in the lipid system, i.e., when the traps can penetrate into the hydrophobic environment where hydroxyl radical is being generated. However, inhibition of NADPH-dependent LPO by $^{\bullet}OH$ traps was observed by Fong et al.[131] and Lai et al.[162] in detergent-free microsomal or reconstituted systems, indicating the presence of detergent may not be critical for hydroxyl radical traps to inhibit LPO.

3.1.2. Superoxide

The superoxide anion radical ($O_2^{\bullet-}$), produced in aerobic organisms during metabolism and in processes involving phagocytosis or responses to xenobiotics, is held to be a major causative agent in pathophysiological events associated with a variety of diseases.[163,164] For example, $O_2^{\bullet-}$ was reported to oxidize the [4Fe−4S] clusters of dehydratases, causing enzyme deactivation and release of iron, and to decrease the activity of antioxidant defense enzymes such as catalase and glutathione peroxidase.[2] However, superoxide is poorly reactive toward most organic compounds,[165] and it is unable to permeate into the liposomal bilayers to react with the bis-allylic moiety of PUFAs; this is due to the negative charges of the phosphatidyl moieties of phospholipids on the membrane surface.[166] The biological effectiveness of superoxide anion in LPO is usually explained by the formation of more reactive species, i.e., superoxide-derived secondary oxidants,[165,167] despite some criticism.[29,30] The reaction between nitric oxide (NO) and $O_2^{\bullet-}$ was found to proceed at a diffusion-limited rate and produce a unique lipid oxidant, peroxynitrite ($ONOO^-$), that undergoes homolytic decomposition at physiological pH to yield a lipophilic oxidant radical species ($NO_2^{\bullet}$).[168] The latter can effectively initiate LPO by H-abstraction from PUFAs and give rise to nitrite ion.[168,169] *In vivo* and *in vitro* experiments have demonstrated that diverse classes of lipid were oxidized where superoxide and nitric oxide were generated simultaneously, especially in endothelial cells, macrophages, europhiles, and neuronal cells. It should be noted that nitrogen dioxide could both oxidize and nitrate unsaturated lipids after the initial H-abstraction reaction,[168] depending on the concentration of ambient O_2 that propagates LPO. At high O_2 concentrations, molecular oxygen will react readily with alkyl radical to produce $LOO^{\bullet}$ radicals; thus, $NO_2^{\bullet}$ will predominantly mediate lipid oxidation. At low O_2 tension, nitration may preferentially occur by an addition reaction between $NO_2^{\bullet}$ and alkyl radical generated from an H-abstraction reaction. These reactions result in formation of a complex mixture of products including nitrated lipid derivatives and alkylnitrites. Moreover, $NO_2^{\bullet}$ can also react at a diffusion-limited rate with peroxyl and alkoxyl radicals, leading to inhibition of peroxidation and formation of novel N-containing lipid derivatives.[168] In addition to $ONOO^-$, the protonated form of $O_2^{\bullet-}$ (hydroperoxyl radical, $HO_2^{\bullet}$) was suggested to be capable of abstracting hydrogen from PUFAs[170] or from the lipids present in low-density lipoproteins.[171] Seemingly, $HO_2^{\bullet}$ could be a good LPO initiator because it is more reactive than superoxide and because it is uncharged and, thus, can permeate into lipid bilayers. Nevertheless, two conflicting facts argue strongly against the role of $HO_2^{\bullet}$ in LPO initiation. First, the pK_a of $HO_2^{\bullet}$ is 4.7 under aqueous conditions; thus, less than 1% of generated superoxide exists in the protonated form under physiological conditions (pH 7.4).[172] Second, superoxide dismutase was shown to have little effect on peroxidation in liposomal or microsomal systems.[31,32] In a comparative study on the ability of $HO_2^{\bullet}$ and superoxide to initiate LPO,[173] LOOH was found to be the kinetically preferred site of $HO_2^{\bullet}$ attack in these systems; the effectiveness of $HO_2^{\bullet}$ was LOOH-dependent, and the $LOO^{\bullet}$ radicals generated from the H-exchange reaction between $HO_2^{\bullet}$ and LOOH could be more effective than $HO_2^{\bullet}$ itself in the LPO initiation; in contrast, superoxide had little initiating activity. Thus, $HO_2^{\bullet}$ seems to stimulate the formation of more reactive lipid peroxyl radicals to initiate the LOOH-dependent LPO, but it is unlikely to play a direct role as the H-abstraction initiator in LPO.[174]

Another way by which superoxide exerts prooxidant functions in LPO is to release “free iron” from iron containing molecules by acting as either a reductant or an oxidant. Superoxide can promote reduction of ferric ion and release of ferrous ion from ferritin (the “free” or “catalytic” form of iron mediates the production of reactive oxygen species),[25,143] and iron delocalization into low-molecular-weight species was seen during postischemic reperfusion.[3,175] Ascorbate stimulated LPO in brain membrane fractions was also shown to be dependent on superoxide radical formation and release of iron from endogenous bound forms, but it was independent of hydroxyl radicals and H_2O_2.[27] A toxicological study on benzene metabolites indicated that the presence of pyrogallol, phloroglucinol, phenylhydrazine, or phenylenediamine resulted in the release of significant amounts of iron from ferritin, which then enhanced LPO in rat brain homogenate and released aldehydic products from bleomycin-dependent degradation of DNA and also caused single-strand nicks to pUC18 DNA.[119] The release of iron by superoxide has also been demonstrated for enzymes containing a [4Fe−4S] cluster such as the dehydratase−lyase family, where the iron−sulfur cluster was oxidized by $O_2^{\bullet-}$ as shown in reaction 5. As a result, the native [4Fe−4S] clusters were transformed into [3Fe−4S] by releasing Fe(II) ions (reaction 6) because the oxidized protein binds the Fe(III) (by sulfur ligands) more tightly.[176] An excess of

superoxide generated in the mitochondria *in vivo* has been reported to mediate iron release from mitochondrial iron−sulfur clusters and lead first to loss of mitochondrial function and then to death;[177] carbon-centered radicals, which were generated in the presence of the iron released from iron−sulfur center proteins by superoxide, were found to initiate LPO during the $O_2^{\bullet-}$-mediated activation of uncoupling proteins.[178]



$$[2Fe(II)2Fe(III)-4S] + O_2^{\bullet-} + 2H^+ \rightarrow [Fe(II)3Fe(III)-4S] + H_2O_2 \quad (5)$$

$$[Fe(II)3Fe(III)-4S] \rightarrow [3Fe(III)-4S] + Fe(II) \quad (6)$$

3.2. The Fe(II)–Fe(III) Complex

Another possible mechanism by which iron could initiate LPO involves the formation of an Fe(III)−Fe(II) complex.[19,33,145] Interest in the initiation of LPO by an iron complex started with the observations of Bucher et al.,[179] who demonstrated that ADP−Fe(II) promoted the peroxidation of phospholipid liposomes only after a lag phase. The lag phase was eliminated by the addition of ADP−Fe(III); therefore, it was concluded that the necessary species generated during the lag phase was Fe(III). A study by Minotti and Aust showed that maximal rates of LPO occurred when approximately 50% of the Fe(II) was oxidized.[19,34] The addition of a second chelator at the end of the lag period resulted in an inhibition dependent on the stability constants of the chelator in ligating Fe(II) and/or Fe(III); a more striking inhibitory effect was observed for the chelators with higher stability constants for either or both Fe(II) and Fe(III) complexes, but much less inhibition was found for those with lower stability constants for both complexes.[180] Other studies showed that iron-dependent LPO in systems comprised initially of Fe(II) and phospholipid liposomes requires Fe(II) oxidation, but systems containing Fe(III) and liposomes require some Fe(III) reduction.[33,145,160] This may imply that the initiator for iron-dependent LPO might be formed during the redox cycling of iron ions. Assuming the "initiator" is formed through the redox cycling of iron ion and finally emerged at the end of the lag period, the inhibitory effect of the second chelator may be explained as the abstraction of either Fe(II) or Fe(III) from the initiator by the additional free chelator and subsequent decomposition of the initiator.[180] A prevailing hypothesis in this case, as proposed by Aust et al., involves the formation of an Fe(II)−dioxygen−Fe(III) complex.[33] That is, the initiation of LPO relies on the oxidation of Fe(II) to Fe(III) or on the reduction of Fe(III) to Fe(II) to form a Fe(II)−Fe(III) complex as the initiator; the maximal LPO was achieved by a 1:1 Fe(II)/Fe(III) ratio. This proposal has been summarized in Figure 4. Starting with Fe(III), reduction would be required but complete reduction would inhibit LPO; starting with Fe(II), oxidation would be essential but complete oxidation would also inhibit LPO. The proposed involvement of oxygen to bridge Fe(III) and Fe(II) in a Fe(II)−oxygen−Fe(III) complex suggests that the presence of different electric charges in iron complexes creates a sort of nidus for oxygen wherein electrons can be captured for oxidizable substrates such as unsaturated lipids.

However, the molecular basis of this elusive initiator is increasingly questioned in addition to the failure to observe, identify, or isolate such a complex.[19,33,145] First, a Fe(II)−Fe(III) combination with the ratio ranging from 1:3 to 2:1

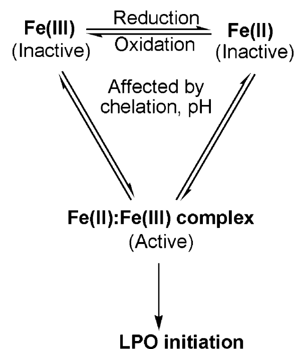


Figure 4. Representative scheme of the formation of a Fe(II)−Fe(III) complex as a LPO initiator. Reprinted with permission from ref 33. Copyright 1987 Elsevier Inc.

was unable to initiate LPO in phosphatidylcholine liposomes that were free of lipid peroxide (LOOH).[36,52] In contrast, the liposome peroxidation was started by $FeCl_2$ and stimulated by Fe(III) when increasing amounts of cumene hydroperoxide were incorporated. Moreover, the Fe(II) autoxidation was stimulated by Fe(III) addition, but the stimulation did not reach a maximum at a Fe(II)/Fe(III) ratio of 1:1. The $FeCl_2$ concentration at which Fe(II) oxidation was maximal depended on the LOOH concentration. Second, the Fe(II)/Fe(III) ratios required for maximal stimulation of LPO vary greatly in the literature instead of being fixed at 1:1.[19,36,37,52,134] In a LPO system composed of Fe(II)−citrate and mitochondrial membranes, the maximal stimulation of LPO was only achieved by Fe(III) when low citrate/Fe(II) ratios were used ($\leq$4:1), and the Fe(II)/Fe(III) ratio was in the range of 1:1 to 1:2.[181] If a critical 1:1 ratio of Fe(II)/Fe(III) is necessary for maximal LPO stimulation via formation of a Fe(II)-O_2−Fe(III) complex, a combination of Fe(II) and Fe(III) (at a ratio of 1:1) should stimulate LPO much faster than a combination of Fe(III) and ascorbate. However, the former showed both a slower rate and a longer lag period in LPO than the latter (Table 3; Figure 5, lines b and e), suggesting that ascorbate could not be acting only by giving an equimolar Fe(II)/Fe(III) ratio for the initiation process.[174] Third, other cations such as Pb(II) and Al(III) can take the place of Fe(III) to stimulate LPO in a similar or more efficient way (Figure 5, lines b and c), but they behaved quite differently from Fe(III) in the study conducted on Fe(II) autoxidation;[174,182,183] unlike Fe(III), Pb(II) did not alter the rate of Fe(II) autoxidation,[174] and aluminum ion markedly inhibited the autoxidation of ferrous ion.[183] These results suggested that Fe(III) is not absolutely required to initiate LPO or forming a complex for LPO initiation. Some researchers hypothesized that the prooxidant properties of aluminum might be due to the formation of a proposed aluminum superoxide semireduced radical ion, but the hypothesis is without direct evidence and remains to be confirmed.[172] Finally, if a Fe(II)−O_2−Fe(III) complex is needed to initiate LPO, a higher rate in Fe(II) autoxidation will allow a higher rate in LPO initiation; nevertheless, this is not the case. In a set of Fe(II) autoxidation experiments, the effects of ligands indicated no direct relation between Fe(II) oxidation and the stimulation or rate of LPO.[160] The presence of citrate led to a rapid rate of Fe(II) oxidation, ADP caused a relatively slower rate, but histidine-chelated Fe(II) did not show appreciable autoxidation even after 10 min of incubation. Surprisingly, citrate-chelated iron showed the lowest rate in LPO; histidine-chelated iron produced a modest LPO rate, whereas ADP-chelated Fe(II) exhibited

Table 3. Action of Fe(III), Al(III), and Pb(II) on Peroxidation of Ox-Brain Phospholipid Liposomes[a]

metal ions added	peroxidation (A_{532})
none	0.000
Fe^{II} [b]	0.095
Fe^{III} (100−400 μM)	0.000
Fe^{II}/Fe^{III} (10 μM)	0.102
Fe^{II}/Fe^{III} (50 μM)	0.114
Fe^{II}/Fe^{III} (80 μM)	0.121
Fe^{II}/Fe^{III} (100 μM)	0.126
Fe^{II}/Fe^{III} (200 μM)	0.127
Fe^{II}/Fe^{III} (300 μM)	0.179
Pb^{II}	0.000
Fe^{II}/Pb^{II}	0.266
$Fe^{II}/Pb^{II}/Fe^{III}$ (300 μM)	0.183
Al^{III} (100 μM)	0.000
$Fe^{II}/Al^{III}/Fe^{III}$ (100 μM)	0.183
ascorbate/Fe^{III} (100 μM)	0.480
Fe^{II}/Al^{III}	0.213
ascorbate/Pb^{II}/Fe^{III} (100 μM)	0.170
ascorbate/Al^{III}/Fe^{III} (100 μM)	0.117

[a] LPO of liposomes was measured at 1−2 min intervals during incubation at 37 °C by the thiobarbituric acid (TBA) test in the presence of butylated hydroxytoluene (pH 7.4). The lag period lasted 10 min, and values after 10 min of incubation are presented. In a series of 32 experiments, peroxidation at the end of the lag period varied from 0.086 to 0.152. Mean percentage stimulations by 100 μM−Fe^{III}, 400 μM−Pb^{II}, and 400 μM−Al^{III} were 67%, 130%, and 89%, respectively. Reproduced with permission from ref 174. Copyright 1989 The Biochemical Society, London. [b] If not specified, the concentrations of Fe^{II}, ascorbate, Pb^{II}, and Al^{III} were fixed at 100, 100, 400, and 400 μM, respectively, when applicable. The concentrations of Fe^{III} were indicated individually in the table.

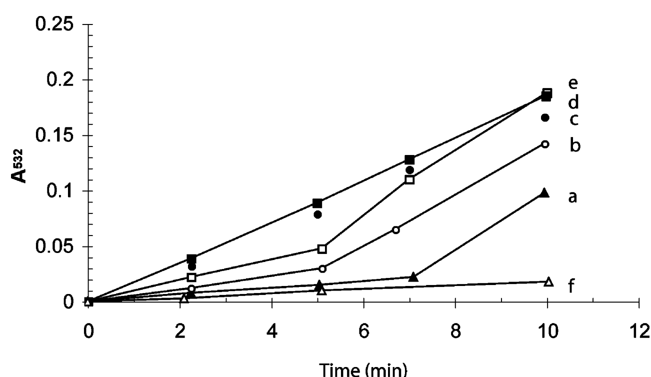


Figure 5. Time course of Fe(II)-stimulated peroxidation in rat liver microsomes at pH 7.4. The reaction mixture contained 0.25 mg of microsome protein, and peroxidation was measured by the thiobarbituric acid (TBA) test. In 15 similar experiments, peroxidation at the end of the lag period was 0.003−0.016 (as A_{532}); the lag period varied from 1 to 7 min. The mean percentage stimulation by Fe(III) (at the end of the lag period) was 242%, that by Pb(II) was 436%, and that by both was 449% (not significantly different from stimulation by Pb^{II} alone). Line a, 100 μM Fe(II); line b, 100 μM Fe(II) + 100 μM Fe(III); line c, 100 μM Fe(II) + 400 μM Pb(II) (●); line d, 100 μM Fe(II) + 400 μM Pb(II) + 100 μM Fe(III); line e, 100 μM Fe(III) + 100 μM ascorbate (■); line f, 100 μM Fe(III) + 100 μM ascorbate + 400 μM Pb(II). Control experiments with Fe(III), or with Pb(II) in the absence of Fe(II) gave no significant peroxidation. Reprinted with permission from ref 174. Copyright 1989 The Biochemical Society, London.

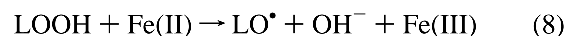
the highest rate in LPO. These reports question the putative Fe(II)−O_2−Fe(III) complex and make it less likely to be involved in LPO initiation.

It should be noted that, since microsomes and liposomes always contain traces of preformed lipid peroxides, iron ions are more likely to stimulate peroxidation by decomposing them to chain-propagating peroxyl and alkoxyl radicals rather than by generating initiating species.[184] As a result, the effects of Fe(III) and other metal cations on LPO can be accounted for by the competition of these cations with Fe(II) for binding sites on the membrane[157,174,185] because considerable evidence showed that the binding of iron to lipid material plays an important role in the LPO development.[4,55,186−191] In fact, divalent iron bound to the membrane surface has been shown to be the only actual regulator of LPO rate in membrane suspensions and related systems, and the effects of iron, chelators, and cations may be a result of the nonlinear dependence of the catalytic action of Fe(II) on its surface concentration[185] and also of the variation of the amount of preformed LOOH in membrane systems under study. This issue will be discussed in detail in the following section.

3.3. Secondary (Lipoperoxide-Dependent) LPO Initiation

In addition to reactive radicals abstracting hydrogen atoms from a lipid moiety, LPO can be triggered by decomposition of preformed LOOH within membranes,[76] where the reaction intermediates (lipid radicals such as alkoxyl and peroxyl radicals) can go on to propagate LPO.[27] The pre-existing LOOH has been shown to occur widely in biological samples or model membranes and plays an essential role in iron-mediated LPO.[36,37,52,184,192−194] In the absence of preformed LOOH, addition of ascorbic acid ($AscH_2$) plus Fe(II) did not induce LPO,[192] and even variable $FeCl_2/FeCl_3$ ratios could not stimulate LPO in phosphatidylcholine liposomes deprived of LOOH by triphenylphosphine (TPP) treatment.[37,52] In the presence of preformed LOOH, however, iron-dependent LPO was diminished by ethanolamine plasmalogen, a strong chelator of transition metal ions.[193] These results implied that LOOH-dependent LPO requires the availability of catalytically active iron[195] and the decomposition of the preformed lipid peroxide[36,52,194] (reactions 7 and 8). Since the reactions of Fe(II) with LOOHs are much faster than those of Fe(III), the propagation of LPO was potentiated by ascorbate to reduce Fe(III) back to Fe(II).[30,87,179,193,196,197]

$$\mathrm{LOOH + Fe(III) \rightarrow LOO^{\bullet} + H^{+} + Fe(II)} \quad (7)$$



$$\mathrm{LOOH + Fe(II) \rightarrow LO^{\bullet} + OH^{-} + Fe(III)} \quad (8)$$

The LOOH-dependent initiation also refers to "secondary initiation" because the chemistry starts from LOOHs other than lipids or unsaturated fatty acids.[195,198−200] Such metal−LOOH reactions initially develop two kinds of free radicals, lipid peroxyl radicals ($LOO^{\bullet}$) and lipid alkoxyl radicals ($LO^{\bullet}$), but a variety of radicals can be formed during the propagation of LPO, such as carbon-centered radicals (lipid alkyl $L^{\bullet}$, β-scission alkyl $R^{\bullet}$, epoxyallylic $OL^{\bullet}$) and oxygen-centered radicals (alkoxyl $LO^{\bullet}/RO^{\bullet}$, peroxyl $LOO^{\bullet}/ROO^{\bullet}$, or epoxyperoxyl $OLOO^{\bullet}$).[201,202] These free radicals can undergo a H-abstraction reaction with PUFAs to initiate new rounds of radical chain reactions.[27,37] Carbon-centered radicals are less active/cytotoxic than the oxygen-centered radicals due to their lower reactivity and to their location.[202] Alkoxyl radicals, although highly reactive, are very short-lived and always undergo extensive cyclization to an epoxyallylic radical or β-scission to an aldehyde and an octenyl radical. It is estimated that the rate constant of cyclization of $LO^{\bullet}$ is approximately 2×10^{7} s^{-1}, while the rate constant for

β-scission is $\sim 1 \times 10^6$ s^{-1}.[203] The cyclization reaction that occurs preferentially transforms alkoxyl radicals predominantly into carbon-centered epoxyallylic radicals that react with O_2 to form epoxyperoxyl radicals, the most stable and highly selective oxygen radicals that abstract the active methylene hydrogens of PUFAs.[50,204−207] The conversion, as shown in Figure 1, suggests that the species LO• is a minor propagating species but that the secondary product OLOO• is the major propagating species of LPO.[202,208,209] That is why little LO•-derived alcohol product was detected in studies of the metal-catalyzed decomposition of PUFA hydroperoxide in the presence of microsomal lipids.[210,211] Notably, unlike LOXs that recognize only free PUFAs as substrate, lipid-derived radicals can peroxidize both free PUFAs and those in the form of phospholipids and esters of cholesterol.[47,212,213]

In iron-mediated LPO systems, a lag/latent period is frequently observed before appreciable peroxidation is detected,[34,37,180,186,214,215] which suggests that lipid-derived radicals do not trigger LPO immediately when they are generated from the decomposition of preexisting LOOH. Conceivably, during the lag period the species responsible for LPO initiation is either formed or accumulated to such a degree that its reaction with lipid becomes dominant over all other competitive reactions. Aust et al.[33,145] ascribed the lag period to the time for Fe(II) oxidation or Fe(III) reduction to form a Fe(II)−O_2−Fe(III) complex as the LPO initiator, but increasing evidence argued against this hypothesis.[19,36,37,52,134,160,174,181] In contrast to the proposed Fe(II)/Fe(III) ratio of 1:1, Tang et al.[37] showed that, either (1) adding 100 μM or 150 μM Fe(II) initially or (2) adding 100 μM Fe(II) initially and then 50 μM Fe(II) later at various intervals during the lag period, the concentration of the remaining Fe(II) at the end of the lag period was almost the same (about 40 μM). Thus, the "critical" Fe(II)/Fe(III) ratio by the end of the lag period was 1:2.5 for 100 μM Fe(II)-initiated LPO and 1:3.75 for 150 μM Fe(II)-initiated LPO. Meanwhile, a second addition of ferrous ions within the lag period lengthened the time lag, and the apparent lag period depended only on the total dose of Fe(II); removal of peroxyl radicals (LOO•) or lipid peroxide by specific scavengers eliminated the LPO. These findings demonstrated that Fe(II) exerted dual functions: to produce lipid radicals by decomposition of preformed LOOH (reactions 7 and 8) and to quench the free radicals by donating electrons (reactions 9 and 10). It has been reported that Fe(II) can scavenge alkoxyl radicals at a rate constant of 3.0×10^8 M^{-1}•s^{-1} (ref 216) and 5.0×10^3 M^{-1}•s^{-1} for peroxyl radicals.[217] The scavenging reactions are fast enough compared to the iron−LOOH reactions ($\sim 1.3 \times 10^3$ M^{-1}•s^{-1} for reaction 8 and ~40-fold lower for reaction 7)[179,218] and can suppress the initial formation of LO• and LOO• or even terminate the chain reaction of LPO at higher concentrations of Fe(II). Only when Fe(II) was oxidized to such a degree that LOO• and LO• are no longer suppressed effectively does LPO start. Therefore, the lag period can be interpreted as the time needed to produce and accumulate the initial lipid radicals at the expense of Fe(II) to reach a sufficiently high level at which their attack on the fatty acid chain of the lipid becomes dominant. When the second dose of Fe(II) is added within the lag period, the lag period is lengthened because more ferrous ions are available to suppress LOO• and LO•.[144,145,186,219−221]

$$\mathrm{LOO^\bullet + Fe(II) + H^+ \rightarrow LOOH + Fe(III)} \quad (9)$$

$$\mathrm{LO^\bullet + Fe(II) + H^+ \rightarrow LOH + Fe(III)} \quad (10)$$

Since the availability and reactivity of catalytically active iron vary with different chelated states,[181,185,191,222] ligands may have a concentration-dependent biphasic function on LPO; chelators at lower concentration improve the solubility of iron ions to stimulate LPO, but at high chelator/iron ratios, strong inhibitory effects occur to iron-mediated LPO. In addition to the decrease in reactivity as already discussed, chelators can decrease the availability of active iron by changing the net charge of the iron complex or by competition with membranes for iron ions.[4,55,157,174,185−191] Tampo et al.[186,189−191] suggested that iron species, incompletely complexed with EDTA, changed from positive charge to negative charge with an increase in EDTA concentration at some critical ratio of EDTA/iron (i.e., between 1:2 and 1:1 in the case of EDTA/Fe^{III}).[191] These iron species acted either as prooxidants or as antioxidants depending on their electrical charges in liposomes: at a low molar ratio of EDTA to iron, they caused LPO only in negatively charged liposomes, but not in neutral or positively charged liposomes, indicating the EDTA−iron complex is positively charged; at a high EDTA/iron ratio, the iron species acquired negative charge and LPO was inhibited accordingly. Meanwhile, removal of the bound active iron from membranes by chelators[181,185,222] or displacing it by other metal cations[157,174,185] also led to pronounced inhibition against iron-induced LPO.

To characterize the availability of active iron and its function, Vladimirov et al. proposed an important parameter that is called "the critical Fe(II) concentration value", [Fe(II)]*.[185] The [Fe(II)]* was found to be dependent on the concentrations of membrane particles in the suspension or, more accurately, on the concentrations of preformed LOOH in the suspensions of lipid particles, although they vary a lot in different membrane samples.[185] When the available Fe(II) is at a concentration above [Fe(II)]*, LPO kinetics show a lag period during which no LPO products are accumulated due to the preferred reactions between Fe(II) and lipid radicals;[144,145,186,219−221] the duration of the lag period is determined by the time necessary for making the effective [Fe(II)] equal to [Fe(II)]*, at which point the lipid radicals are no longer scavenged by Fe(II) and the LPO chain reaction occurs and becomes dominating. Conversely, addition of Fe(II) can start the LPO chain reaction immediately when [Fe(II)] < [Fe(II)]*. In most cases, however, a lag period of different duration was observed because the applied [Fe(II)] was above the corresponding [Fe(II)]*.[185] As a result, an ideal picture can be outlined to explain the lag period and the effects of iron chelators, metal cations, and the second addition of Fe(II) (Figure 6). Factors that make the [Fe(II)] approach the [Fe(II)]* will definitely shorten the lag period and stimulate LPO. Such factors include (1) oxidation of Fe(II) by H_2O_2 and LOOH to remove excessive Fe(II) or reduction of Fe(III) to Fe(II) by ascorbate and superoxide to generate Fe(II) necessary for LPO stimulation;[27,55,223] (2) displacement of the Fe(II) bound to the membrane surface by added Fe(III), Al(III), or Pb(II); (3) removal of Fe(II) by chelators; and (4) reduction of the amount of added Fe(II), which allows a lower concentration of the membrane surface bound Fe(II). On the other hand, factors that make the [Fe(II)] differ from [Fe(II)]* will lengthen the lag period and inhibit the initiation of LPO. These factors include (1) a second addition of Fe(II), (2) the use of a low concentration

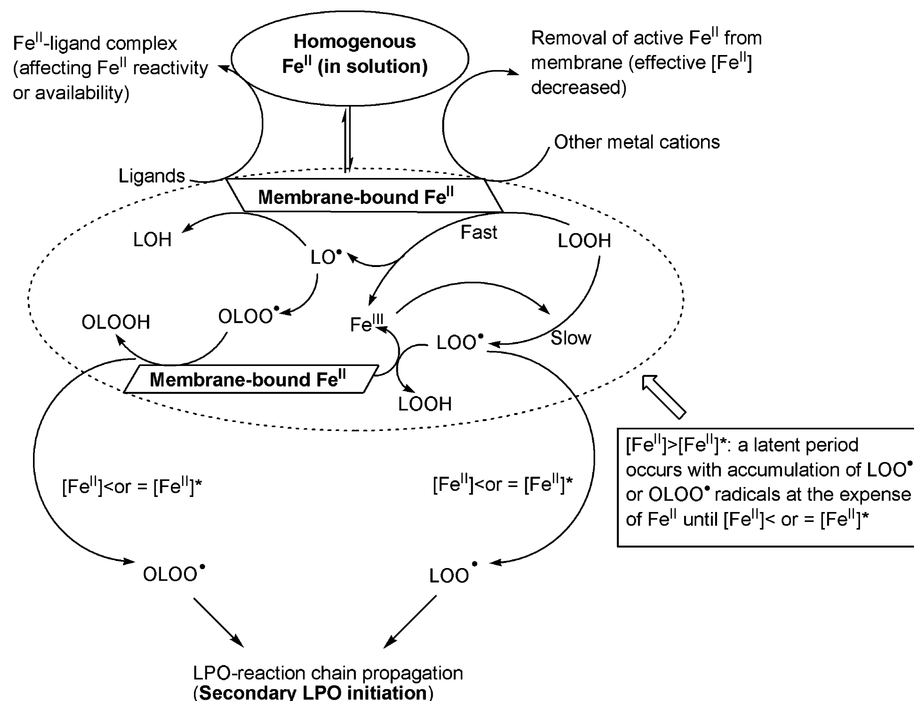
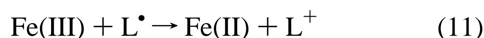


Figure 6. The dual functions of Fe^{II} in LOOH-dependent (secondary) LPO initiation. When the effective iron concentration, $[Fe^{II}]$, is greater than the critical Fe^{II} concentration, $[Fe^{II}]^*$, a lag period occurs, during which $LOO^•$ or $OLOO^•$ radicals (for secondary LPO initiation) are accumulated at the expense of Fe^{II} until $[Fe^{II}] \leq [Fe^{II}]^*$. Chelators or metal cations (such as Al^{III}, Fe^{III}, or Pb^{II} can replace/remove Fe^{II} from the membrane and, thus, promote or inhibit LPO, depending on whether $[Fe^{II}] > [Fe^{II}]^*$ or $[Fe^{II}] < [Fe^{II}]^*$.

of lipid particles to allow a smaller [Fe(II)]*, and (3) extreme oxidation of Fe(II) to Fe(III) or reduction of Fe(III) to Fe(II). Simply speaking, when the concentration of effective [Fe(II)] is different from [Fe(II)]*, LPO will show various lag periods during which either Fe(II) or lipid radicals are accumulated for LPO initiation and propagation. When [Fe(II)] is approaching [Fe(II)]*, LPO will be triggered/stimulated immediately. On the basis of such a principle, it is not difficult to understand the controversial roles of catalase and SOD in LPO (Table 2 and refs 33 and 34). $O_2^{•-}$ can reduce Fe(III) to Fe(II) (reaction 2) and release Fe(II) for the LPO initiation; the addition of SOD inhibited LPO, presumably because it would decompose superoxide, thus decreasing the level of iron release that was necessary for LPO propagation. Also, H_2O_2 is an excellent oxidant that oxidizes Fe(II) to Fe(III) via the Fenton reaction (reaction 4) and maintains iron in the ferric state. This may shorten the lag period observed in the secondary LPO initiation, but extreme conversion of Fe(II) into Fe(III) by H_2O_2 could also block LPO because excessive Fe(III) may quench alkyl radicals to form carbocationic intermediates or it may act as a Lewis acid to neutralize LOOH and to yield carbonyls and ferric hydroxide (reactions 11 and 12).[224] The removal of H_2O_2 by catalase could make more Fe(II) available and enhance the LPO by making [Fe(II)] approach [Fe(II)]* for LPO propagation.

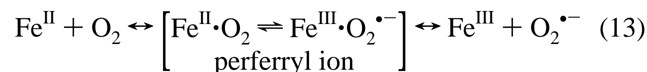


$$\mathrm{Fe(III)} + \mathrm{L}^{\bullet} \rightarrow \mathrm{Fe(II)} + \mathrm{L}^{+} \qquad (11)$$

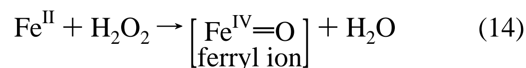
$$2\mathrm{Fe(III)} + 4\mathrm{LOOH} \rightarrow 2\mathrm{Fe(OH)_3} + 4\mathrm{LO}^{-} \qquad (12)$$

3.4. Iron–Oxygen Species

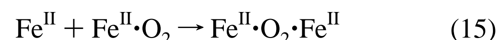
The oxidizing species formed by Fe(II) + dioxygen (ferryl or perferryl ions) have recently attracted increasing interest due to the oxidative damage via "Fe(II) + O_2" chemistry.[26,57,144,225−227] The high ratio of $[O_2]/[H_2O_2]$ ($\geq 10^3$) under physiological conditions allows oxidation of reducing substrates at rates 10^6-10^8 fold faster by "Fe(II) + O_2" chemistry than by the Fenton reaction. In addition to hydroxyl radicals, the cascade of reactions between iron complexed with hydrogen peroxide or O_2 can produce high-valent iron−oxygen species (reactions 13 and 14), although the relative rates of the processes depend on chelator, stoichiometry, solvent, and pH.[136−138,144,227−229] Evidence has been obtained for oxygen transfer to Fe(III)−EDTA in methanol to yield formally FeO^{3+}−EDTA.[228] The iron(IV) species, expected to be a strong one-electron oxidizing agent, can also be obtained with simple ligands in aqueous media at alkaline pH.[135] So far, the formation of ferryl ion has been suggested to follow two routes:[144,230] the first relies on superoxide-dependent reduction of Fe(III) to Fe(II) and subsequent reaction of Fe(II) with H_2O_2. Under conditions of proper pH and polarity, Fenton reagents will produce ferryl ion rather than hydroxyl radical (reaction 14);[144,227] a second route to ferryl ion formation is the reaction of perferryl ion with Fe(II) (reactions 15 and 16).



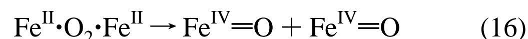
$$\mathrm{Fe^{II}} + \mathrm{O_2} \leftrightarrow \left[\begin{matrix}\mathrm{Fe^{II}{\cdot}O_2} \rightleftharpoons \mathrm{Fe^{III}{\cdot}O_2^{\bullet-}} \\ \text{perferryl ion}\end{matrix}\right] \leftrightarrow \mathrm{Fe^{III}} + \mathrm{O_2^{\bullet-}} \qquad (13)$$



$$\mathrm{Fe^{II}} + \mathrm{H_2O_2} \rightarrow \left[\begin{matrix}\mathrm{Fe^{IV}{=}O} \\ \text{ferryl ion}\end{matrix}\right] + \mathrm{H_2O} \qquad (14)$$



$$\mathrm{Fe^{II}} + \mathrm{Fe^{II}{\cdot}O_2} \rightarrow \mathrm{Fe^{II}{\cdot}O_2{\cdot}Fe^{II}} \qquad (15)$$



$$\mathrm{Fe^{II}{\cdot}O_2{\cdot}Fe^{II}} \rightarrow \mathrm{Fe^{IV}{=}O} + \mathrm{Fe^{IV}{=}O} \qquad (16)$$

Due to their high electron affinities, perferryl and ferryl ions could be important oxidants in detrimental biological oxidations.[144] For example, they were found to oxidize a

number of organic chemicals and to catalyze the hydroxylation of aromatic compounds.[41,231−233] Compared to hydroxyl radicals, these complexes are less reactive toward benzoate, *tert*-butyl alcohol, acetate ion, arginine, and serine; they were scavenged by compounds with readily oxidizable functional groups such as ethanol and isopropyl alcohol.[41] Meanwhile, ferryl complexes liganded by hydroxide and EDTA had appreciable lifetimes in aqueous solution,[234] and the formation of the ferryl species predominates in hydrophobic media, suggesting the potent role of ferryl species in the oxidative degradations of membranes.[229] In a study on iron-loaded human liver HepG2 cells, Huang et al.[219] found that Fe(II) autoxidation produced no detectable hydroxyl radical signal with DMPO as the spin trapping agent, but strong DMPO/$CO_2^{\bullet-}$ signals were observed in the presence of formate, indicating oxidants other than hydroxyl radical were formed. In the absence of O_2 or in the presence of *o*-phenanthroline (an Fe^{II} chelator with molar ratio to Fe^{II} of 2:1) or DFO (an Fe^{III} chelator with molar ratio of 1:1); however, no EPR signal was detected. These results implied that either a strong Fe(II) or Fe(III) chelator could inhibit the formation of the oxidizing species, and their oxidative activity depended on the interaction with O_2. Further investigation indicated that the oxidizing species have similar reactivity to that of hydroxyl radicals in terms of the reactions with simple alcohols but are less reactive to benzoate or *tert*-butanol.[26,41,121,233,235,236] Given the reactivity and structural features, a high-valent iron−oxygen complex ($Fe^{IV}{=}O$) should be responsible for the LPO induced by "Fe(II) + O_2" chemistry.[219,230] These iron−oxygen complexes are capable of extracting hydrogen atoms from the PUFAs of membranes to form alkyl radicals which take up oxygen yielding the peroxyl radical and propagate the chain reactions of LPO.[28,106,222,237−242] Some investigators[26,43,44,236,243,244] have proposed that the iron−oxygen complex involved in LPO can be best described as the perferryl ion ($Fe^{3+}\cdot O_2^{\bullet-}$); the requirement of both Fe(II) and Fe(III) was proposed to underlie LPO initiation by perferryl iron for at least two reasons.[241,245] First, perferryl iron formation and reaction with lipids best occur with chelators and at chelator/Fe(II) ratios that favor moderate Fe(II) oxidation and generate an equilibrium between $Fe^{2+}\cdot O_2$ and $Fe^{3+}\cdot O_2^{\bullet-}$.[241] Second, excessive Fe(II) would compete with lipids as electron donors for $Fe^{3+}\cdot O_2^{\bullet-}$, degrade the complex into less oxidizing Fe(III), and, thereby, inhibit hydrogen abstraction and LPO (reaction 17).[241,245] The proposed competition between lipids and excessive Fe(II) for perferryl ion seems consistent with Aust's findings that LPO is maximal when a critical amount of Fe(II) is oxidized to Fe(III),[33] whereas addition of Fe(III) may eliminate the antioxidant effects of Fe(II) by pushing reaction 17 backward to produce the LPO initiator.

$$Fe^{III}\cdot O_2^{\bullet-} + Fe^{II} \rightarrow 2Fe^{III} + H_2O_2 \qquad (17)$$

It should be noted that several reports are not consistent with the proposal of ferryl or perferryl ion as LPO initiator. For example, SOD or catalase did not inhibit LPO induced by Fe(II) or Fe(III) chelates plus ascorbate or glutathione,[179,246,247] but it showed significant stimulating effects in some cases.[33,34] These phenomena could be explained at least in part by the secondary-initiation mechanism where SOD or catalase would function in regulating [Fe(II)] vs [Fe(II)]* as discussed above. Furthermore, ferryl and perferryl ions can both be derived directly from Fe(II) and molecular oxygen (see reactions 13−16); superoxide and hydrogen peroxide are not imperative for hypervalent iron formation. In fact, Yin et al.[26] has reached a similar conclusion in their investigation of the LPO system where the Fenton reagent is present, and they suggested that perferryl ions or chelator−Fe−O_2 complexes were responsible for the first chain initiation of LPO. Some theoretical study questioned the role of hypervalent iron−oxygen species in LPO initiation, since the calculated redox potential of perferryl ion indicated a thermodynamic barrier in the H-abstraction reaction for LPO.[248] However, this calculation should be reconsidered because it is based only on an approximation of myoglobin−oxygen binding and also because the effective redox potential under actual reaction conditions would be quite different from the theoretical approximation.[26,249] For example, the effective redox potential for the P450−$(Fe{=}O)^{3+}/(Fe{=}O)^{2+}$ couple was defined as an average value of 1.85 V (vs SCE at pH 7.0), whereas the values measured for model mangano ranged from 1.00 to 1.75 V and those for horseradish peroxidase ranged from 0.73 to 0.75 V (ref 250 and the references therein). Further evidence indicated that an enhancement of the oxidation potential of P450 occurs due to a Coulombic factor which arises from the gain or loss of electrostatic free energy upon transfer of the electron in the transition state. The Coulombic interactions between the charged heme−oxo radical anion and the dimethylaniline radical cation, subsequent to electron transfer, could cause an enhanced redox potential of P-450, with the magnitude of the electrostatic interaction being approximately 0.5−0.8 V and building up the intrinsic oxidation potential for the $(Fe{=}O)^{3+}$ porphyrin core of the enzyme.[250] Unlike some other peroxidases, horseradish peroxidase does not appear to involve tyrosinyl cations, and the measured redox potential is believed to reflect the redox changes of the heme−oxo species.

4. The Most Likely Initiators of LPO

The disassociation energy of the C−H bond at the bis-allylic position, where the abstraction of a hydrogen atom takes place to initiate LPO, was estimated as 75 kcal/mol and corresponds to a redox potential of +0.60 V at neutral pH.[130,251] Such a redox potential seemingly implies that LPO could be easy initiated by iron-induced ROS.[2,249] A serious argument that must be taken into consideration, however, is the observation that LOOH and LOH derived from natural sources contain an excess of one enantiomer.[252,253] Further evidence was reported by Herold and Spiteller, who detected only one single isomeric LPO product immediately after cell destruction.[87,254] As is known, $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ are not chiral; consequently, they are unable to generate LOOHs or LOHs in enantiomeric excess. In contrast, the iron−oxo species (e.g., the ferryl complex) is much more selective in its reactions,[41] with a reduction potential (ferryl/ferric couple) estimated theoretically to be in excess of 0.9 V at pH 7.0;[135,255] thus, we can be sure that it is responsible for initiation of the first chain of LPO (the initial H-abstration for LOOH-independent initiation of LPO).[135,256] At the same time, the enzymes (e.g., LOX) that utilize free PUFAs as their reductant substrates are bound to trigger LPO because they are both powerful enough and stereospecific.[87,208,257−259]

4.1. Hypervalent Iron−Oxygen Species

Three hypervalent oxidation states, Fe(VI), Fe(V), and Fe(IV), have been extensively studied in both aqueous and

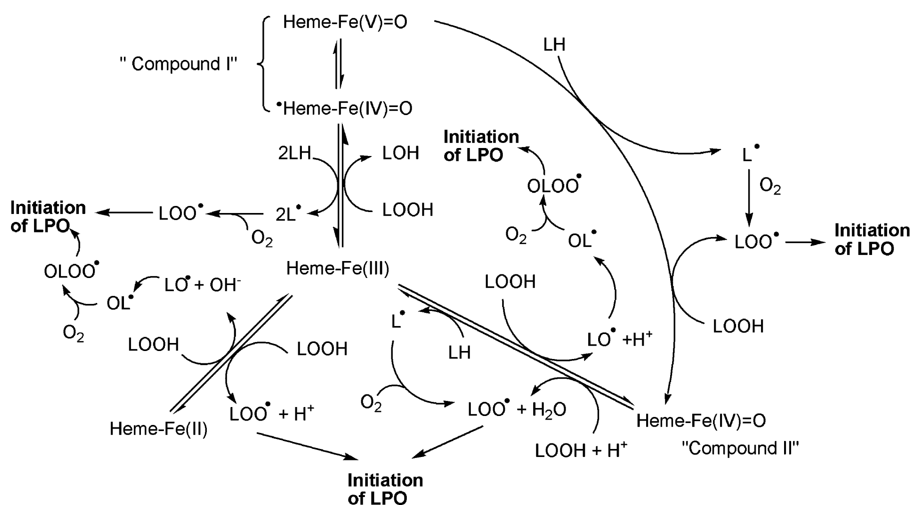
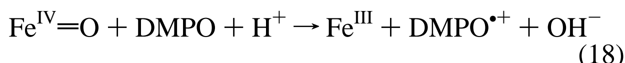


Figure 7. Heme-iron induced LPO. Heme-iron induces LPO via two mechanisms: to develop the lipid radicals (e.g., LOO• and OLOO•) and to produce hypervalent iron complexes. The products of the two pathways are effective agents to abstract hydrogen atoms from PUFAs and initiate LPO.

organic media.[260] Due to a substantial free-radical feature of the Fe−O bond in the Fe(V) and Fe(IV) species, they have been shown to be significantly more reactive than Fe(VI). The reactions between ferryl species and some spin traps (e.g., DMPO) have been found to give rise to an EPR-detectable radical that is similar to hydroxyl radical (reactions 18 and 19).[121,261] The final product of DMPO oxidation by ferryl species (DMPO/HO•) may explain why hydroxyl radicals were detected in some iron-mediated LPO systems, but the corresponding scavengers could not inhibit/eliminate the LPO.[55,132]



$$Fe^{IV}{=}O + DMPO + H^+ \rightarrow Fe^{III} + DMPO^{\bullet+} + OH^- \quad (18)$$

$$DMPO^{\bullet+} + H_2O \rightarrow DMPO/HO^{\bullet} + H^+ \quad (19)$$

Because Fe(V) and Fe(IV) species commonly act as catalytically active intermediates of some enzymes or simple iron complexes,[262,263] it is no wonder that they are regarded as powerful agents in the stereospecific H-abstraction reactions for LPO initiation.[2,34,241,260,264−267] In a reconstituted system comprised of purified NADPH-P450 reductase, P450, and isolated microsomal lipid or pure L-α-phosphatidylcholine diarachidoyl, the generation of the perferryl moiety $P450{-}Fe^{III}{\cdot}O_2^{\bullet-}$ was found to initiate LPO by abstracting a methylene hydrogen from polyunsaturated lipid to form a lipid radical, which then combined with oxygen to produce the chain propagating peroxyl radical for subsequent formation of lipid peroxides.[268] Ferrylmyoglobin, which contains the oxoferryl complex, showed a reduction potential of 0.85 V (pH 7.0) for the $Mb{-}Fe^{IV}{=}O/Mb{-}Fe^{III}$ couple.[269] Its interaction with isolated erythrocyte membranes resulted in an increase in their fluidity and a significant rise in the level of LPO.[270] The oxidative cleavage of LOOH by Mb−Fe(IV)=O would regenerate Mb−Fe(III) and produce LOO•;[271] the LOO• radicals can participate in another chain reaction of LPO. The redox cycling of heme proteins among different oxidation states of heme iron, as indicated in Figure 7, demonstrated their essential role in LPO initiation and propagation in two ways. The electron-transfer processes between LOOH and heme proteins lead to the formation of peroxyl and epoxyallylic radicals that are active in propagating the LPO chain reactions as discussed in section 3.3; on the other hand, the ferryl and perferryl species, which correspond to compound II and compound I in the case of peroxidases,[272−274] can oxidize the PUFA moieties of lipid to generate alkyl radicals and initiate LPO in a pseudoperoxidase mechanism.[15] The perferryl species, i.e., the “Heme−Fe(V)=O complex of a heme iron”, often refers to $Heme{-}Fe^{IV}{=}O$ together with a porphyrin π cation radical because the two electrons come from heme Fe(III) and the porphyrin ring, respectively. Thus, a $^{\bullet}Heme{-}Fe^{IV}{=}O$ species is formed. For myoglobin (Mb) and hemoglobin (Hb), the porphyrin radical cation immediately oxidizes an amino acid residue of the surrounding peptide chain, leaving the perferryl species as a protein radical with iron in the oxidation state of +4.[275,276] When a “compound I-like species” accepts one electron, generating a “compound II-like species” (the $Heme{-}Fe^{IV}{=}O$ complex), the electron donator, such as lipid or peroxide, is transformed into an active free radical and triggers LPO. In turn, “compound II” can oxidize LOOH or LH to stimulate LPO.[73,237,267,277−280] It should be noted that, although the cycling pathway of myoglobin and hemoglobin is generally the same as that of real heme peroxidases, their peroxidase activity is several orders of magnitude lower than that of the latter, which has been ascribed to the difference in the coordination state of the heme iron and the microenvironment around heme group.[15] Since charged amino acid residues close to the heme group in the heme pocket favor the reactions of the peroxidase cycle through hydrogen bonding and protonization,[281] the protonation of the ferryl species at lower pH values was found to enhance the pseudoperoxidase and pro-oxidant activities of Mb and Hb, and alkalinization treatment may be effective in detoxifying the pro-oxidant activities of Mb or Hb by stabilizing the ferryl species and making it less reactive (ref 282 and the references therein).

In addition to heme proteins, less exotic iron complexes such as iron−EDTA or iron−ADP have been proposed to form ferryl ions and account for the high specificity of Fenton-type reagents.[260] The first spectroscopic evidence, for complexes containing iron formally in the IV and V oxidation states, was obtained by Rush et al. with simple ligands (i.e., OH^- and $P_2O_7^{4-}$) in alkaline solution, and the preliminary results indicated that both Fe(IV) and Fe(V) had significant lifetimes when complexed with the ligand and were thus plausible intermediates in iron-catalyzed oxidations of or-

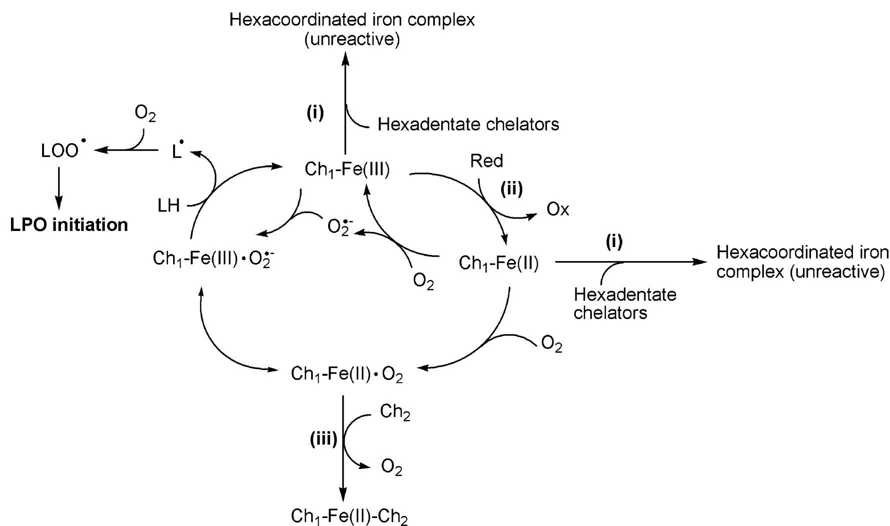


Figure 8. Scheme of the LPO initiation by non-heme hypervalent iron−oxygen complexes and the three related antioxidant mechanisms: (i) to inactivate iron by forming a hexacoordinated iron complex (e.g., DFO); (ii) to prevent the formation of perferryl ion or the iron−oxygen complexes by maintaining iron in its reduced state (e.g., eugenol compounds); and (iii) to avoid the formation of perferryl species by dislodging molecular oxygen from the iron−oxygen species (e.g., 7,8-dihydroxy-4-methylcoumarin). Ch_1 refers to a physiological iron chelator (non-hexadentate) such as ADP; Ch_2 represents an antioxidant that is also a non-hexadentate chelator of iron such as 7,8-dihydroxy-4-methylcoumarin (DHMC). DHMC can dislodge molecular oxygen from the ADP−iron−oxygen species to form a stable ternary, mixed-ligand complex (ADP−Fe−DHMC). As a result, the formation of the ADP−perferryl is prevented, and the LPO initiation by this mechanism is thereby avoided.

ganic compounds.[234] During the oxidation of organic substrates by the Fenton reagent, Wink et al.[283] suggested two strongly oxidizing intermediates (X, Y) generated from Fenton reagents that showed similar spectral properties to those identified by Rush et al.[234] Moreover, neither of the intermediates showed the competitive reactivity expected for the hydroxyl radical,[236] but both showed great selectivity in the attack on organic compounds.[283] Studies using rapid-reaction (stop-flow) kinetics suggested that intermediate X was a peroxo complex and that Y would be a Fe(IV)−oxo complex. Since peroxo and Fe(IV)−oxo complexes would be capable of undergoing H-abstraction reactions, it is easily imagined how such species might be mistaken for hydroxyl radicals if a narrow range of substrates were examined for competitive reactivity.[236,284] In the systematic comparative studies, Nakano et al. have shown that in LPO hydroxyl radicals are not important but the presence of perferryl ion complexes is very important.[285,286] A system that contained NADPH, cytochrome P-450 reductase, and an Fe(III)−ADP−adriamycin complex in Tris-HCl buffer was found to possess a strong activity to initiate LPO of exogenously added phospholipid micelles. Mechanistic investigation indicated the LPO was initiated by a perferryl ion complex which was generated by the interaction of the Fe(II)−ADP−adriamycin complex with O_2 or produced during the enzymatic reduction of the Fe(III)−ADP−EDTA complex in the presence of air.[285,286] The LPO induced by such a hypervalent iron complex has been implicated in the pathology of various human diseases;[225,287] compounds capable of inhibiting the generation of ferryl or perferryl species were shown to be powerful antioxidants in attenuating the LPO. As depicted in Figure 8, good antioxidants against perferryl-induced LPO can be grouped into three classes: (i) hexadentate chelators such as DFO that can make iron inert or inactive by forming a hexacoordinated iron complex; (ii) reducing compounds such as eugenol compounds that can effectively inhibit Fe(II) autoxidation and iron-mediated LPO because they maintain iron in its reduced state and, thus, inhibit the formation of perferryl ion or the iron−oxygen complex;[288] and (iii) compounds such as 7,8-dihydroxy-4-methylcoumarin (DHMC) that can dislodge molecular oxygen to prevent the formation of the ADP−perferryl, thereby inhibiting membrane LPO.[289] DHMC has been reported to effectively inhibit *in vivo* LPO in rat tissues. A sensitive pH metric technique revealed DHMC could form a very stable ADP−Fe−DHMC ternary complex, while Fe−DHMC and ADP−DHMC had negligible stability.[289] These findings indicated that DHMC prevented the generation of an ADP−perferryl complex by forming a stable ternary, mixed-ligand complex (ADP−Fe−DHMC). As a result, molecular oxygen was dislodged from the iron−oxygen species, and LPO was inhibited due to a failure to form the ADP−perferryl species as the initiator.

4.2. Enzymatic LPO Initiation

As mentioned above, lipid hydroperoxides have a chiral center at the carbon atom substituted with the −OOH group. LOOHs are easily reduced to LOHs in the milieu of a biological system, and this reduction does not influence the configuration at the carbon that carried the original −OOH group.[290] Two independent groups have recognized that the *S*-isomer predominates in mixtures of LOHs and of LOOHs obtained from biological sources.[252,253] Because iron-induced ROS such as hydroxyl radical, superoxide, and its protonated form are achiral and can only produce a racemic mixture when reacting with PUFAs,[47,87,291,292] the partial involvement of specific LOXs in LPO production must be seriously considered in addition to the case of the hypervalent iron complexes as discussed above. The lipoxygenase superfamily is found widely in plants, fungi, and animals, the suitable substrates for which are PUFAs containing a series of *cis* double bonds.[88] Structural studies indicate LOXs have an N-terminal β-barrel domain and a larger catalytic domain containing a unique non-heme iron cofactor that is liganded to conserved histidines and to the carboxyl group of a conserved isoleucine at the C-terminus of the protein.[88−90] Agents that interact with histidine residues such as 4-hydroxyl-2(*E*)-nonenal have been shown to cause suicide

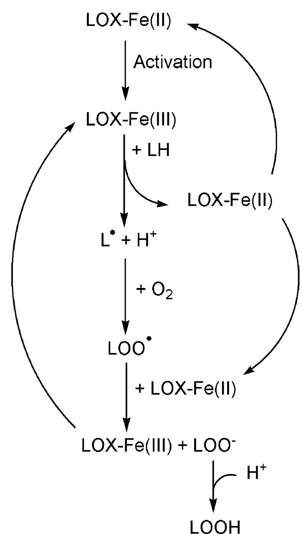


Figure 9. Enzymatic LPO induced by activated lipoxygenase (LOX). Reprinted from ref 47, Copyright 2001, with permission from Elsevier.

inhibition of LOX-1 by modification of the active site via formation of a Michaelis adduct.[293] Under conditions of stress, some structural changes occur due to cell damage and induce the activation of phospholipases and lipoxygenases; phospholipids are thus cleaved by membrane-bound phospholipases to liberate PUFAs, the substrates for lipoxygenases.[294−296] In turn, the liberated PUFAs (LH) are transformed into LOOHs with concomitant formation of alkyl, alkoxyl, and peoxyl radicals in a highly regiospecific and stereospecific reaction.[87,257,259,297,298]

In inactivated LOX, the iron exists as Fe(II), and the complex is activated by transforming Fe(II) into Fe(III); Fe(III) is reduced within the complex by reaction with PUFAs (LHs) to produce alkyl radicals ($L^{\bullet}$). The mesomeric $L^{\bullet}$ reacts in a regio- and stereospecific way with oxygen to form peroxyl radical ($LOO^{\bullet}$), which undergoes a one-electron-transfer reaction with LOX−Fe(II) and is converted to peroxyl anion (LOO^-). As a result, LOX−Fe(III) is regenerated to start the next cycle, with release of the final product, LOOH (Figure 9) (refs 47, 294, and 296 and the references therein). The enzymatic LPO appears to follow a fairly controlled pathway to "discharge" lipid radicals and to produce chiral lipid hydroperoxide; thus, the generation of LOOHs could be terminated by depletion of substrate. However, this is not the case. Due to the existence of labile histidines in the active sites, LOXs have been found to be disabled and release toxic iron by the oxidative modification of histidine residues, as happens to superoxidase dismutase (SOD) and human growth hormone (hGH).[91,92,299] A recent investigation by Spiteller et al. suggested that the lipid radical intermediates could oxidize the histidines to 2-oxo-histidines and cause iron release from the active site because 2-oxo-histidine has no iron-binding capacity.[299] In sensing plant tissues,[300,301] LOX was found to induce ROS such as singlet oxygen that can react with PUFAs directly to produce hydroperoxide and lipid-derived radicals. This process would potentiate the oxidative deactivation of LOX and the escape of the complexed iron from the active sites. In turn, the released iron ions induce a switch from enzymatic to nonenzymatic generation of LPO by catalyzing the cleavage of LOOH.[294,296]

In fact, enzymatic and nonenzymatic LPO in biomembranes does not occur independently; rather, there is a mutual triggering of these processes.[302] Once the enzymatic reaction starts, LOX will produce more hydroperoxyl compounds so that an autocatalytic process is initiated. That is, a LOX-catalyzed reaction can initiate nonenzymatic LPO in biomembranes by several modes of action. (a) During the catalytic cycle of LOX, enzyme-bound intermediate radicals are formed, which under certain circumstances can be released from the active site of the enzyme. In turn, the free radicals will start nonenzymatic LPO by abstracting hydrogen atoms from the bis-allylic methylenes present in the PUFA moieties of phospholipids. (b) The final product of the LOX-catalyzed reaction (LOOH) can be converted to reactive free radicals ($LOO^{\bullet}$ or $OOLO^{\bullet}$) in the presence of certain metal catalysts (heavy metal complexes). These reactive free radicals are ready to initiate LPO via H-abstraction.[302] Another type of

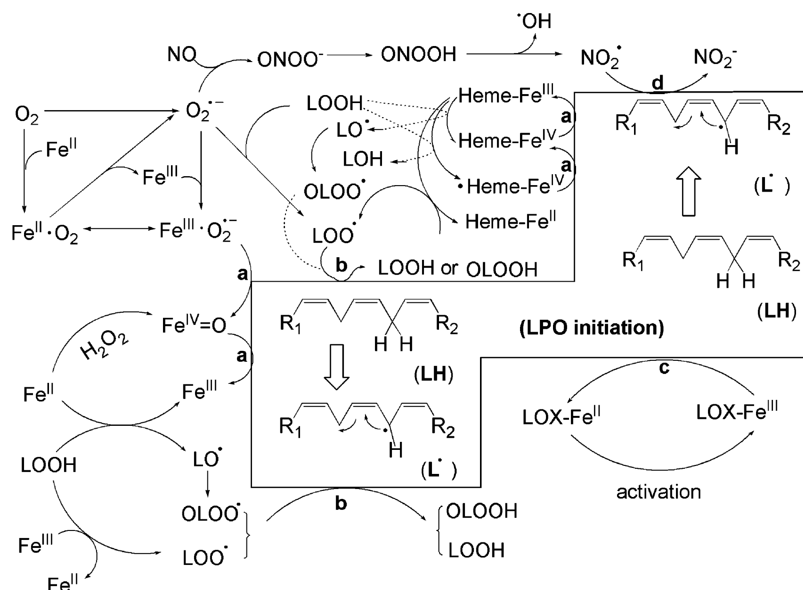


Figure 10. Summary of the initiating (H-abstraction to produce alkyl radicals) pathways of iron-mediated LPO: (a) hypervalent iron species as LPO initiator; (b) lipid-derived radicals as LPO initiators (secondary LPO initiation); (c) lipoxygenase (LOX)-catalyzed H-abstraction to initiate LPO; (d) peroxynitrite ion and its decomposition product ($NO_2^{\bullet}$) as LPO initiators. Superoxide may play a role in forming secondary oxidant species (e.g., $LOO^{\bullet}$, perferryl ions and $ONOO^-$) that are more reactive in LPO initiation.

LPO, which includes both enzymatic and nonenzymatic steps, is the NADPH- and ADP-dependent LPO in the endoplasmic reticulum of rat liver.[303,304] This system consists of NADPH−cytochrome P450 reductase and cytochrome P450 that form a branched electron-transfer system. Unlike LOXs, which activate the lipid substrate, cytochrome P-450 activates the dioxygen molecule via an ADP−perferryl radical that in turn abstracts hydrogen from the lipid.

5. Conclusions

LPO involves complex chemical reactions. The relationship of these diverse processes to various diseases will provide a challenging future for this area of research.[3,305] Interpretation of the results from the studies reviewed here have been plagued by such factors as pH, chelating effects, and the presence of oxidant substrates, as well as the variation in assays; this may also account for much of the contradictory information on the chemistry of LPO initiation.[121,185,202,266,306−309] The variation in amounts of preexisting LOOH in biochemical samples as well as the contamination by metal ions in buffers, enzyme preparations, and tissue extracts must also have led to artifacts in the experiments.[19,29,185,192,306,310−314] Keeping these in mind, we may gain a better understanding of LPO and the mechanisms of LPO-related diseases, with the development of reliable and discriminating methods.[26,202,314,315]

The redox chemistry of iron plays an important role in the occurrence of LPO. Several different kinds of species have been proposed for the first chain initiators despite some criticism. The accumulated data in the past few decades have shown that hydroxyl radicals can be dismissed as the LPO initiator. These data also indicate that the elusive Fe(II)−Fe(III) complex or an optimal Fe(II)/Fe(III) ratio of 1:1 is unlikely to account for the LPO initiation. The most likely initiators of LPO are the hypervalent iron complexes, since these oxidation states have a very high redox potential and are stereospecific oxidants in H-abstraction. In accordance with the enantiomeric excess in LPO products, LOXs should be the right initiators because they generally mediate LPO via regio-, stereo- and enantiospecific mechanisms. The role of superoxide in LPO, if any, is to form more reactive species for LPO initiation or to release/activate iron from its inactive and complexed forms. Since homogenization activates LOXs and proteases (which can induce LOOH formation and contribute to iron release, respectively), biomembrane fractions may already contain trace amounts of LOOH and metal ions that are essential for nonenzymatic LPO. As a result, the secondary (or LOOH-dependent) LPO initiation also plays an important role in LPO propagation. The most likely initiators and the pathways by which they trigger LPO are summarized in Figure 10. It is to be hoped that future investigators will take seriously the great complexity of the chemistry and biology of the systems reviewed here.

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7. Note Added after Print Publication

Reference 45 and a formula in the caption for Figure 10 were incorrect in the version posted on the Web February 28, 2007 (ASAP) and printed in the March 14, 2007 issue (Vol. 107, No. 3, pp 748−766); the corrected electronic version of the paper was published on March 20, 2007, and an Addition and Correction is posted on the Web (http://dx.doi.org/10.1021/cr078201+) and appears in the May 9, 2007 issue (Vol. 107, No. 5).

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