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POSSIBLE SOURCES OF IRON FOR LIPID PEROXIDATION

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Possible sources of iron for lipid peroxidation are described and discussed. In particular, evidence is presented that microsomes contain ferric nonheme iron which may participate in formation of lipid oxidants, provided reductants are available to favor its mobilization from membrane binding sites. Aging- and tumor-associated changes of this microsomal pool of nonheme iron are also described and discussed from biochemical and biomedical viewpoints.

KEY WORDS: Iron, lipid peroxidation, ferritin, nonheme-nonferritin iron, aging, tumors.

ABBREVIATIONS: PUFA, polyunsaturated fatty acids; $O_2^{\cdot-}$, superoxide; H_2O_2, hydrogen peroxide; $^{\cdot}OH$, hydroxyl radical; SOD, superoxide dismutase; ADR, adriamycin; $ADR^{\cdot-}$, adriamycin semiquinone; t-BOOH, tert-butyl hydroperoxide.

INTRODUCTION

The peroxidative decomposition of polyunsaturated fatty acids (PUFA) is frequently invoked as a possible cause of cell damage.[1] Several lines of evidence indicate that iron is required for initiation of lipid peroxidation but the exact nature of such requirement remains unknown. It has been proposed that lipid peroxidation is promoted by $^{\cdot}OH$ and that iron catalyzes $O_2^{\cdot-}$ – and H_2O_2-dependent reactions yielding $^{\cdot}OH$. Under certain conditions, however, $^{\cdot}OH$ can effectively be replaced by iron-oxygen complexes like ferryl/perferryl ions or by as yet uncharacterized species that involve both Fe^{2+} and Fe^{3+}. Mechanisms for the formation of all of these initiators of lipid peroxidation are described in Refs.[2-4]. For several years lipid peroxidation has been studied *in vitro* by incubating subcellular fractions or commercially available PUFA with low-molecular-weight iron complexes and enzymatic or nonenzymatic sources of $O_2^{\cdot-}$ and H_2O_2. These studies have helped to understand important aspects of lipid peroxidation, e.g., the effects of chelators on iron redox behavior and iron-lipids sterical interactions.[2,4,5] Nonetheless, it must be recognized that most of these studies rely on artificially high concentrations of iron and hence may not adequately reproduce any *in vivo* situation, even if one assumes that tissue damage is preceded or accompanied by disruption of iron homeostasis/storage.[6] There is therefore growing interest in the identification of intracellular sites from which lower but more "realistic" amounts of iron can be mobilized to promote peroxidation. Studies by Gutteridge *et al.*[7] and by Aust's[8-11] and Winterbourn's[12,13] groups have shown that ferritin is a possible source of iron for lipid peroxidation. Ferritin is a shell-shaped multi-subunit

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protein with narrow channels leading to a core in which iron can be stored in the ferric form and from which iron can be released upon reduction to the ferrous form. The involvement of ferritin in lipid peroxidation is therefore contingent on availability of reductants, which have been found to include, among others, the semiquinone radical of cardiotoxic antitumor anthracyclines like ADR;[14] and the monocation radical of paraquat, a widely used but highly toxic herbicide.[15] The microsomal flavoenzyme NADPH-cytochrome P-450 reductase catalyzes formation of these reducing radicals via one-electron addition to the parent compounds. Ferritin iron mobilization will in turn occur directly, via reoxidation of radicals at expense of iron; or indirectly, via reoxidation of radicals at expense of oxygen and formation of another reductant like $O_2^{\cdot-}$.[14,15] In addition to ferritin, a small pool of microsome-bound nonheme iron has been recently identified as a possible catalyst of lipid peroxidation.[16–19] In this article we briefly report our studies of this latter source of iron for lipid peroxidation.

MATERIALS AND METHODS

Chemicals

Adiamycin was purchased from Farmatalia-Carlo Erba (Milano, Italy). All other chemicals were purchased form Sigma Chemical Co. (St. Louis, MO), with exception of Chelex 100 ion-exchange resin, that was from Bio-Rad (Richmond, CA) and was used to remove contaminating metals from solutions and reagents.

Microsome Isolation, Chromatography and Assays

Isolation of microsomes (by ultracentrifugation or low-speed Ca^{2+} aggregation) and subsequent chromatography on Sepharose Cl-2B were carried out as described in.[16,20] Protein content, enzymes, ferritin and nonheme iron were assyed as outlined in.[16–19] Microsomal reductive release of iron was monitored spectrophotometrically as bathophenanthroline-Fe^{2+} formation according to Ulvik,[21] with minor modifications.[16] Where indicated, anaerobiosis was achieved by means of argon plus glucose and glucose oxidase.[16]

Other details are given in legends to tables.

RESULTS AND DISCUSSION

Nonheme-Nonferritin Iron: General Concepts

Rat liver microsomes isolated by usual method of ultracentrifugation contain ferritin and hence substantial amounts of ferric nonheme iron.[20,22] Ferritin can be removed from microsomes by means of Sepharose CL-2B chromatography[16,22] or by replacing ultracentrifugation with low-speed Ca^{2+} aggregation.[20] Table I shows that separate or combined application of these procedures completely removes ferritin but fails to "clean" microsomes of all of their nonheme iron. This has led to propose that microsomes contain "endogenous" iron, tentatively referred to as nonheme-nonferritin.[16–19,20,22] Nonhemosiderin- nonferritin-iron has also been found to associate with the membrane of sickle erythrocytes.[23] This is quite indicative for the ubiquitous occurence of alternate means of iron storage. At this time, however, we have no data to speculate on possible analogies between the nonheme-nonferritin iron pools of membranes with different structure and functions.

TABLE I
Ferritin and nonheme iron content of different rat liver microsome preparations.

Isolation procedure	Ferritin	Nonheme iron
	μg/mg prot.	nmoles/mg prot.
Ultracentrifugation	4.9	15.1
+ chromatography	0.0	4.0
Ca^{2+} aggregation	0.0	4.9
+ chromatography	0.0	4.0

Note. Six weeks old male Wistar rats were used for these experiments.

As a general feature, the microsomal content of nonheme-nonferritin iron is inversely related to that of cytochrome P-450. For example, pretreatment of young rats with phenobarbital, an inducer of cytochrome P-450, causes simultaneous increase of the hemeprotein and depletion of nonheme-nonferritin iron (Table II). An opposite situation, i.e. increase of nonheme-nonferritin iron and decrease of cytochrome P-450, can be found in microsomes from the liver of untreated aging rats. It would also appear that pretreatment of aging rats with phenobarbital modifies the cytochrome P-450: nonheme-nonferritin iron balance in a manner similar to but less pronounced than that observed with young rats (see also Table II). Phenobarbital-induced depletion of nonheme-nonferritin iron and increase of cytochrome P-450 are counteracted by simultaneous administration of inhibitors of heme synthesis.[17] This has suggested that nonheme-nonferritin iron is utilized to form the heme iron group of cytochrome P-450.[17]

Mechanisms for the Release of Nonheme-Nonferritin Iron and its Involvement in Lipid Peroxidation

As observed with ferritin-bound iron, microsome-bound ferric nonheme-nonferritin iron can be mobilized upon reduction to the ferrous form. Three mechanisms have been thus far described for the reductive release of nonheme-nonferritin iron. Thomas and Aust[22] first observed that addition of NADPH to microsomes causes SOD-insensitive release of nonheme-nonferritin iron. They have therefore proposed that NADPH-supplemented microsomal electron transport culminates in $O_2^{\cdot-}$-independent reduction of nonheme-nonferritin iron. Studies with low-molecular-weight chelates and either microsomes or reconstituted microsomal systems point to the reductase and cytochrome P-450 as possible sites for the direct reduction of iron.[22,24,25]

TABLE II
Effects of phenobarbital on cytochrome P-450 and nonheme-nonferritin iron content of microsomes from the liver of 6 weeks or 21 months old Wistar rats.

Treatment	cytochrome P-450		nonheme-nonferritin iron	
	nmoles/mg protein			
	6 weeks	21 months	6 weeks	21 months
None	1.1	0.7	4.0	5.3
Phenobarbital	3.3	1.4	1.2	4.7

Note. Where indicated, animals were treated with phenobarbital for 3 days (30 mg i.p./kg body wt/day) and sacrificed 24 hours after the last injection. Chromatographed microsomes were used for these experiments.

The two remaining mechanisms of release have been studied in our laboratory. Nonheme-nonferritin iron can be reductively released upon reaction of t-BOOH with cytochrome P-450.[17] ADP is required for the mobilization of iron, perhaps to facilitate sterical and/or redox interactions of reductant(s) with iron itself.[17] From a mechanistic viewpoint t-BOOH- and cytochrome P-450-dependent release of iron can be mediated by: (i) heterolytic cleavage of t-BOOH and formation of cytochrome P-450 peroxidaselike intermediates that undergo continuous self-reduction/reoxidation at expense of iron, as it has been shown to occur between similar intermediates of horseradish peroxidase and other metal complexes; or (ii) homolytic cleavage of t-BOOH, with formation of t-BO$^{\cdot}$ radicals and their subsequent β-scission and oxygen addition to yield a reductant like hydroxymethyl radical.[17] Finally, SOD-insensitive release of nonheme-nonferritin iron has been observed in incubations containing NADPH and ADR.[16] This has led to propose that iron is reduced by ADR$^{\cdot-}$ rather than by $O^{\cdot-}_2$ formed upon reaction of ADR$^{\cdot-}$ with molecular oxygen. This proposal is consistent with the repeated finding that free radical intermediates of redox cycling xenobiotics can reduce iron more effectively via direct one-electron transfer than via formation of $O^{\cdot-}_2$.[26,27]

The reductive mobilization of nonheme-nonferritin iron is invariably paralleled by lipid peroxidation. In a NADPH-dependent system, SOD-insensitive release of Fe^{2+} causes equally SOD-insensitive lipid damage.[22] Lipid peroxidation is also insensitive to addition of catalase, thus suggesting that formation of an iron-oxygen complex can substitute for $O^{\cdot-}_2$- and H_2O_2-dependent $^{\cdot}OH$ formation in initiation reactions.[22] In a t-BOOH-dependent system, the addition of ADP, which is required for Fe^{2+} release, stimulates background peroxidation mediated by t-BO$^{\cdot}$ radicals or peroxidaselike intermediates of cytochrome P-450.[18] Given the peculiar nature of this system, which generates reductants/oxidants other than $O^{\cdot-}_2$ and H_2O_2, neither basal lipid peroxidation nor the ADP-promoted release of Fe^{2+} and concurrent stimulation of lipid peroxidation are affected by SOD and catalase (Minotti, G., unpublished data). This implies that stimulation of background t-BOOH-dependent lipid peroxidation is mediated by formation of some type of complex between oxygen and the released Fe^{2+}. In a NADPH- and ADR-driven system, lipid peroxidation is strictly contingent on the release of nonheme-nonferritin iron yet the two reactions are governed by very distinct mechanisms. Thus, while iron release is SOD- and catalase-insensitive, lipid peroxidation best occurs when both catalase and SOD are available to microsomes.[19] The ineffectiveness of SOD and catalase on iron release is consistent with a role for ADR$^{\cdot-}$ as direct reductant for iron, and suggests that H_2O_2 does not interfere with ADR$^{\cdot-}$-iron interaction. On the other hand, stimulation of lipid peroxidation by SOD and catalase indicates that the $O^{\cdot-}_2/H_2O_2$ couple would paradoxically inhibit formation and/or reactivity of initiators. These results highlight the complexity of lipid peroxidation and the number of reactive species possibly involved in the initiation step.

Biomedical Implications

In a previous study, Floyd *et al.*[28] have found that brain iron content increases with age yet brain homogenates from either young or aging animals yield comparable amounts of lipid peroxidation by-products when incubated under oxygen tensions. The authors could not therefore obtain clearcut relationship between aging-associated cell modifications (e.g., lipofuscin accumulation) and increased availability of iron for generation of lipid oxidants. Our finding that "aging" liver microsomes are

TABLE III

Cytochrome P-450 and nonheme-nonferritin iron content of microsomes from untreated or phenobarbital-treated rats and from Morris hepatomas (H).

Microsomes	cytochrome P-450		nonheme-nonferritin iron	
	nmoles/mg protein			
		%		%
Phenobarbital liver[a]	3.3	100	1.2	17
Liver[a]	1.1	33	4.0	57
H 9618A[b] (slow-growing, well-differentiated)	0.3	9	5.6	80
H 3924A[b] (fast-growing, poorly differentiated)	0.0	0	7.0	100

Note. In (a) liver microsomes were isolated from 6 weeks old male Wistar rat. "Phenobarbital microsomes" indicates microsomes from rats treated as in legend to Table II. In (b) Morris hepatomas 9618A and 3924A were grown in inbred male rats from Buffalo and ACI/T strains, respectively, as described in Ref. [29]. Chromatographed Ca^{2+}-aggregated microsomes were used for these experiments.

TABLE IV
ADR-dependent anaerobic release of iron from human lung tumor microsomes

Microsomes	Cytochrome P-450	Nonheme-nonferritin iron	NADPH-cytochrome P-450 reductase	ADR	Fe^{2+} Release
	nmoles/mg prot.		mU/mg prot.	(μM)	nmoles/mg prot.
Lung	0.0	147.1	39	25	0.05 (aerobic)
Lung	0.0	127.1	42	25	0.06 (aerobic)
undifferentiated				25	1.25 (anaerobic)
carcinoma				10	0.55 (anaerobic)

Note. Normal or neoplastic tissues (5–13 grams) were removed during surgical intervention and ferritin-free microsomes were obtained by ultracentrifugation and Sepharose CL-2B chromatography. Incubations for measurements of iron release (1 ml final volume) contained microsomes (2 mg prot./ml), NADPH (1 mM), and bathophenanthroline (0.5 mM) in 50 mM NaCl, pH 7.0, 37 °C. Values of iron release are those determined at 5 minutes. All other conditions were as described in Refs. [16,21].

characterized by net increase of nonheme-nonferritin iron and by decreased susceptibility of this iron to phenobarbital-induced conversion to cytochrome P-450 confirms that nonheme iron does accumulate during senescence (cf. Table II). However, our studies on the mechanisms for the involvement of nonheme-nonferritin iron in formation of lipid oxidants point out that differences in the peroxidation of tissue homogenates or subcellular fractions from young and aging rats should be sought for in the presence of reductants that mobilize iron from membrane binding sites.[16-19] Changes in the microsomal content of nonheme-nonferritin iron can also occur in tumor cells. Galeotti *et al.*[29] have shown that experimental tumors may lack cytochrome P-450 either partially or completely, depending on tumor growth rate and degree of dedifferentiation. We now report that in microsomes from rat Morris hepatomas growth- and dedifferentiation-related decrease of cytochrome P-450 is paralleled by consistent increase of nonheme-nonferritin iron (Table III). This is most evident when a comparison is made with liver microsomes of phenobarbital-treated rats, in which the pool of nonheme-nonferritin iron has been depleted to form cytochrome P-450 (see also Table III). It would appear, therefore, that tumor growth interferes with nonheme-nonferritin iron → cytochrome P-450 conversion. In light of this result, we have made similar assays in microsomes from human lung tumors. Surprisingly, we have found that both microsomes from tumors and microsomes from the "normal" neighboring lung are characterized by absence of cytochrome P-450 and accumulation of very high but nearly comparable amounts of nonheme-nonferritin iron (see Table IV for a representative case). "Normal" and tumor microsomes also share similar reductase activities (Table IV). At this time, we have no explanation for such a striking similarity between lung tumors and their"normal" parent tissues. Low but significant amounts of cytochrome P450 should in fact be detected in microsomes of truly normal lung.[30] Nonetheless, we have data to suggest that tumor microsomes may represent a pharmacologically important site of iron release. In fact, one must consider that solid-growing tumors have insufficient vascularization and hence contain hypoxic cells in which $ADR^{\cdot}$ would reduce iron via direct one-electron transfer rather than via reaction with oxygen and formation of a weaker reductant like $O_2^{\cdot}$. This is exemplified in Table IV, which shows that lung tumor microsomes incubated anaerobically with ADR release much higher amounts of iron than do "normal" microsomes incubated aerobically. Tumor microsomes can release more iron even when incubated with less than one-half of the ADR available to normal microsomes (see also Table IV). This latter *in vitro* situation tentatively reproduces situations occurring *in vivo* when insufficient vascularization results not only in low oxygen supply but also in low drug distribution within the tumor burden. Studies are in progress to establish whether hypoxia-normoxia transitions may facilitate reaction of the released iron with oxygen and *in situ* formation of lipid oxidants which mediate tumor cell killing.[31] Mobilization of iron from within tumor cells into systemic circulation might also account for the severe cardiotoxicity of ADR, reportedly mediated by lipid peroxidation or equally detrimental iron-dependent reactions.[32]

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