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Forum Review

Role of Lipid Hydroperoxides in Photo-Oxidative Stress Signaling

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ABSTRACT

Photosensitized peroxidation of membrane lipids has been implicated in skin pathologies such as phototoxicity, premature aging, and carcinogenesis, and may play a role in the antitumor effects of photodynamic therapy. Lipid hydroperoxides (LOOHs) are prominent early products of photoperoxidation that typically arise via singlet oxygen ($^1O_2$) attack. Nascent LOOHs can have several possible fates, including (i) iron-catalyzed one-electron reduction to chain-initiating free radicals, which exacerbate peroxidative damage, (ii) selenoperoxidase-catalyzed two-electron reduction to relatively innocuous alcohols, and (iii) translocation to other membranes, where reactions noted in (i) or (ii) might take place. In addition, LOOHs, like other stress-associated lipid metabolites/peroxidation products (*e.g.,* arachidonate, diacylglycerol, ceramide, 4-hydroxynonenal), may act as signaling molecules. Intermembrane transfer of LOOHs may greatly expand their signaling range. When photogenerated rapidly and site-specifically, *e.g.,* in mitochondria, LOOHs may act as early mediators of apoptotic cell death. This review will focus on these various aspects, with special attention to the role of LOOHs in photooxidative signaling. *Antioxid. Redox Signal.* 6, 301–310.

INTRODUCTION

UNSATURATED MEMBRANE LIPIDS IN MAMMALIAN CELLS, including glycolipids, phospholipids (PLs), and cholesterol (Ch), are well known targets of damaging and potentially lethal peroxidative modification (24, 27). Lipid peroxidation can be provoked by numerous physical/chemical challenges that give rise to reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\bullet$), or singlet oxygen ($^1O_2$). O_2^- and H_2O_2 can be generated metabolically, *e.g.,* during neutrophil activation or mitochondrial electron transport, and can give rise to strongly oxidizing $HO^\bullet$ via Fenton chemistry (16, 47). These ROS, as well as $^1O_2$, may also derive from the action of various extracellular agents, important examples being ionizing and nonionizing radiation (47, 55). Of special interest in the latter category are UVA (320–400 nm) and visible (400–700 nm) radiations, which, in the presence of photosensitizing agents and ground-state oxygen ($^3O_2$) can oxidize lipids and other biomolecules through photodynamic action (13, 55, 60). Endogenous UVA-absorbing sensitizers include tetrapyrroles, flavins, and reduced pyridine nucleotides, whereas exogenous sensitizers can be found in certain food additives, cosmetics, and drugs (8, 60). Similarly, many natural and synthetic sensitizers exist that are photoactivated by visible light. One important example is protoporphyrin IX (PpIX), the immediate metabolic precursor of heme. Abnormal accumulation of PpIX, as in the genetic disorder protoporphyria, can result in severe skin photosensitivity (2). On the other hand, numerous visible light-absorbing sensitizers, including PpIX, can be exploited for therapeutic purposes, the most notable example being antitumor photodynamic therapy (PDT) (10). All photodynamic reactions commence with photon absorption by the sensitizer (S), converting it to a singlet and thence triplet excited state (^3S). The immediate postexcitation chemistry is classified as either type I or type II (12). A typical type I reaction is characterized by electron or hydrogen transfer from a substrate (RH) to ^3S, with free radical (*e.g.,* O_2^-, $HO^\bullet$) generation, whereas a type II re-

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action involves energy transfer from ^3S to $^3O_2$ to give delta-state $^1O_2$. The relative importance of these two mechanisms in a cellular system depends on factors such as sensitizer localization, kinetics of the ^3S reaction with RH versus $^3O_2$, and relative concentrations of RH and $^3O_2$ in the vicinity of ^3S (12). Inhibitory effects of $^1O_2$ or free radical scavengers are often used for distinguishing between type I and type II reactions, but with many uncertainties due to lack of absolute specificity (18, 55). Likewise, direct physical measurement of $^1O_2$ produced in cells has proven difficult because of its short lifetime relative to that in homogeneous aqueous solution (<50 ns versus 4–5 μs) (11, 42). These difficulties can be largely overcome by using certain characteristic lipid hydroperoxides (LOOHs) as mechanistic "reporters" (see below).

PHOTODYNAMIC LIPID PEROXIDATION: PRIMARY LOOH FORMATION

Most PDT-related sensitizers are amphiphiles and, by binding to cell membranes that are O_2-enriched relative to aqueous compartments, tend to catalyze lipid peroxidation via the type II ($^1O_2$) pathway (18, 21). $^1O_2$ also reacts with membrane proteins, and with rate constants that are considerably higher on average than those for lipids (9); however, if the sensitizer preferentially localizes in the lipid domain, this will greatly enhance $^1O_2$ reactivity there. In contrast to HO• and other radical oxidants, which induce lipid peroxidation via H abstraction, $^1O_2$ can add directly to Ch or to a PL unsaturated *sn*-2 fatty acyl group to give a primary LOOH (Fig. 1) with its double bond shifted to the allylic position (18). $^1O_2$-generated LOOHs accumulate linearly with light dose in the absence of reductants and redox-active metal ions, but rapidly turn over in the presence of these agents, thereby triggering free radical-mediated chain reactions (see below). Hydroperoxides generated by reaction of $^1O_2$ with unsaturated fatty acids or simple PLs in homogeneous solution have been well characterized (15, 59). For example, reaction with 1-palmitoyl-2-linoleoyl-phosphatidylcholine gives four *sn*-2-positioned hydroperoxides, two conjugated (9-OOH, 13-OOH) and two nonconjugated (10-OOH, 12-OOH), in approximately equal yields (59) (Fig. 2). By contrast, free radical attack gives only 9-OOH and 13-OOH, so identifying the conjugated species in addition to the nonconjugated would specify $^1O_2$ involvement in a reaction, but not necessarily sole involvement. A similar pattern holds for more unsaturated PLs. Thus, $^1O_2$ attack on an arachidonyl group gives up to eight hydroperoxide species, only two of which are nonconjugated (15) and thus specific for $^1O_2$. All of the nonconjugated and most of the conjugated LOOHs described are unique to nonenzymatic lipid oxidation, *e.g.,* they are not produced by lipoxygenases.

Like PLs, Ch is found in all membrane compartments of eukaryotic cells, but most of it (typically >80%) is located in the plasma membrane, where it accounts for ~45 mol % of the total lipid (5), making it an excellent target for membrane-bound sensitizers. Unlike PLs, Ch exists as a single molecular species, and its relatively few peroxidation products are much easier to separate and characterize than those of PLs (22, 31). Ch oxidation products (ChOX) generated by chemical oxi-

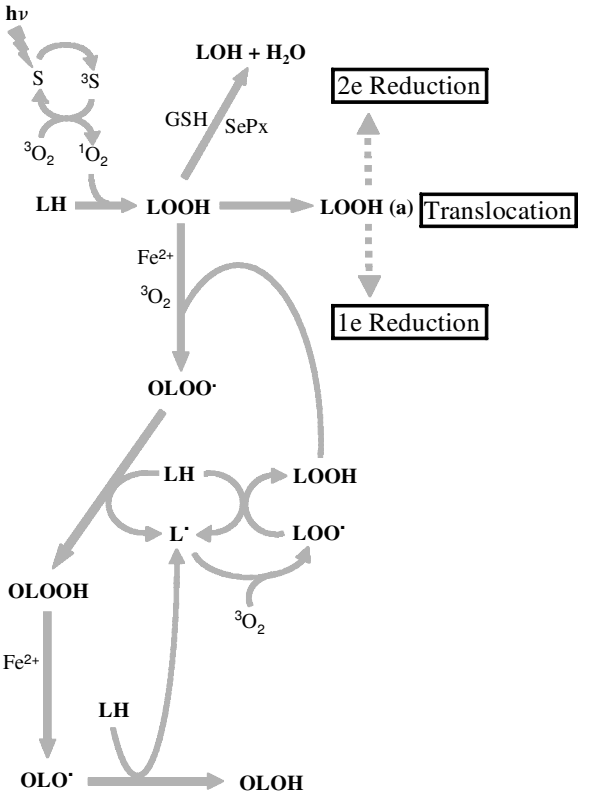

FIG. 1. Type II ($^1O_2$-mediated) photogeneration of LOOHs in cell membranes and their possible subsequent reactions. The latter include: (i) iron-catalyzed one-electron reduction with induction of chain peroxidation (toxicity expansion); (ii) selenoperoxidase (SePx)-catalyzed two-electron reduction (toxicity containment); and (iii) transfer to another membrane (a), where one- or two-electron reduction might occur. LH, LOOH, OLOOH, and OLOH denote an unsaturated lipid, lipid hydroperoxide, epoxyallylic hydroperoxide, and epoxyallylic alcohol, respectively. L•, LOO•, OLOO•, and OLO• denote a lipid radical, peroxyl radical, epoxyallylic peroxyl radical, and epoxyallylic oxyl radical, respectively.

dants, ionizing radiation, and photodynamic action have been well characterized in a variety of reaction systems, ranging from model membranes to cultured cells (19). In $^1O_2$-mediated reactions such as type II photoreactions, three characteristic Ch hydroperoxides (ChOOHs) are produced (36): 5α-OOH, 6α-OOH, and 6β-OOH (Fig. 2). The yield of 5α-OOH in a peroxidizing membrane typically exceeds that of 6α- and 6β-OOH by at least fivefold, making 5α-OOH a more sensitive indicator of $^1O_2$ intermediacy (31). In free radical-mediated reactions, including type I photoreactions, two ChOOHs typically predominate: 7α-OOH and 7β-OOH, the latter being the more thermodynamically stable epimer (54). The 7-OOHs arise via H abstraction at the C-7 position of Ch, typically by reaction of a strong oxidizing radical at this position. This can occur either as a primary event (*e.g.,* attack by HO•) or subsequently during chain propagation (see Fig. 1). Several other ChOX species are generated during propagation reactions, most of which are stable nonperoxide end products, *e.g.,* 5,6-epoxides and the 7α-OH and 7β-OH diols (54). Mechanistic

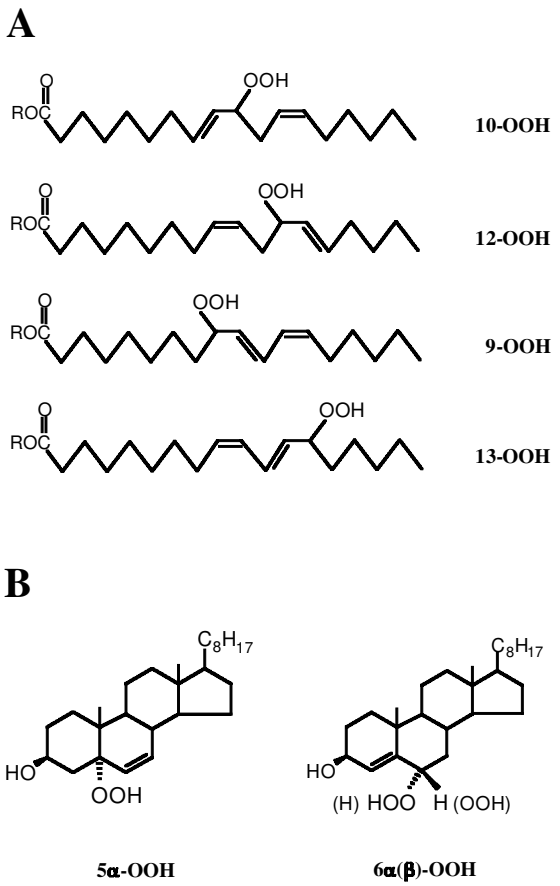

FIG. 2. Characteristic hydroperoxide species produced by $^1O_2$ attack on phospholipid linoleoyl (9,12-octadecadienoyl) moieties (A) and on cholesterol (B). In (A), R represents the remainder of the phospholipid molecule; possible stereochemical configurations are ignored.

information based on detection of 5α-OOH as a $^1O_2$ reporter and 7α/7β-OOH as a free radical reporter has been acquired for a variety of isolated membrane and cellular systems (19, 22, 31). 5α-OOH was recently detected in the skin of rats exposed to intense visible light after ingesting a sensitizing agent (70), the first solid evidence for the occurrence of $^1O_2$ *in vivo*.

High-sensitivity/specificity analytical techniques are necessary for monitoring the LOOHs described. One of these is reverse-phase high-performance liquid chromatography with mercury cathode electrochemical detection [HPLC-EC(Hg)]. Developed in this laboratory (32), HPLC-EC(Hg) is extremely sensitive [detection limits ~0.2 pmol and ~1 pmol for ChOOHs and phospholipid hydroperoxides (PLOOHs), respectively] and is well suited for analysis of complex samples, *e.g.,* lipid extracts from cells exposed to UVA radiation or to sensitizers and visible light (22, 31, 35).

LOOH ONE-ELECTRON REDUCTION: DAMAGE EXPANSION

In addition to perturbing membranes directly because of increased polarity, LOOHs are susceptible to iron-catalyzed reduction in the presence of electron donors such as ascorbate or glutathione (GSH) (18, 20). One-electron reduction to oxyl ($LO^\bullet$) radicals is kinetically much more favorable than one-electron oxidation to peroxyl ($LOO^\bullet$) radicals. Studies with fatty acid hydroperoxides (FAOOHs) in solution (67) have indicated that $LO^\bullet$ is far less likely to initiate chain peroxidation via H abstraction than is $OLOO^\bullet$, an epoxyallylic peroxyl radical formed by rearrangement of $LO^\bullet$, followed by rapid addition of $^3O_2$. Although $OLOO^\bullet$ intermediacy has not yet been demonstrated for membrane systems, it nevertheless has gained wide acceptance and, thus, is represented in the one-electron mechanism depicted in Fig. 1. $OLOO^\bullet$ species would trigger rounds of free radical-mediated lipid peroxidation during which new LOOHs are generated and feed into the overall process (Fig. 1). These downstream postphotoexcitation reactions would exacerbate the effects of primary LOOH formation alone, and thus expand the range of peroxidative damage and/or signaling. Accordingly, situations could exist in which $^1O_2$-mediated photoperoxidation evolves into more conventional (nonphotodynamic) chain peroxidation if primary LOOHs encounter properly ligated Fe^{2+} (20). Under these conditions, 7α/7β-OOH would be plentiful, but 5α-OOH might turn over faster than it is generated, leading one to mistakenly conclude, based on analysis of these ChOOHs, that $^1O_2$ plays no role in the reaction. That chain reactions induced by one-electron reduction of primary photoperoxides are important and relatively prolonged after a toxic light dose was demonstrated by showing that postirradiation introduction of chain-breaking antioxidants such as butylated hydroxytoluene (BHT) (17) or nitric oxide (44) is significantly cytoprotective.

Several approaches for monitoring LOOH-initiated chain peroxidation are available, ranging from the relatively simple thiobarbituric acid assay to sophisticated spectroscopic methods such as spin-trapping EPR (20, 21). A new technique recently developed in this laboratory (34) has several advantages over other existing approaches. In this technique, [^{14}C]Ch is used as an *in situ* probe for free radical activity in its membrane surroundings. Chain reactions set off by one-electron reduction of 5α-OOH or other primary LOOHs result in the accumulation of radiolabeled ChOX species that derive from free radical chain reactions, namely, 7α-OOH, 7β-OOH, 7α-OH, 7β-OH, and 5,6-epoxides. These "reporter" molecules can be conveniently resolved from one another and parent Ch by normal-phase high-performance thin-layer chromatography (HPTLC) and detected by phosphorimaging (34). As demonstrated in recent studies with ChOOH- and $^1O_2$-challenged cells (26, 44), this approach (HPTLC-PI) is especially well suited for assessing plasma membrane peroxidation because most of the [^{14}C]Ch probe localizes in this compartment.

LOOH TWO-ELECTRON REDUCTION: DAMAGE CONTAINMENT

Scavenging of O_2^- by superoxide dismutases and H_2O_2 by catalase or GSH-dependent selenoperoxidases (SePXs) can limit formation of strong oxidants such as $HO^\bullet$ and peroxynitrite ($ONOO^-$), and thereby inhibit initiation of chain peroxidation by these species (16, 20). Unlike O_2^- and H_2O_2, $^1O_2$

has no known enzymatic scavengers. Consequently, secondary (reparative) reactions are necessary for cytoprotection against $^1O_2$-generated LOOHs, and SePX(s) appear to play a key role in this (20) (Fig. 1). At least two types of intracellular SePXs have been implicated in LOOH detoxification: "classical" GSH peroxidase (GPX1, ~82-kDa homotetramer) and phospholipid hydroperoxide GSH peroxidase (PHGPX or GPX4, ~20-kDa monomer) (6, 27). These enzymes are widely distributed in mammalian tissues and can be found in the cytosol, nuclei, and mitochondria (50). General SePX involvement in cytoprotection against $^1O_2$-mediated photodamage was first demonstrated by showing that Se-deficient leukemia cells (exhibiting severely depressed GPX1 and GPX4 activities) accumulate LOOHs more rapidly than Se-satisfied controls under a photooxidative challenge, and die off faster in an iron-sensitive fashion (37, 38). GPX1 and GPX4 both contain an active-site selenocysteine, which participates in the two-electron reduction of peroxides to alcohols (Fig. 1), but are strikingly different with regard to LOOH reactivity and specificity. Thus, GPX4 can act directly on PLOOHs in membranes (50), whereas GPX1 cannot unless *sn*-2 fatty acyl bonds are first hydrolyzed to liberate FAOOHs (23). Disposal of membrane PLOOHs could involve sequential action of (i) phospholipase A_2 (PLA_2) and GPX1 or (ii) GPX4 and PLA_2, followed in either case by reacylation of the resulting lysolipids (20). The biological importance of the first pathway (excision/reduction/repair) compared with the second (reduction/excision/repair) is not certain, although most of the evidence to date favors the latter pathway (3). Consistent with this are studies showing that GPX4-overexpressing transfectant cells detoxify PLOOHs more effectively than wild-type controls and are correspondingly more resistant to a PLOOH challenge (69).

The question of ChOOH detoxification in a mammalian cell is also important, especially for the plasma membrane, where Ch comprises nearly half of the lipid population. Unlike FAOOHs, ChOOHs (whether membrane-bound or Triton-solubilized) are completely resistant to GPX1-catalyzed reduction (58), ruling out any involvement of this enzyme in cytoprotection against ChOOH effects. GPX4, on the other hand, does react with and detoxify ChOOHs (albeit more slowly than PLOOHs) and is the only enzyme known to do this (58). The various ChOOHs described are reduced at significantly different rates in detergent micelles or membrane bilayers, their first-order rate constants decreasing in the following order: 6β-OOH > 7α/7β-OOH > 6α-OOH >> 5α-OOH (33). This was observed with purified enzyme and also homogenates of Se-replete cells, whereas Se-deficient counterparts had relatively little activity (31). By contrast, all of these peroxides were found to undergo one-electron reduction at the same rate, at least in liposomal membranes (34), suggesting equal chain-initiating potency. However, their order of toxicity toward leukemia cells under equivalent uptake conditions was found to be diametrically opposite that of GPX4-mediated detoxification (see above). Thus, 6β-OOH, with a relatively short metabolic lifetime based on GPX4 reactivity, was the least toxic of the ChOOHs examined, whereas 5α-OOH, with the longest lifetime, was the most toxic (31, 33). Rapid generation of 5α-OOH under $^1O_2$ pressure, coupled with relatively slow catabolism and ability to translocate (see below), makes this species potentially the most important stress mediator among the ChOOHs (and perhaps all the LOOHs) considered.

LOOH TRANSFER BETWEEN MEMBRANES

LOOHs in photodynamically stressed cells may induce chain peroxidation near their sites of origin (see above) if detoxification capabilities are overwhelmed. Recent studies have demonstrated, however, that chain induction is not necessarily limited to a nascent LOOH's immediate membrane surroundings, but can extend to other membranes by way of LOOH transfer through the aqueous compartment (62). Spontaneous or protein-mediated transfer of LOOHs (facilitated by their increased polarity) could disseminate peroxidative stress and stress signaling both intracellularly and extracellularly, depending on factors such as relative availability of redox iron, chain-breaking antioxidants, and GSH/GPX4 at acceptor sites versus donor sites (see Fig. 1). Model studies involving photoperoxidized erythrocyte membrane donors and liposome acceptors have shown that total ChOOH translocates much faster than parent Ch, desorption from the donor being the rate-limiting step (62). Interestingly, the first-order rate constants for spontaneous transfer of individual ChOOH species decreased in the following progression: 7α/7β-OOH > 5α-OOH >> 6α-OOH > 6β-OOH, which correlates with their order of decreasing polarity (63). The same trend has been observed for several different transfer systems, including cultured cells as either donors or acceptors. Experiments with GPX4-deficient cells have shown that the time-dependent degree of toxicity for these ChOOHs decreases in parallel with their rates of transfer uptake, thus demonstrating that transfer-limited cytotoxicity is possible (63). Similar observations have been made with various PLOOH families, namely, PCOOH, PEOOH, PSOOH, and SMOOH (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and spingomyelin hydroperoxides, respectively), all of which translocate more slowly than ChOOHs (64). ChOOH and PLOOH intermembrane transfer in model systems can be markedly accelerated by SCP-2 (A. Vila and A.W. Girotti, unpublished observations), a nonspecific intracellular carrier protein implicated in Ch and PL trafficking and metabolism (56). Whether other lipid transfer proteins can act similarly is presently unknown. Intermembrane transfer could broaden the signaling and/or toxic range of LOOH species. For example, toxicity could be amplified by spontaneous or SCP-2-facilitated transfer to relatively sensitive sites such as mitochondria or nuclei, where peroxidative damage to the respiratory apparatus or to nuclear DNA might ensue. On the other hand, movement to sites at which GSH and/or GPX4 is more available could provide a means for more efficient LOOH detoxification. The relative importance of these hypotheses awaits critical examination.

ROLE OF LIPIDS AND PEROXIDIZED LIPIDS IN STRESS SIGNALING

The extent of oxidative injury to a cell, *e.g.,* a photodynamically stressed cell, may determine whether it ultimately survives or succumbs to apoptotic or necrotic death (20). Apoptosis (characterized by chromatin condensation and cell shrinkage, but minimally impaired metabolism) can be physiologically beneficial because it removes unnecessary or un-

desirable (*e.g.,* mutated) cells from the population, whereas necrosis (characterized by membrane lysis and metabolic shutdown) can produce noxious effects associated with inflammation (30). The effects of progressively intensifying oxidative pressure on cells, *e.g.,* by membrane-targeted photodynamic action, can be conceptualized as follows (20): (i) no net damage if constitutive antioxidant capacity is sufficient to prevent or repair oxidative lesions; (ii) relatively mild (sublethal) injury, which may signal the up-regulation of antioxidant proteins to fortify cytoprotection; (iii) more extensive damage, which triggers apoptosis because constitutive or inducible repair capacity is exceeded; and (iv) very extensive damage with membrane disruption, which preempts the metabolic requirements of apoptosis and results in necrosis. Apoptosis or necrosis could develop if one-electron reduction of membrane LOOHs outpaces two-electron reduction (see Fig. 1).

Phospholipid metabolites as second messengers

There are numerous reports that mammalian cells overexpress cytoprotective proteins in response to a sublethal or marginally lethal [category (ii)] oxidative insult. In some cases, lipid peroxidation has been assigned a specific role in the underlying signal transduction. Induction of heme oxygenase (HO-1) and ferritin in UVA-exposed skin fibroblasts is an important example (65). Up-regulation of these proteins is believed to protect against free heme- and iron-amplified oxidative damage triggered by UVA-generated $^1O_2$, *e.g.,* membrane peroxidative damage (4). Lipid metabolites such as arachidonate and diacylglycerol (DAG) have been implicated in HO-1 induction, suggesting a role for eicosanoids and protein kinase C (PKC) in the induction process (4). Up-regulation of arachidonate and DAG has been ascribed to peroxidation–activation of PLA_2 and phospholipase C (PLC) (see below).

Category (iii) oxidative pressure may induce factors that promote the apoptotic process. A well studied example in the lipid category is ceramide (Cer), a precursor of sphingomyelin (SM) and other sphingolipids (25, 39). *De novo* synthesis of Cer starts with serine palmitoyl transferase (SPT)-catalyzed condensation of serine and palmitate, the rate-limiting step in the overall pathway. Cer can also be generated by sphingomyelinase (SMase)-catalyzed hydrolysis of SM. On the catabolic side, Cer can react with phosphatidylcholine to give SM and DAG, as catalyzed by SM synthase (25). DAG is a potent activator of PKC and transcription factor nuclear factor-κB, which promote cell survival and proliferation. Thus, Cer and DAG have opposing cytoregulatory effects, and their levels are tightly controlled so as to foster apoptosis on the one hand and cell growth on the other. Intracellular Cer levels are elevated under a variety of oxidative stress conditions, including PDT stress, and this has been implicated in phthalocyanine Pc4-sensitized apoptosis (52, 68). Cer might stimulate apoptosis by activating protein phosphatases, which dephosphorylate and inactivate antiapoptotic proteins like Bcl-2 (51). ISP-1, an SPT inhibitor, was found to attenuate both Cer elevation and apoptosis of Pc4/light-treated A431 cells, and these effects could be reversed by exogenous C16-H_2Cer. This is consistent with the involvement of *de novo* Cer synthesis in apoptotic signaling in these cells (68). On the other hand, Pc4/light-treated acid SMase-knockout cells exhibited increased Cer and apoptosis similar to that of wild-type cells, supporting the argument that this Cer derives from SPT rather than SMase induction/activation (68). The exact mechanism of SPT modulation by photodynamic action is unknown, although some type of posttranslational redox regulation, *e.g.,* glutathionylation, has been postulated (52). This could conceivably occur downstream of LOOH formation and turnover (see Fig. 1).

Peroxidized lipids and peroxidation by-products as stress mediators

As indicated above, certain protein kinases (*e.g.,* PKC, mitogen-activated protein kinases) and phospholipases (*e.g.,* PLC, PLA_2) play a crucial role in oxidant-induced signaling that could culminate in cytoprotection, apoptosis, or various other responses. There is growing interest in ROS-generated LOOHs as early intermediates in these pathways, which can occur in cells exposed to cytokines and growth factors, as well as ionizing radiation and photodynamic action (57). LOOHs are viewed in this context as second messengers, and recent evidence that these species can rapidly translocate between membranes (62, 63) suggests that their signaling range may be far broader than suspected up to now. PLA_2 is more active on peroxidized than nonperoxidized membranes, and this has been investigated extensively in relation to eicosanoid-mediated signal transduction. Model studies with the cytosolic and secreted forms of the enzyme have shown that hydrolytic activity increases as a function of membrane PLOOH content (49). Importantly, the hydrolysis of PLOOHs *per se* greatly exceeded that of unoxidized PLs, *i.e.,* FAOOHs were liberated more rapidly than fatty acids (49). By mimicking natural eicosanoids and/or activating cyclooxygenases or lipoxygenases through an elevated peroxide "tone," these species may play a key role in the flow of oxidative signaling. With regard to PDT signaling, it has been reported (1) that photosensitized apoptosis of lymphoma cells is blunted by PLA_2 and PLC inhibitors (the latter also blocking Ca^{2+} elevation), consistent with the notion that photoperoxidation of membrane lipids "activates" apoptosis via these enzymes. $^1O_2$-derived LOOHs can be considered as relatively early signaling mediators. However, certain late formed species, *e.g.,* by-products of chain peroxidation (15, 47), have also been implicated in stress signaling. A well documented example is 4-hydroxynonenal, which has been reported to play a role in UVA-induced HO-1 overexpression (4) and also in triggering apoptosis via loss of mitochondrial membrane potential, release of cytochrome *c* (cyt *c*), and activation of various caspases (28, 61).

Cellular resistance to LOOH-mediated stress signaling

Oxidative signaling culminating in apoptotic cell death can be triggered by a wide variety of agents/conditions, including H_2O_2, ionizing radiation, UVA radiation, and sensitizer/visible light exposure. Involvement of lipid peroxidation in general and LOOHs in particular has been deduced in several instances. For example, radiation-induced internucleosomal fragmentation of DNA was shown to be inhibited by Trolox, a chain-breaking vitamin E analogue (14), and also by ebselen, a GPX4 mimetic (48). Exogenous or endogenous organoperoxides can also trigger SePX-inhibitable apoptosis. Thus, HL-60 cells made Se-deficient by growing in 1% serum and, *i.e.,* express-

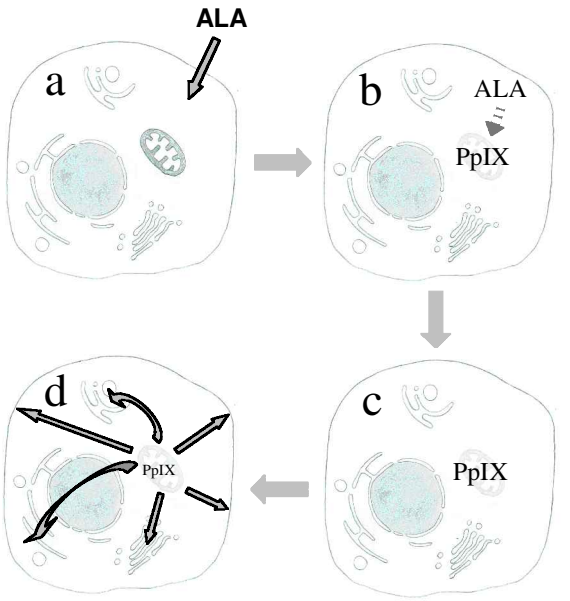

FIG. 3. Photosensitization of tumor cells with ALA-induced PpIX. Scheme depicts entry of ALA into cells (**a**), metabolic conversion to PpIX precursors in the cytosol, and then to PpIX in mitochondria (**b**). Following removal of exogenous ALA, cells can be photochallenged when PpIX is mainly in mitochondria (**c**) or after it redistributes to peripheral sites, including plasma membrane (**d**).

ing minimal overall SePX activity, underwent more extensive DNA fragmentation than Se-replete controls during exposure to *tert*-butyl hydroperoxide (A.W. Girotti, unpublished observations). This was accompanied by greater LOOH accumulation and loss of clonogenicity in the deficient cells. Similar results were obtained when LOOHs were generated endogenously using an internalized photosensitizer. It is apparent that apoptotic signaling in these systems was mediated at least in part by SePX-reactive LOOHs. Although the enzyme in question was most likely GPX4, this was not established in these experiments. More recent work involving GPX4-overexpressing cell lines has provided more definitive evidence for this enzyme's ability to modulate LOOH-induced apoptosis (45, 66, 69). Highly noteworthy are studies carried out with rat basophil transfectants that overexpressed either the mitochondrial-targeted (L-form) or nonmitochondrial (S-form) GPX4 (45). The L-form cells were found to be markedly more resistant to apoptotic killing under a metabolic oxidative stress (2-deoxyglucose treatment) than S-form or vector control cells. Thus, the L-cells were less sensitive to a loss of mitochondrial membrane potential, release of cyt *c,* and activation of effector caspases while exhibiting lower ROS and LOOH levels than S-counterparts (45). Importantly, inner membrane cardiolipin (Cl), which interacts strongly with cyt *c,* thus influencing its electron transfer as well as proapoptotic activity (41), underwent significantly less peroxidation [conversion to cardiolipin hydroperoxide (ClOOH)] in L-cells (46). This correlated with the diminished proapoptotic release of cyt *c* in these cells. These findings strongly implicated mitochondrial GPX4 in cytoprotection against oxidant-induced apoptosis, which presumably resulted from accelerated respiration in response to 2-deoxyglucose inhibition of glycolysis.

Recent studies in this laboratory have demonstrated that overexpressed GPX4 can also protect cells against photodynamically induced apoptosis, and in a site-specific fashion with respect to location of enzyme relative to damaging LOOHs (35). A GPX4-deficient human breast tumor line (COH-BR1) was transfected with a construct encoding the L-form of the enzyme, and several stably overexpressing clones were selected out (26), one of which, 7G4, exhibited nearly 90 times greater GPX4 activity than a vector control (VC). As expected, most of this activity (~80%) was associated with mitochondria (35). Sensitization of 7G4 and VC cells was accomplished via the metabolism of administered 5-aminolevulinic acid (ALA) to PpIX, using two different approaches: *protocol-1*, in which PpIX was localized mainly in mitochondria, where it is generated; and *protocol-2*, in which PpIX at the same total intracellular concentration was allowed to diffuse to peripheral sites, including plasma membrane (Fig. 3) (35). When irradiated with broad-band visible light, protocol-1 cells died almost exclusively by apoptosis, whereas protocol-2 cells died via necrosis (35). As shown in Fig. 4, however, protocol-1 7G4 cells were substantially more resistant to apoptosis than VC counterparts; protocol-2 7G4 and VC cells, on the other hand, were equally sensitive to photoinduced necrosis (35). The well known proapoptotic agents staurosporine and A23187 (a Ca^{2+} ionophore) also provoked COH-BR1 apoptosis, but effects of GPX4 overexpression were different. As observed with protocol-1 ALA/light treatment, 7G4 cells were significantly more resistant to staurosporine than VC cells, consistent with evidence that this agent acts by eliciting a mi-

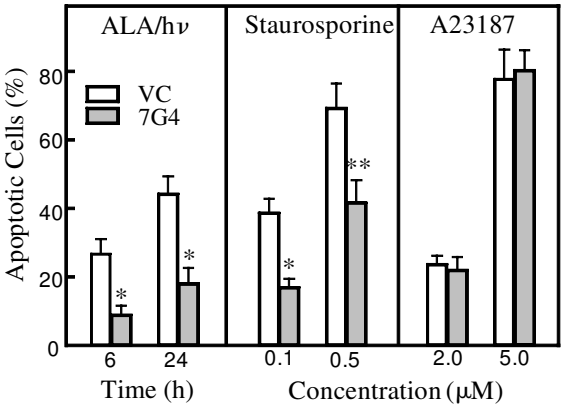

FIG. 4. Relative sensitivity of VC and 7G4 cells to apoptotic death induced by ALA/light, staurosporine, or calcimycin (A23187). *ALA/light*: Cells treated with 1 m*M* ALA via protocol-1 were irradiated for 30 min with broad-band visible light (delivered fluence ~2 J/cm^2) and then checked for apoptosis 6 h and 24 h later. *Staurosporine*: Cells were incubated in the presence of 0.1 μ*M* or 0.5 μ*M* staurosporine for 16 h, and then without it for an additional 8 h, after which apoptosis was assessed. *A23187*: Treatment was similar to that described for staurosporine, except that 2 μ*M* or 5 μ*M* A23187 was used. Apoptotic cells were visualized by fluorescence microscopy, using Hoechst 33258 to stain nuclei. Approximately 100 cells in each of five fields were scored. White bars: VC cells; gray bars: 7G4 cells. Mean values from duplicate experiments are plotted. *$p < 0.001$, compared with concurrent VC; **$p < 0.05$, compared with concurrent VC.

tochondrial peroxidative stress (7). On the other hand, Ca^{2+} does not act oxidatively (43), thus explaining why 7G4 cells were not more resistant to A23187 (Fig. 4). Further examination of photokilling revealed that apoptosis of protocol-1 cells was preceded by cyt *c* accumulation in the cytosol and caspase 3 activation, both of which were much reduced in 7G4 versus VC cells (T. Kriska and A.W. Girotti, unpublished observations). By contrast, there was hardly any cyt *c* release or caspase 3 activation in irradiated protocol-2 cells, and no obvious difference between 7G4 and VC. Of added importance was the observation that 7G4 cells accumulated HPLC-EC(Hg)-detectable LOOHs more slowly than VC during apoptotic photokilling, but at the same rate during necrotic photokilling (35). Representative results for different PLOOH families in irradiated protocol-1 cells are shown in Fig. 5. As can be seen, the corrected level of PCOOH, PEOOH, or SMOOH in 7G4 cells was <20% of that in VC cells, indicating that mitochondrial GPX4 was highly effective in detoxifying endogenous PLOOHs. Importantly, the ClOOH level (determined by an HPTLC method) was also substantially suppressed in 7G4 cells (T. Kriska and A.W. Girotti, unpublished observations), consistent with apoptosis being diminished due to preservation of mitochondrial cyt *c*–Cl interactions. In striking contrast to this, irradiated protocol-2 7G4 cells accumulated all PLOOHs, including ClOOHs, to the same extent as VC (35), indicating that GPX4 was relatively ineffective in this case. The same general trends were observed for ChOOHs. From these findings, which have important bearing on ALA-based PDT (10), one can deduce the following: (i) that the death mechanism of ALA/light-challenged cells is site-specific with respect to subcellular location of PpIX, a shift from apoptosis to necrosis occurring when the porphyrin moves from mitochondria to peripheral sites; (ii) that mitochondrial GPX4 also acts site-specifically, protecting against apoptosis, but not necrosis; and (iii) that GPX4 selectively inhibits mitochondrial LOOH buildup, which is observed long before any signs of apoptosis (see Fig. 5). Our evidence supports the hypothesis that LOOHs produced by $^1O_2$ attack on mitochondrial lipids (most notably Cl) are important upstream mediators of photoinduced apoptosis. That this hypothesis may also apply to other types of LOOH-generating stresses is suggested by numerous recent studies (29, 40, 45, 46, 53).

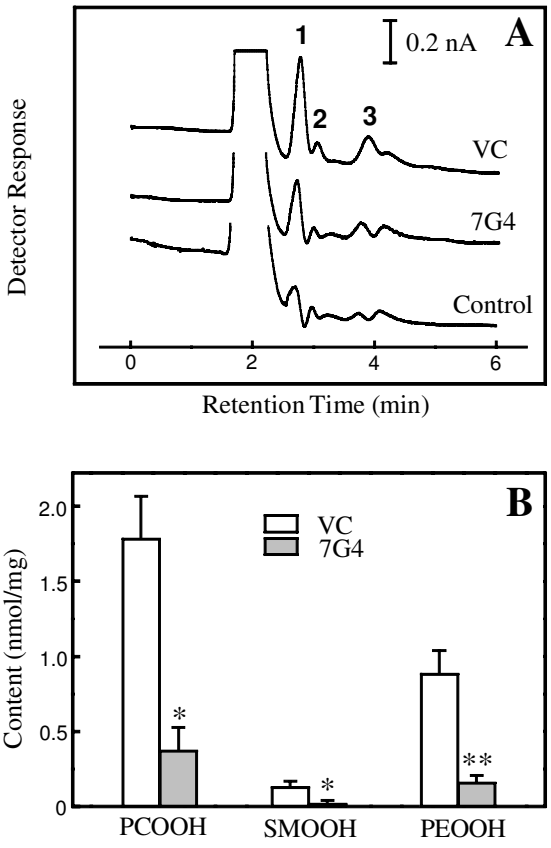

FIG. 5. PLOOH accumulation in protocol-1 ALA/light-treated COH-BR1 tumor cells. Near-confluent 7G4 and VC cells in serum-free medium (~8×10^6 per 10-cm dish) were exposed to 1 m*M* ALA for 90 min (protocol-1), then rinsed, overlaid with ALA-free medium, and irradiated for 30 min (~2 J/cm^2). Immediately thereafter, the cells were rinsed, recovered in ice-cold phosphate-buffered saline containing chelators, BHT, and Triton X-100, and extracted. Recovered lipid fractions were analyzed for PLOOHs by means of HPLC-EC(Hg), using an LC-NH_2 column (see Ref. 35 for additional details). (**A**) Chromatographic profiles for ALA/light-treated VC and 7G4 cells, and for a control (irradiated, non–ALA-treated VC). Each injected sample represented ~3×10^6 cells. Peak assignments are as follows: (1) PCOOH; (2) SMOOH; (3) PEOOH. (**B**) Content of each PLOOH family in VC versus 7G4 cells. Plotted values (nmol/mg of cellular protein, corrected for control levels) are means ± SEM ($n = 3$). *$p < 0.001$; **$p < 0.05$.

SUMMARY AND PERSPECTIVES

As key intermediates in peroxidative reactions, LOOHs are typically much longer-lived than free radical precursors or products. This makes LOOHs potentially more provocative/dangerous to mammalian cells than other reactive lipid oxidation products. Primary LOOHs generated by $^1O_2$ and other ROS attack on unsaturated membrane lipids have several alternative fates that could have negative or positive biological impact, including (i) intermembrane translocation within or between cells, (ii) nonenzymatic one-electron reduction, which can expand cytotoxicity, and (iii) enzymatic two-electron reduction, which suppresses/contains cytotoxicity. In addition, there is growing awareness that LOOHs can function as signaling molecules, eliciting cytoprotective or apoptotic responses in cells, depending on the severity of the oxidative stress, *e.g.,* stress associated with photodynamic action. An important goal for future research is to better characterize these diverse reactions and functions of LOOHs, and in particular their signaling functions, which are still poorly understood.

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ABBREVIATIONS

ALA, 5-aminolevulinic acid; BHT, butylated hydroxytoluene; Cer, ceramide; Ch, cholesterol; ChOOH(s), cholesterol hydroperoxide(s); ChOX, cholesterol oxidation product(s); Cl, cardiolipin; ClOOH(s), cardiolipin hydroperoxide(s); cyt *c*, cytochrome *c*; DAG, diacylglycerol; FAOOH(s), fatty acid hydroperoxide(s); GPX4 (PHGPX), glutathione peroxidase isotype-4; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; $HO^{\cdot}$, hydroxyl radical; HO-1, heme oxygenase-1; HPLC-EC(Hg), high-performance liquid chromatography with mercury cathode electrochemical detection; HPTLC, high-performance thin-layer chromatography; LOOH(s), lipid hydroperoxide(s); O_2^{-}, superoxide; $^1O_2$, singlet oxygen; $^3O_2$, ground-state oxygen; PCOOH(s), PEOOH(s), PSOOH(s), and SMOOH(s), phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin hydroperoxides, respectively; PDT, photodynamic therapy; PKC, protein kinase C; PL(s), phospholipid(s); PLA_2, phospholipase A_2; PLC, phospholipase C; PLOOH(s), phospholipid hydroperoxide(s); PpIX, protoporphyrin IX; ROS, reactive oxygen species; SePX(s), selenoperoxidase(s); SM, sphingomyelin; SMase, sphingomyelinase; SPT, serine palmitoyl transferase; VC, vector control; 5α-OOH, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide; 6α/6β-OOH, 3-hydroxycholest-4-ene-6α/6β-hydroperoxide; 7α/7β-OOH, 3β-hydroxycholest-5-ene-7α/7β-hydroperoxide.

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