Intracellular Dissemination of Peroxidative Stress

INTERNALIZATION, TRANSPORT, AND LETAL TARGETING OF A CHOLESTEROL HYDROPEROXIDE SPECIES BY STEROL CARRIER PROTEIN-2-OVEREXPRESSING HEPATOMA CELLS

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Sterol carrier protein-2 (SCP-2) plays a crucial role in the trafficking and metabolism of cholesterol and other lipids in mammalian cells. Lipid hydroperoxides generated under oxidative stress conditions are relatively long-lived intermediates that damage cell membranes and play an important role in redox signaling. We hypothesized that SCP-2 facilitates translocation of lipid hydroperoxides in oxidatively stressed cells might enhance cytotoxicity if highly sensitive sites are targeted and detoxification capacity is insufficient. We tested this using a clone (SC2A) of rat hepatoma cells that overexpress mature immunodetectable SCP-2. When challenged with liposomal cholesterol-7α-hydroperoxide (7α-OOH), SC2A cells were found to be much more sensitive to viability loss than vector control (VC) counterparts. Correspondingly, SC2A cells imported [14C]-7α-OOH more rapidly. The clones were equally sensitive to tert-butyldihydroperoxide, suggesting that the 7α-OOH effect was SCP-2-specific. Fluorescence intensity of the probes 2′,7′-dichlorofluorescein and C11-BODIPY increased more rapidly in SC2A than VC cells after 7α-OOH exposure, consistent with more rapid internalization and oxidative turnover in the former. 1[14C]7α-OOH radioactivity accumulated much faster in SC2A mitochondria than in VC, whereas other subcellular fractions showed little rate difference. In keeping with this, 7α-OOH-stressed SC2A cells exhibited a faster loss of mitochondrial membrane potential and development of apoptosis. This is the first reported evidence that peroxidative stress damage can be selectively targeted and exacerbated by an intracellular lipid transfer protein.

Non-esterified cholesterol (Ch) and various phospholipids are known to move from one membrane compartment to another in mammalian cells as needed for metabolic processing and membrane biogenesis/homeostasis (1–3). Desorption from the donor membrane is typically the rate-limiting step in a spontaneous lipid translocation process (1). Numerous studies with model systems have shown that natural lipids (particularly those in the phospholipid family) translocate relatively slowly on their own but that this is accelerated by specific lipid transfer proteins (4, 5). A well studied example is intracellular sterol carrier protein-2 (SCP-2), also known as nonspecific lipid transfer protein. SCP-2 not only facilitates the intermembrane translocation of Ch and other sterols but also various fatty acids and fatty acyl-CoAs as (6–8). SCP-2 is a relatively small (13.2 kDa) translation product of a fusion gene encoded for 58-kDa SCP-x (~45 kDa of which represents a peroxisomal 3-ketoacyl-CoA thiolase) and 15-kDa pro-SCP-2 (7–10). Mature SCP-2 appears to arise mainly from post-translational cleavage of pro-SCP-2, although some direct formation by cleavage at the SCP-x level is also possible (7, 8). Examination of subcellular distribution by immunodetection methods has revealed that SCP-2 is at the highest concentration in peroxisomes, although significant levels are also found in mitochondria, lysosomes, and cytosol, presumably reflecting the wide-ranging trafficking activity of this protein (7). In mouse L-cells, for example, ~50% of the total immunoreactivity is extraperoxisomal (11). Numerous studies with model systems have shown that recombinant SCP-2, like the natural protein, can greatly accelerate the transfer of various sterols and phospholipids from donor to acceptor membranes (12–15). Various mechanisms for this have been proposed including one in which SCP-2 binds desorbed lipids in the aqueous compartment and another in which the protein binds resident lipids upon interacting with the donor membrane (7, 15). Support for the latter mechanism derives from evidence that the N-terminal α-helical segment of SCP-2 with several lysyl residues can interact with anionic phospholipids on donor/acceptor membrane surfaces (7, 16).

GPx1 and GPx4, glutathione peroxidase isotype 1 and 4; Ho258, Hoechst 33258; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; JC-1, 5,5′,6′,6′-tetrachloro-1,1′,3′,3′-tetrathyylbenzimidazolylcarbozyme iodide; LOOH, lipid hydroperoxide; MTT, 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide; PBS, Chlex-treated phosphate-buffered saline (25 mM sodium phosphate/125 mM NaCl (pH 7.4)); PI, propidium iodide; ROS, reactive oxygen species; SCP-2, sterol carrier protein-2; SUV, small unilamellar vesicle; t-BuOOH, tert-butyl hydroperoxide; VC, vector control.

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Oxidative stress-induced peroxidation of unsaturated lipids in cell membranes gives rise to a large number of reactive intermediates and end products (17–19). Highly prominent in the former category are lipid hydroperoxide (LOOH) species (19). Under redox-constrained conditions, LOOHs generated by singlet oxygen addition, for example, can accumulate and perturb membrane structure/function directly due to their increased hydrophilicity. However, in the presence of reductants and catalytic iron, LOOHs undergo one-electron reduction to oxy radicals, which either directly or after conversion to epoxycyclic peroxyl radicals (20) can exacerbate damage by triggering chain peroxidation reactions (17–19). Opposing this is two-electron detoxification catalyzed by certain selenium- and non-selenium-dependent peroxidases (21, 22). Our recent studies have indicated that these reactions are not necessarily limited to a LOOH membrane of origin but can extend to other membranes via LOOH translocation through the aqueous phase (23–25). Using model membrane systems, we have shown that Ch- and phospholipid-derived hydroperoxide species translocate more rapidly than the respective parent lipids and that this is further accelerated by SCP-2 (25). Cytotoxic relevance was demonstrated by showing that SCP-2 accelerates transfer of 7α-OOH (a free-radical-derived ChOOH) from liposomes to isolated mitochondria, which enhances peroxide-induced loss of mitochondrial membrane potential (25).

In this study we have examined the effects of SCP-2 overexpression in rat hepatoma cells on uptake, distribution, and cytotoxicity of exogenous 7α-OOH delivered in liposomal form. Our findings are novel in that they are the first to implicate an intracellular lipid trafficking protein in the dissemination of lethal damage under peroxidative stress conditions.

EXPERIMENTAL PROCEDURES

Materials—Ch, cholesteryl linoleate, Ho258, JC-1, MTT, PI, Dulbecco’s modified Eagle’s medium, phenol red-free RPMI medium, fetal bovine serum, and other cell culture materials were from Sigma. Molecular Probes (Eugene, OR) supplied the following: C11-BODIPY581/591, DCFH-DA, and MitoTracker Deep Red 633. 4-[14C]Ch (50 mCi/ml) was prepared by dye-sensitized photoperoxidation of 7α-OOH (2.9 mm M, respectively). After various periods of incubation at 37 °C of up to 30 min, cells in each dish were washed extensively with cold PBS, recovered by scraping, centrifuged, and resuspended in 0.5 ml of PBS containing 0.1 mM EDTA. Samples were checked for protein content (30) and then extracted with 0.8 ml of cold chloroform/methanol (2:1) using sonication. Membranes were then hypotonic buffer, and lysed by sonication. Lysates were centrifuged at 100,000 g for 1 h at 4 °C. Recovered supernatant fractions were examined for protein content (30) and then subjected to Western analysis using a 15% polyacrylamide gel for electrophoresis and 0.45-µm polyvinylidene difluoride membrane for transblotting. Blots were blocked, treated with rabbit anti-mouse SCP-2 (10) and anti-actin antibodies and then with peroxidase-conjugated anti-rabbit IgG, and analyzed using enhanced chemiluminescence (31).

Cell Culture—An SCP-2-overexpressing transfected clone (SC2A) of rat McA-RH777 hepatoma cells (28) along with a vector control clone (VC) were grown in Dulbecco’s modified Eagle’s medium containing 10% serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml), and Geneticin (G418, 0.35 mg/ml) using standard culture conditions (29). The SC2A cells had been transfected with a construct encoded for 15-kDa pro-SCP-2, which is post-translationally converted to 13.2 kDa SCP-2 (7, 28). Cells were taken off Geneticin 5 days before an experiment, reseeded 3 days later, and grown back to 50–60% confluence before beginning experimental manipulations.

Immunoblot Analysis—The level of SCP-2 protein expressed in SC2A and VC cells was determined by immunoblotting. Cells were recovered by trypsinization, washed with PBS and then hypotonic buffer, and lysed by sonication. Lysates were centrifuged at 100,000 × g for 1 h at 4 °C. Recovered supernatant fractions were examined for protein content (30) and then subjected to Western analysis using a 15% polyacrylamide gel for electrophoresis and 0.45-µm polyvinylidene difluoride membrane for transblotting. Blots were blocked, treated with rabbit anti-mouse SCP-2 (10) and anti-actin antibodies and then with peroxidase-conjugated anti-rabbit IgG, and analyzed using enhanced chemiluminescence (31).

Cellular Enzymatic Activities and Glutathione Levels—GPx4 activity was determined by measuring the GSH-dependent decay kinetics of 7α-OOH using HPLC with electrochemical detection (26, 29). GPx1 was determined by coupled enzymatic assay using t-BuOOH as the peroxide substrate (29). Total glutathione (GSH + 2GSSG) was quantified by Tietze assay (32).

Determination of [14C]7α-OOH and [14C]Ch Uptake by Cells and Subcellular Distribution of Radioactivity—Two days before an experiment SC2A and VC cells were seeded into 10-cm dishes (5 × 105 cells each), and 12 h before the medium was changed to RPMI containing 5% lipoprotein-deficient serum to minimize Ch supply. This reduced Ch content of both cell types by ~30%, as determined by HPLC analysis (23). Small unilamellar liposomes (50 nm SUVs) consisting of 5.5 mM 1,2-dimyristoyl-sn-glycero-3-phosphocholine, 2.5 mM [14C]7α-OOH (2.9 µCi/ml), 2.0 mM Ch, and 0.06 mM dicetyl phosphate in bulk phase PBS (pH 7.4) were prepared by an extrusion process as described (23). Identical composition SUVs containing [14C]Ch (2.5 µCi/ml) and unlabeled 7α-OOH were prepared alongside. Cells were first washed free of lipoprotein-deficient serum, then overlaid with [14C]7α-OOH- or [14C]Ch-containing SUVs in RPMI medium, with starting sterol concentrations being 25 and 20 µM, respectively. After various periods of incubation at 37 °C of up to 30 min, cells in each dish were washed extensively with cold PBS, recovered by scraping, centrifuged, and resuspended in 0.5 ml of PBS containing 0.1 mM EDTA. Samples were checked for protein content (30) and then extracted with 0.8 ml of cold chloroform/methanol (2:1, v/v) (27). Lipid-containing fractions were dried under N2, dissolved in hexane/isopropanol (97:3, v/v), applied to a TLC plate section in a hairline N2 stream, and subjected directly to phosphorimagining using a Storm 860 storage phosphor system (33).

[14C]Ch and [14C]7α-OOH standards of known specific radioactivity were used for calibration.

For determining subcellular distribution of radioactivity from incoming [14C]Ch and [14C]7α-OOH, cells were incubated with [14C]7α-OOH-containing SUVs for 3 h, then washed with PBS, resuspended in ice-cold hypotonic buffer (34) for 5 min, and Dounce-homogenized. Immediately thereafter, osmolarity was restored (34), and the lysate was layered onto a buffered 10-step
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SCP-2 Protein Expression in SC2A Versus VC Cells—The SCP-2 content of SC2A cells was found to be substantially higher than that of VC, consistent with previous findings (28). Actin-normalized band integration for the immunoblot shown in Fig. 1 indicated an ~10-fold elevation of SCP-2 in the overexpressing cells. No significant difference in the levels of other immunodetectable proteins, including SCP-x and an undefined ~32-kDa protein, was apparent, which also agrees with earlier results (28). Little if any pro-SCP-2 could be detected, indicat-
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FIGURE 1. Immunoblot showing SCP-2 levels in transfectant SC2A and VC cells. Solubilized cell samples (each containing 60 ng of total cellular protein) were loaded onto a 4–15% SDS-PAGE gel, electrophoresed, transferred to a polyvinylidene difluoride membrane, and probed with anti-SCP2 and anti-actin antibodies. A 10-ng sample of human recombinant SCP-2 was used as a standard (Std).

FIGURE 2. Comparative sensitivity of SC2A and VC cells to peroxide-induced killing. SC2A (∆) and VC (○) cells were incubated with SUV 7α-OH in increasing concentrations for 3 h (A) or at a fixed concentration (25 μM) for increasing times (B), then washed and checked for viability by MTT assay 20 h later. Either clone (∆) was also exposed to SUV 7α-OH in increasing concentrations for 3 h and checked 20 h later (A). The same time schedule was used for testing toxicity of t-BuOOH on SC2A (∆) and VC (○) cells (C). Data points in each panel are the means ± S.E. (n = 6). was tracked, the rate in this case being ~35% greater for SC2A cells (Table 1). In agreement with previous findings based on use of cholesterol oxidase for analysis (28), the [14C]Ch results probably reflect more rapid internalization by the SCP-2-overexpressing cells rather than mere surface association. A similar deduction is made for [14C]7α-OH uptake (Table 1), and our data relating to the appearance of radioactivity from this hydroperoxide in subcellular compartments supports this (see below).

Comparative Kinetics of Sterol Uptake—Using [14C]7α-OH-containing SUVs as donors and cells that had been deprived of Ch by growing in lipoprotein-deficient medium, we monitored uptake of radioactivity as a function of incubation time. As shown in Table 1, specific radioactivity of cellular lipid extracts increased progressively and nearly linearly over a 30-min period, the rate for SC2A cells being ~25% greater than that for VC. The same trend was observed when [14C]Ch uptake...

Glutathione Peroxidase Activities and Glutathione Contents—Cellular levels of selected antioxidants were determined, viz. glutathione and the GSH-dependent selenoperoxidases GPx1 and GPx4. Measured values (means ± S.D., n = 3) are as follows: GPx4 activity (units/mg of protein), 0.78 ± 0.12 (SC2A), 0.86 ± 0.09 (VC); GPx1 activity (units/mg of protein), 8.7 ± 1.1 (SC2A), 8.3 ± 0.6 (VC); GSH + GSSG (nmol/mg of protein), 29.2 ± 2.0 (SC2A), 26.3 ± 3.3 (VC). Thus, there was no significant difference between SC2A and VC cells with respect to these particular antioxidant levels, ruling this out as a possible contributing factor in any observed difference to a peroxidative challenge (see below).

Cytotoxic Effects of 7α-OH and t-BuOOH—When challenged with liposomal 7α-OH, SC2A cells were found to be substantially more sensitive to MTT-assessed killing than VC. This was observed in both an increasing [7α-OH]/fixed time (Fig. 2A) and fixed [7α-OH]/increasing time (Fig. 2B) format. The LC50 values for SC2A and VC cells were ~19 and ~75 μM, respectively (Fig. 2A). Control SUVs in which 7α-OH was replaced by reoxid-inactive 7α-OH exhibited no significant cytotoxicity over the concentration range used (Fig. 2A). This indicates that the observed lethality was not merely a generalized oxysterol effect but required the OOH group specifically. In contrast to the 7α-OH response, SC2A and VC cells were found to be equally sensitive to t-BuOOH (Fig. 2C), with the LC50 (~165 μM) significantly higher than those for the sterol hydroperoxide. Not seeing a toxicity difference with t-BuOOH (a non-lipid hydroperoxide) suggests that the effects observed with 7α-OH were SCP-2-specific.

Subcellular Distribution of [14C]7α-OH Radioactivity—In a separate experiment, cells prepared as described in Table 1 were exposed to liposomal [14C]7α-OH for 3 h and then homogenized. Density gradient-separated fractions containing (i) cytosol, (ii) microsomes, (iii) mitochondria (with some microsomes), and (iv) mitochondria (with some microsomes...
**TABLE 1**

Time-dependent sterol uptake by SC2A and VC transfectants

| Sterol        | Incubation time | Uptake pCi/mg of protein |
|---------------|-----------------|--------------------------|
| [14C]Ch       | 15 min          | 44.3 ± 5.3               |
|               | 30 min          | 77.1 ± 4.2               |
| [14C]7α-OOH   | 15 min          | 19.8 ± 3.7               |
|               | 30 min          | 37.1 ± 4.3               |

*Significantly different from 30 min VC value, p < 0.05.

![Graph showing sterol uptake by SC2A and VC cells](image)

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**FIGURE 3.** Subcellular distribution of [14C]7α-OOH radioactivity in SC2A versus VC cells. Cells preincubated in lipoprotein-deficient serum-containing medium were exposed to SUV [14C]7α-OOH (25 μM; 30 nCi/ml) for 3 h. After washing and homogenization, subcellular fractions were separated by sucrose density gradient centrifugation. Protein-based specific radioactivity of the lipid extract from each fraction was determined. Plotted values (corrected for cross-contamination where necessary) are the means ± S.E. (n = 4).

**FIGURE 4.** Accumulation of [14C]7α-OOH turnover products as a function of incubation time with SC2A versus VC cells. Lipids extracted from cells incubated with [14C]7α-OOH-containing SUVs were analyzed by HPTLC with phosphorimaging detection. Each sample lane on the chromatogram represents ~1.3 × 10^6 cells. Incubation times for VC cells (lanes 1–3) and SC2A cells (lanes 4–6) were 1.5 h (lanes 1 and 4), 3.0 h (lanes 2 and 5), and 4.5 h (lanes 3 and 6). A mixture of non-radiolabeled 7α-OOH, 7α-OH, 7-one, 7α-OOH, and CE standards (Std) was run alongside and detected by spraying with 9N H2SO4 with warming. O, origin; F, solvent front. Time courses for accumulation of radiolabeled cellular 7α-OOH, 7α-OH, 7-one, and CE (standardized to cell protein for each sample) are shown in the plots: △, SC2A; ○, VC. Values are the means ± S.E. (n = 3) (at 4.5 h, 7α-OOH represents 14.6 and 4.5% of the total radioactivity in VC and SC2A, respectively).

and nuclei), based on immunodetection of marker enzymes, were analyzed for protein content and extracted. Phosphorimaging of recovered lipid material with correction for cross-contamination in fractions iii and iv showed no significant difference between SC2A and VC cells in the level of specific radioactivity associated with the nuclear, microsomal, and cytosolic fractions (Fig. 3). However, there was a striking difference in specific radioactivity of the mitochondrial fractions, the SC2A being ~2.5 times greater than the VC (Fig. 3). Therefore, SCP-2 overexpression resulted not only in more rapid internalization of 7α-OOH but also selectively greater localization of the hydroperoxide and/or metabolites thereof in the mitochondrial compartment.

**Turnover and Fate of Internalized 7α-OOH**—Like all other LOOHs, 7α-OOH would be expected to turn over in various ways upon entering cells, undergoing e.g. iron-catalyzed one-electron reduction, enzyme-catalyzed two-electron reduction (detoxification), or transesterification to give a peroxyester ester (19, 41). To assess this in a comparative manner for [14C]7α-OOH-treated SC2A and VC cells, we analyzed lipid extracts at various time points by means of HPTLC with phosphorimaging detection (33). As shown by the chromatogram in Fig. 4, four prominent radiolabeled analytes were identified based on co-migration with authentic standards, 7α-OH, 7-one, 7α-OOH, and CE, in order of increasing relative mobility (the CE standard was cholesteryl linolate; however, cellular CE could be any number of unresolved acyl-CoA:cholesterol acyltransferase-generated esters of 7α-OOH or species derived from it). Intensity of all analyte bands increased with time of cell incubation with [14C]7α-OOH. However, there was a striking difference in the rate of intensification between SC2A and VC cells, with 7α-OOH, 7-one, and CE increasing more rapidly in the latter but 7α-OH more slowly (Fig. 4). This is seen graphically in the time course plots for integrated 7α-OOH, 7α-OH, 7-one, and CE radioactivity (Fig. 4). The slower 7α-OOH and faster 7α-OH accumulation in SC2A cells is attributed to relatively rapid SCP-2-facilitated transfer of the hydroperoxide to redox-active subcellular sites. One-electron (free radical) reductive turnover at such sites would give rise to 7α-OH and other redox-inactive products (33, 41, 42). 7α-OH could also arise from two-electron (non-radical) reduction catalyzed by cytoprotective GPx4 (19, 41). No significant 7β-OH was
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Table 1

| Time (h) | VC | SC2A |
|----------|----|------|
| 0.0      | 2.5±0.2 | 2.9±0.4 |
| 1.5      | 3.6±0.4 | 6.6±0.8 |
| 3.0      | 5.2±0.5 | 56.2±7.4 |

FIGURE 5. Reactive oxidant generation in 7α-OOH-treated cells. SC2A and VC cells were washed after the indicated times of exposure to 25 μM SUV 7α-OOH, then treated with 10 μM DCFH-DA, washed again, and analyzed by fluorescence microscopy using 488-nm excitation and 510-nm emission. Images of representative cell populations are shown. Integrated fluorescence intensity is indicated below each panel. Values are the means ± S.E. (n = 4).

FIGURE 6. Oxidation of endogenous lipids in 7α-OOH-treated cells. Cells were incubated with 25 μM SUV 7α-OOH for the indicated periods, washed, treated with 5 μM C11-BODIPY, washed again, and examined by fluorescence microscopy using 488-nm excitation and appropriate cutoff filters for detecting non-oxidized (red) and oxidized (green) probe. A, representative viewing fields. B, integrated fluorescence intensities plotted as percent oxidized C11-BODIPY versus contact time with 7α-OOH; means ± S.E. (n = 4). *, significantly greater than corresponding VC value, p < 0.001. C, confocal microscopy of SC2A cells stained with 2 μM C11-BODIPY and 0.5 μM MitoTracker Deep Red after 3 h of contact with 25 μM 7α-OOH. Images represent a single focal layer. The merged frame shows dye co-localization. Bar, 10 μm.

More direct evidence that 7α-OOH cytotoxicity is mediated by free radical lipid peroxidation was sought by using the fluorophore C11-BODIPY. This probe enters cells, intercalates with membrane lipids, and acts as an in situ “sensor” of peroxidative damage by changing from red emission in its non-oxidized form to green emission in its oxidized form (40). As shown by the fluorescence micrographs in Fig. 6A, C11-BODIPY-detected lipid peroxidation progressed more rapidly in SC2A cells than VC during 7α-OOH exposure, as indicated by faster disappearance of red fluorescence in the former with reciprocal appearance of green fluorescence. Thus, SC2A cells were more susceptible to this specific type of 7α-OOH-primed oxidative damage. In quantitative terms, the amount of probe in its oxidized form averaged ~3 times higher in the SCP-2-overexpressing cells after 3 h (Fig. 6B). Closer scrutiny revealed that the strongest signal from oxidized probe was concentrated in perinuclear zones (see SC2A at 3 h in Fig. 6A). To learn whether these might represent mitochondria, we examined the fluores-
FIGURE 7. Effect of 7α-OOH exposure on mitochondrial membrane potential. SC2A (△) and VC (○) cells were washed after the indicated times of incubation with 25 μM SUV 7α-OOH, then treated with 2 μM JC-1, washed again, and examined by fluorescence plate reader using 488-nm excitation and 590- or 530-nm emission. Time-dependent changes in RFI (fluorescence intensity at 590 nm relative to that at 530 nm) are plotted. Values are the means ± S.E. (n = 4).

FIGURE 8. Relative cell susceptibility to 7α-OOH-induced apoptosis. Immediately after incubating with 25 μM liposomal 7α-OOH for the indicated times, SC2A and VC cells were treated with 5 μM H(2)O(2)/50 μM PI and examined for apoptotic (bright blue) versus necrotic (red) nuclei by fluorescence microscopy. Images of representative cell populations after various peroxide exposure times are shown. Percentages of apoptotic (A) and necrotic (N) cells are indicated below each panel. Values are the means ± S.E. (n = 8).

DISCUSSION

Free radical-mediated oxidation of unsaturated lipids in cell membranes and lipoproteins gives rise to numerous oxidized intermediates and products (18, 19). In the case of Ch oxidation, these are collectively referred to as Ch oxides. Among the latter, the epimeric hydroperoxides 7α-OOH and 7β-OOH are prominent intermediates whose lifetimes are significantly greater than those of free radical precursors or products (19, 41). We predicted earlier (23, 45) that this attribute along with greater hydrophilicity compared with parent lipids would allow 7-OOHs to depart membranes of origin relatively rapidly and translocate to other membranes. Similar to parental lipid translocation (1–3), this could be fostered by favorable intermembrane concentration gradients or by large acceptor compartments serving as sinks. Initial supporting evidence came from model membrane studies (23, 24). These showed that a group of photochemically generated ChOOHs translocated spontaneously with a rate constant at least 60 times greater than that of parent Ch, the rate-limiting step being desorption from the donor bilayer. As in the case of Ch itself (1), spontaneous ChOOH transfer was found to occur mainly via aqueous diffusion rather than membrane collision (23). We subsequently showed that three individual ChOOH isomers, including 7α-OOH, translocate at different rates and that these increase proportionately with degree of hydrophilicity (24). Experiments with a GPx4-deficient cell line (24) revealed that the time-dependent degree of cell killing by SUV-borne 7α-OOH and two other ChOOHs increased in parallel with their rates of spontaneous transfer uptake, thus demonstrating that transfer-limited cytotoxicity is possible. More recent in vitro studies (25)
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showed that intermembrane ChOOH transfer could be further accelerated by a recombinant SCP-2, raising the possibility that this protein plays a role not only in the intracellular trafficking of Ch but also potentially damaging ChOOH species. Similarly, SCP-2 further enhanced the intermembrane shuttling of several phospholipid hydroperoxide families (25), suggesting broad range LOOH applicability. We postulated on the basis of these findings that transfer protein-facilitated dissemination of stress-generated LOOHs would greatly expand their range of prooxidant cytotoxic and signaling activity (23–25, 45). In initial testing of direct relevance to the present work, we showed that transfer of SUV-borne 7α-OOH to isolated mitochondria with the resultant loss of membrane potential was significantly enhanced by SCP-2 (25).

This study is a natural extension of our work with isolated SCP-2 (25) and provides the first known evidence that LOOH cytotoxicity can be enhanced by an endogenous lipid transfer protein. SCP-2-enriched SC2A cells exhibited no obvious difference in morphology or growth rate compared with VC counterparts nor were their levels of protective antioxidants such as GSH, GPx1, and GPx4 any different. It was important to check at least some key antioxidants to be certain that interpretation of our cytotoxicity results would not be complicated by any down-regulation of these. We found that SC2A cells internalized 7α-OOH more rapidly than VC and were substantially more sensitive to 7α-OOH-induced killing. On the other hand, SC2A and VC cells were equally sensitive to the non-lipid hydroperoxide t-BuOOH (Fig. 2), suggesting that enhanced cytotoxicity of 7α-OOH depended on its ability to interact with and be transported by SCP-2 similarly to parent Ch (5–7). It is reasonable to assume that delivery of internalized 7α-OOH to vital redox-active membrane sites played a key role in the underlying mechanism of SC2A hypersensitivity. At such sites the hydroperoxide could undergo iron-catalyzed one-electron reduction with generation of oxyl and/or epoxyallylic peroxyl radicals (19, 20), which trigger damaging chain peroxidation. In support of this, we showed that 7α-OOH-challenged SC2A cells (i) accumulated DCFH-detectable ROS more rapidly than VC, (ii) oxidized the lipid-like reporter C11-BODIPY faster, and (iii) effected a faster turnover of internalized [14C]7α-OOH, as monitored by HPTLC with phosphorimaging detection. The latter was manifested by accumulation rates of 7α-OOH and its reduction product 7α-OH, which were substantially lower and higher, respectively, than those for VC (Fig. 4). 7α-OH derives not only from one-electron but also two-electron reduction of 7α-OOH (19, 41); therefore, some of it observed in Fig. 4 may have arisen from detoxification rather than toxicity-expanding free radical reactions. How these reactions were proportioned in our system is not known. However, identification of 7-one provides unambiguous evidence for free radical activity, and the higher level of 7-one relative to 7α-OOH observed in stressed SC2A versus VC cells (Fig. 4) is consistent with more vigorous radical activity in the former. It should be stressed that the Fig. 4 data represent only the turnover fate of internalized [14C]7α-OOH, the “priming” hydroperoxide. One-electron reduction of this ChOOH in an acceptor compartment with ensuing chain peroxidation would give rise to various endogenous LOOHs, including phospholipid-derived species and Ch-derived 7α-OH. These downstream LOOHs might also be translocated by SCP-2, thereby increasing the possibility of peroxidative membrane damage at key subcellular sites. It is noteworthy in this regard that t-BuOOH, which would be expected to give rise to cellular LOOHs via its own one-electron decomposition (46), was not hypertoxic toward SC2A cells. One possible explanation is that t-BuOOH-induced lipid peroxidation was slow compared with its diffusion to vital subcellular sites.

We postulate that binding to SCP-2 sheltered 7α-OOH from redox degradation, presumably because catalytic iron or detoxifying enzyme access to the peroxide was restricted. Although this remains to be investigated, a recent report indicated that C11-BODIPY is protected against oxidative attack by Cu2+/H2O2-generated hydroxyl radical when bound to SCP-2 (47). Similar redox protection of bound 7α-OOH or other LOOHs would prolong their lifetimes in transit and also minimize free radical damage to the protein.

The data in Fig. 4 represent a composite of overall internalized [14C]7α-OOH turnover. We have not yet examined its turnover profile in separated subcellular compartments, e.g. nuclear, cytosolic, and mitochondrial fractions. However, a key observation with clear implications for this is that SC2A mitochondria incorporated [14C]7α-OOH radioactivity much more rapidly than VC (Fig. 3). The uptake rate in other compartments was the same, suggesting that mitochondria were special targets for SCP-2-mediated hydroperoxide transfer. This is consistent with previous evidence (i) that SCP-2 can enhance 7α-OOH transfer to isolated mitochondria with damaging consequences and (ii) that cellular mitochondria can harbor significant amounts of SCP-2, presumably for internal lipid trafficking (7, 48). Because mitochondria are iron-rich and highly redox-active, more rapid peroxide delivery to them would explain their greater extent of lipid peroxidation damage and loss of membrane potential in SC2A cells (Figs. 6 and 7). Significant mitochondrial targeting in these cells may account for fact that they showed early signs of dying apoptotically, supposedly via the intrinsic mitochondrial pathway (49).

Immunohistochemical studies have revealed that steady state levels of SCP-2 vary in different tissues, liver, and adrenal cortex, for example, exhibiting much higher amounts than lung and kidney (7, 50, 51). In adrenocortical cells the protein has been reported to play a crucial role in delivering Ch from endoplasmic reticulum and lipid droplet depots to mitochondria for steroid hormone synthesis (48, 52). If oxidatively stressed, such cells might be susceptible to mitochondrial injury due to SCP-2-mediated transfer of ChOOHs along with Ch to mitochondria. Similar reasoning could apply to other cell types expressing relatively high constitutive levels of SCP-2.

In view of the present findings, we have focused up to this point on the prooxidant cytotoxic potential of SCP-2-dependent LOOH translocation. However, one can imagine situations in which LOOH transfer by constitutive SCP-2 could have the opposite effect, i.e. act cytoprotectively by easing peroxidative stress. This might occur, for example, if LOOHs are translocated to compartments whose content of redox-active metal ions is low, but content of antioxidants such as GSH and GPx4 is relatively high, leading to more efficient LOOH detoxifica-
tion than toxicity enhancement (19). One can predict that this situation would require relatively modest peroxidative pressure and antioxidant-rich compartments that already contain SCP-2 or are readily accessed by it. The existence of SCP-2-facilitated LOOH detoxification and its importance relative to toxicity enhancement under various stress conditions awaits critical examination.

In summary, we have shown for the first time that up-regulation of the low specificity lipid transfer protein SCP-2 puts affected cells at greater risk of lethal injury from LOOHs that can interact with and be translocated by this protein. Mitochondria were found to be special targets of priming hydroperoxide (7α-OOH) delivery and damaging turnover in SCP-2-overexpressing cells. These findings provide further support for the idea (25, 45) that under stress conditions, which overwhelm detoxification capacity, the cytotoxic and signaling ranges of LOOHs can be greatly expanded by SCP-2-mediated transfer. Whether similar effects can be seen with other intracellular lipid transfer proteins, e.g. those with greater specificity for phospholipid ligands (4, 5), remains to be explored.

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REFERENCES
1. Phillips, M. C., Johnson, W. J., and Rothblat, G. H. (1987) Biochim. Biophys. Acta 906, 223–276
2. Dawidowicz, E. A. (1987) Curr. Top. Membr. Transp. 29, 175–202
3. Brown, R. E. (1992) Biochim. Biophys. Acta 1113, 375–389
4. Zilversmit, D. B. (1984) J. Lipid Res. 25, 1563–1569
5. Wirtz, K. W. A. (1991) Annu. Rev. Biochem. 60, 73–99
6. Noland, B. J., Arebalo, R. E., Hansbury, E., and Scallen, T. J. (1980) J. Lipid Res. 21, 274–289
7. Gallegos, A. M., Atshaves, B. P., Storey, S. M., Starodub, O., Petrescu, A. D., Huang, H., McIntosh, A. L., Martin, G. G., Chao, H., Kier, A. B., and Schroeder, F. (2000) Prog. Lipid Res. 40, 498–563
8. Stolowich, N. I., Petrescu, A. D., Huang, H., Martin, G. G., Scott, A. I., and Schroeder, F. (2002) Cell. Mol. Life Sci. 59, 193–212
9. Ohba, T., Holt, J. A., Billheimer, J. T., and Strauss, J. F. I. (1995) Biochemistry 34, 10660–10668
10. Atshaves, B. P., Petrescu, A., Starodub, O., Roths, J., Kier, A. B., and Schroeder, F. (1999) J. Lipid Res. 40, 610–622
11. Van Amerongen, A., Demel, R. A., Westerman, J., and Wirtz, K. W. A. (1989) Biochim. Biophys. Acta 1004, 36–43
12. Nichols, J. W. (1987) J. Biol. Chem. 262, 14172–14177
13. Billheimer, J. T., and Gaylor, J. L. (1990) Biochim. Biophys. Acta 1046, 136–143
14. Woodford, J. K., Colles, S. M., Myers-Payne, S. C., Billheimer, J. T., and Schroeder, F. (1995) Chem. Phys. Lipids 76, 73–84
15. Huang, H., Ball, J. M., Billheimer, J. T., and Schroeder, F. (1999) Biochemistry 38, 13231–13243
16. Huang, H., Gallegos, A. M., Zhou, M., Ball, J. M., and Schroeder, F. (2002) Biochemistry 41, 12149–12162
17. Halliwell, B., and Gutteridge, J. M. C. (1990) Methods Enzymol. 186, 1–85
18. Porter, N. A., Caldwell, S. E., and Mills, K. A. (1995) Lipids 30, 277–290
19. Girotti, A. W. (1998) J. Lipid Res. 39, 1529–1542
20. Wilcox, A. L., and Marnett, L. J. (1993) Chem. Res. Toxicol. 6, 413–416
21. Brigelius-Flohe, R. (1999) Free Radic. Biol. Med. 27, 951–965
22. Flohe, L., Budde, H., and Hofmann, B. (2003) Biofactors 19, 3–10
23. Vila, A., Korytowski, W., and Girotti, A. W. (2000) Arch. Biochem. Biophys. 380, 208–218
24. Vila, A., Korytowski, W., and Girotti, A. W. (2001) Biochemistry 40, 14715–14726
25. Vila, A., Levchenko, V. V., Korytowski, W., and Girotti, A. W. (2004) Biochemistry 43, 12592–12605
26. Girotti, A. W., and Korytowski, W. (2004) Methods Enzymol. 390, 23–33
27. Girotti, A. W., and Korytowski, W. (2000) Methods Enzymol. 319, 85–100
28. Nishimura, Y., and Lemasters, J. J. (2001) Free Radic. Biol. Med. 31, 1051–1065
29. Bradfod, M. M. (1976) Anal. Biochem. 72, 248–254
30. Kriska, T., Korytowski, W., and Girotti, A. W. (2005) Arch. Biochem. Biophys. 433, 435–446
31. Tietze, F. (1969) Anal. Biochem. 27, 502–522
32. Korytowski, W., Wrona, M., and Girotti, A. W. (1999) Anal. Biochem. 270, 123–132
33. Srinivas, K. S., Chandrasekar, G., Srivastava, R., and Puvanakrishnan, R. (2004) J. Biochem. Biophys. Methods 60, 23–27
34. Vila, A., Levchenko, V. V., and Korytowski, W. (2004) Free Radic. Biol. Med. 37, 1389–1402
35. Reers, M., Smiley, S. T., Motolla-Harthorn, C., Chen, A., Lin, M., and Chen, L. B. (1995) Methods Enzymol. 260, 406–417
36. LeBel, C. P., Ischiropoulos, H., and Bondy, S. C. (1992) Chem. Res. Toxicol. 5, 227–231
37. Pap, E. H. W., Drummen, C. P. C., Winter, V. J., Kooij, T. W. A., Rijken, P., Wirtz, K. W. A., Op den Kamp, I. A. F., Hage, W. J., and Post, J. A. (1999) FEBS Lett. 453, 78–82
38. Drummen, G. P. C., Van Liebergen, L. C. M., Op den Kamp, I. A. F., and Post, J. A. (2002) Free Radic. Biol. Med. 33, 437–490
39. Girotti, A. W. (2002) in Sterols and Oxysterols: Chemistry, Biology, and Pathobiology (Fliesler, S.J., ed) pp. 121–139, Research Signpost, Trivandrum
40. Smith, L. L., Kulig, M. J., Miller, D., and Ansari, F. A. S. (1978) J. Am. Chem. Soc. 100, 6202–6211
41. Teng, J. L., Kulig, M. J., and Smith, L. L. (1973) J. Chromatogr. 75, 108–113
42. Nishimura, Y., and Lemasters, J. J. (2001) Cell Death Differ. 8, 850–858
43. Girotti, A. W., and Kriska, T. (2004) Antioxid. Redox Signal. 6, 301–310
44. Masaki, N., Kiy, M. E., and Farber, J. L. (1989) Arch. Biochem. Biophys. 269, 390–399
45. Danse, T. B., Kops, G. J. P. L., Denis, S., Jelluma, N., Wanders, R. J. A., Bos, J. L., Burgering, B. M. T., and Wirtz, K. W. A. (2004) J. Lipid Res. 45, 81–88
46. Gallegos, A. M., Schoer, J. K., Starodub, O., Kier, A. B., Billheimer, J. T., and Schroeder, F. (2000) Chem. Phys. Lipids 105, 9–29
47. Newmeyer, D. D., and Ferguson-Miller, S. (2003) Cell 112, 481–490
48. Baum, C. L., Kansal, S., and Davidson, N. O. (1993) J. Lipid Res. 34, 729–739
49. Ossendorp, B. C., Voorhout, W. F., van Amerongen, A., Brunink, F., Batenburg, J. J., and Wirtz, K. W. A. (1996) Arch. Biochem. Biophys. 334, 251–260
50. Stocco, D. M. (2000) Biochim. Biophys. Acta 1486, 184–197