Roles of multiple oxidized LDL lipids in cellular injury: dominance of 7β-hydroperoxycholesterol

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Abstract The relative toxicities of several lipid oxidation products formed on oxidized LDL, their presence on oxidized LDL, and potential mechanisms of cell injury compared to oxidized LDL were examined. Toxicities to fibroblasts, with lipoprotein-deficient serum supplementation, were: 7β-hydroperoxycholesterol > 7β-hydroxycholesterol ≈ 4-hydroxynonenal > 7-ketocholesterol > 5α,6α-epoxycholesterol. Lysophosphatidylcholine was only significantly cytotoxic in the absence of lipoprotein-deficient serum. Without serum, relative toxicities were: 7β-hydroperoxycholesterol > lysophosphatidylcholine > 4-hydroxynonenal > 7β-hydroxycholesterol. Similar relative potencies were observed in smooth muscle and endothelial cell cultures. 7β-Hydroperoxycholesterol accumulated on oxidized LDL to greater amounts than other oxysterols and 4-hydroxynonenal, but less than lysophosphatidylcholine. Cell injury by 7β-hydroperoxycholesterol and oxidized LDL was inhibitable by antioxidants but not by exogenous cholesterol or cycloheximide. In contrast, *a)* toxicities by 7β-hydroxycholesterol, 7-ketocholesterol, 5α,6α-epoxycholesterol, and 4-hydroxynonenal were not inhibited by antioxidants; *b)* 7β-hydroxycholesterol and lysophosphatidylcholine toxicities were inhibited by exogenous cholesterol; and *c)* 7β-hydroxycholesterol toxicity was inhibited by cycloheximide. Injury by lysophosphatidylcholine was reduced by vitamin E and not affected by altering the cellular exposure to selenium; reduced selenium enhanced toxicity by oxidized LDL and 7β-hydroperoxycholesterol. The high relative toxicity of 7β-hydroperoxycholesterol, the level of its accumulation on oxidized LDL, and its mechanism of action similar to oxidized LDL suggest that it is the compound predominantly responsible for oxidized LDL induced cytotoxicity.—**Colles, S. M., K. C. Irwin, and G. M. Chisolm.** Roles of multiple oxidized LDL lipids in cellular injury: dominance of 7β-hydroperoxycholesterol. *J. Lipid Res.* 1996. **37:** 2018–2028.

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Studies on oxidatively modified LDL have attempted to define cell interactions linked to the development of atherosclerotic lesions. Oxidized LDL has been shown to injure a variety of cell types including endothelial cells, smooth muscle cells, and fibroblasts (1–3). In addition, oxidized LDL is cytotoxic whether it is oxidized in cell-free systems (with or without added metal ion) or by a variety of cells (1, 3–10). During oxidation of LDL the various classes of lipids undergo modification. This includes the oxidation of cholesterol to oxysterols, oxidation of polyunsaturated fatty acids to form various aldehydes, including 4-hydroxynonenal, and the conversion of phospholipids to lysophospholipids by a phospholipase A_2-like activity (3, 7, 11, 12).

Several compounds borne by oxidized LDL have been reported to be cytotoxic, including 7β-hydroperoxycholesterol (7β-OOH chol), 7-hydroxycholesterol (7-OH chol), 7-ketocholesterol (7-keto chol), cholesterol epoxides, lysophosphatidylcholine (lysoPC), and 4-hydroxynonenal (13–18). Identification of the principal component(s) of oxidized LDL responsible for cellular injury in vitro and the mechanism by which injury occurs have been sought with the hope that these would aid in evaluating whether cell injury by oxidized LDL plays a role in vivo in atherosclerotic lesion development and suggest means of intervening in the process.

The toxicity of oxysterols has been studied extensively (14); however, the toxicity of the precursor to some of these stable oxysterols, 7β-OOH chol, has received less attention, in part, due to difficulty in isolating this compound. We recently reported that 7β-OOH chol was the most potent of the toxic lipids found on metal ion-oxidized LDL (13, 15) and that the mechanism of

Abbreviations: 7β-OOH chol, 7β-hydroperoxycholesterol; 7-OH chol, 7-hydroxycholesterol; 7-keto chol, 7-ketocholesterol; lysoPC, lysophosphatidylcholine; LPDS, lipoprotein-deficient serum; DPPD, N,N′-diphenyl-1,4-phenylenediamine; desferal, deferoxamine mesylate; GSH, reduced glutathione; Tiron, 4,5-dihydroxy-1,3-benzene-disulfonic acid; KTBA, α-keto-γ-methiolbutyric acid; EGF, epidermal growth factor; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; LDL, low density lipoprotein.

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cell injury appeared to involve peroxidation of cellular lipids (15). However, others using various cell systems have attributed the cytotoxicity of oxidized LDL to 7β-OH chol, 7-keto chol, lysoPC, and 4-hydroxynonenal (16, 18–21).

In this study we sought to clarify the importance of various lipids formed during oxidation of LDL to cell injury using two separate approaches. First, the relative toxicities of these lipid products of lipoprotein oxidation were directly compared on a molar basis in vitro. This was coupled with assessments of the relative amounts of these lipids formed during LDL oxidation. In the second approach we imposed a diverse group of inhibitors of a variety of cellular pathways to aid in distinguishing the mechanisms of toxicity of selected oxysterols and other products of lipoprotein oxidation from the mechanism of oxidized LDL toxicity. 7β-OOH chol and oxidized LDL appeared to injure cells similarly, via cellular lipid peroxidation (15). The other putative toxins appeared to injure cells by mechanisms distinct from that of oxidized LDL and 7β-OOH chol.

METHODS

Materials

7β-Hydroxycholesterol, 7α-hydroxycholesterol, 7-ketocholesterol, and 5α,6α-epoxycholesterol were purchased from Steraloids Inc. (Wilton, NH). Lysophosphatidylcholine was purchased from Serdary, (Englewood Cliffs, NJ) and Avanti Polar Lipids, Inc., (Alabaster, AL), and 4-hydroxynonenal was purchased from Biomol (Plymouth Meeting, PA). [^{14}C]adenine was purchased from Sigma Chemical Co. (St. Louis, MO) and ICN (Costa Mesa, CA). EGF was purchased from Clonetics (San Diego, CA). Solvents for HPLC were Optima Grade purchased from Fisher Scientific (Pittsburgh, PA).

3β-Hydroxy-5-cholesten-7-β-hydroperoxide (7β-OOH chol) synthesis

7β-OOH chol was synthesized using soybean lipoxidase and ethyl linoleate as previously described (22, 23) or through incubation of cholesterol in solution at 85°C (24). Briefly, 4 mg of re-crystallized cholesterol and 120 mg of ethyl linoleate (Sigma) were dissolved in acetone. Lipids were then dried in a reaction flask under a stream of N_2. The combined lipids were resuspended in 250 ml of 50 mM Tris (pH 9.0) that had been oxygenated by bubbling O_2 for 15 min. Soybean lipoxidase (30 mg; Sigma) was added and the mixture was incubated overnight at 30°C. Alternatively, cholesterol (100 mg in 4 ml ethanol) was added to 100 ml distilled water (85°C) containing 60 mg ethyl linoleate (1 ml ethanol) and incubated for 4 h. In either case, lipids were extracted using four equal volumes of ethyl acetate. The ethyl acetate was pooled and dried over anhydrous Na_2SO_4. Ethyl acetate was removed from the sample using a Büch Rota-Vap under reduced pressure and in the absence of additional heat. When heat was used to oxidize cholesterol, the extracted sterols were eluted on a silica column to separate oxysterols from the bulk of cholesterol remaining (25). The eluents were hexane–ether 90:10, hexane–ether 50:50, ether, and finally methanol. The ether fraction contained the oxysterols and was dried under nitrogen. The lipids were then dissolved in acetonitrile–isopropanol 1:1 for chromatography on a preparative HPLC C18 column.

7β-Hydroperoxycholesterol purification and quantification

7β-OOH chol was purified as previously described (13) using sequential HPLC steps involving C18 and CN columns. The sample was first resolved on a preparative C18 column (Waters) using a Waters HPLC (5 ml/min), with a gradient of water–acetonitrile 40:60 to 100% acetonitrile at 5 min, and to 100% isopropanol at 50 min. The fraction between 21.5 to 24.5 min contained the 7-hydroperoxycholesterols, among other lipids, and was dried under a stream of N_2. The lipids were resuspended in hexane–isopropanol 97:3 and resolved on a CN column (3.9 mm × 300 mm; Waters) using isocratic hexane–isopropanol 97:3 at 1 ml/min. Thin-layer chromatography using silica plates and ethyl acetate–heptane 1:1 for elution, before and after borohydride reduction (25), was performed in addition to the above chromatographic steps to verify the identity of 7β-OOH chol and also to ascertain the approximate purity of purchased oxysterols.

Isolation of LDL and LPDS and oxidation of LDL

Human LDL and lipoprotein-deficient serum (LPDS) were isolated from plasma using sequential ultracentrifugation (5, 26). LDL was oxidized by dialysis against 3 μM $CuSO_4$ or 5 μM $FeSO_4$ at 25°C for up to 48 h (5, 27). After oxidation LDL was extensively dialyzed against 0.15 M NaCl/0.5 mM EDTA, pH 8.5, to remove metal ions. Relative oxidation was indexed by quantifying electrophoretic mobility relative to native LDL.

Isolation and maintenance of cultured cells

Human dermal fibroblasts were isolated from neonatal foreskins as previously described (4). Cells were maintained in DME/Ham's F12 supplemented with 5–10% FBS in a humidified 5% CO_2 and air environment. Bovine aortic endothelial cells were harvested by techniques previously described (28). The exposed intimal surface of a bovine aorta was rinsed gently with serum-free media to remove adherent blood cells and then treated with collagenase. Detached endothelial cell

patches were gently collected and seeded with 5–10% FBS in DME/Ham's F12 (1:1). Human and rabbit smooth muscle cell cultures were obtained from explants by a method similar to that described by Ross (29). Endothelial cells were enzymatically removed and the media–adventitial layer was peeled away from the intimal–medial layer. The latter was minced into 1-mm^2 pieces. DME/F12 media with 10% FBS was gently added to explants that adhered to plastic by light drying. Smooth muscle cells begin migrating from explants as early as 5–10 days. Cells were maintained as described above for fibroblasts; media was changed every 3–5 days until each passage by trypsinization.

Measurement of cellular injury

The degree of cellular injury was measured by the specific release of ^{14}C from proliferating cells previously loaded with [^{14}C]adenine (15, 30). Briefly, cells were trypsinized and seeded on 48-well plates in the presence of 0.2 μCi/ml [^{14}C]adenine in DME/F12 containing 5% fetal bovine serum. After overnight incubation, the cells were washed twice with either media or Dulbecco's PBS to remove excess [^{14}C]adenine. The cells were then exposed to the lipids to be tested in fresh DME/F12 media containing lipoprotein-deficient serum (LPDS, 2 mg protein/ml) and 100 ng/ml EGF for 24 h unless otherwise indicated. Cell injury was expressed as specific ^{14}C release, determined by subtracting basal release of ^{14}C from cells treated with solvent-containing control media (0%) and comparing it to the total release of ^{14}C obtained with detergent (0.5% Triton) or 0.25 N NaOH lysis of cells (100%). Oxysterols and 4-hydroxynonenal were added in ethanol to DME/F12 media. For oxysterols, media was supplemented with 2 mg/ml LPDS and 100 ng/ml EGF. Final concentration of ethanol was maintained below 1.5%. Solvents at matched concentrations were included in control media where appropriate. LysoPC and 4-hydroxynonenal were added either with or without 2 mg/ml LPDS and EGF as stated. Exposure of cells to antioxidants was initiated prior to, and continued after the addition of putative toxins.

Formation of oxysterols during oxidation of LDL

Oxysterol formation was quantified during the oxidation of [^{14}C]cholesterol-loaded native LDL. [^{14}C]cholesterol was transferred to native LDL by the method of Nilsson and Zilversmit (31). LDL was then oxidized as described above and performed previously (13). Aliquots were removed as a function of time, as indicated. Samples were lyophilized, extracted with degassed acetone, and then dried under N_2. Extracted oxidized sterols from LDL and, in parallel, commercial standards were separated by TLC using diethyl ether–cyclohexane 9:1 and/or ethyl acetate–heptane 1:1 (23, 25). Selected oxysterols were then quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Enhancement of cholesterol in cultured cells

Cells were "loaded" with exogenous cholesterol as previously described (32). Briefly, 1 ml of cholesterol solution (20 mg/ml acetone) was injected into 2 ml of albumin (20 mg/ml, DME/F12) that had been sterile-filtered through a 0.2-μm filter. After mixing, the acetone was evaporated under a stream of N_2. The resulting mixture of cholesterol–albumin was sonicated for 60 sec in an ice bath. Exogenous cholesterol was provided by adding cholesterol to the media as either a cholesterol–albumin complex or as free cholesterol dissolved in ethanol; controls received albumin or ethanol, respectively. Cells were exposed to cholesterol for 24 h, followed by washing of the cells (2 × with media) prior to addition of the lipids of interest. Total cell cholesterol increased up to approximately 2-fold (15, 33).

Altering cellular selenium content in cultured cells

Human fibroblasts were depleted of selenium using the technique of Thomas, Geiger, and Girotti (8). As the principal source of selenium for cultured cells is serum, cellular selenium was lowered by growing cells in low amounts of serum. We incubated cells in 1% FBS for at least 14 days prior to assessing cellular injury. Selenium-supplemented cells were grown in 1% FBS with 5 ng Na_2SeO_3/ml.

RESULTS

Relative toxicities of oxysterols associated with oxidized LDL

The relative capacity of compounds carried by oxidized LDL to injure cells may depend not only on which compound is the most potent toxin on a molar basis, but also on the relative concentration to which the compound accumulates in the lipoprotein during its oxidation. We sought to compare the toxicities of putative toxins directly and to examine the relative potency taking into consideration the amounts of these compounds formed on oxidized LDL.

To determine the relative toxicities of these lipids, the release of ^{14}C from [^{14}C]adenine-loaded cells was quantified after oxysterol exposure. **Figure 1** shows that 7β-OOH chol, which was also representative of 7α-OOH chol (data not shown), was able to induce significant release of ^{14}C from fibroblasts at lower concentrations than other oxysterols, 7β-OH chol, 7-keto chol, and 5α,6α-epoxycholesterol. The molar concentration required to achieve half-maximal release of ^{14}C ($C_{½}$) for 7β-OOH chol was 1.4 ± 0.8 μM (n = 22). This was over

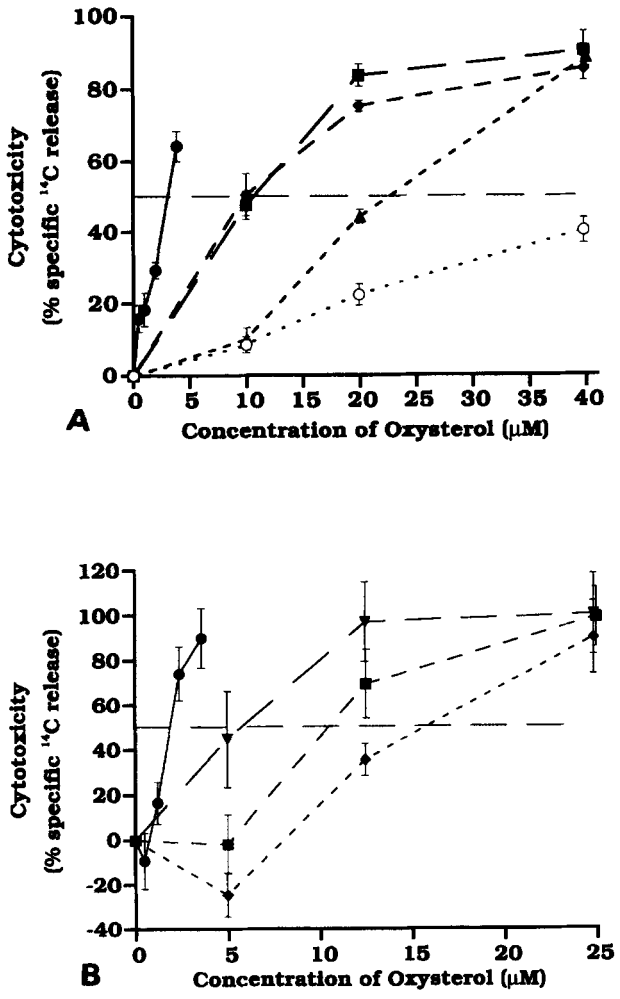

Fig. 1. Relative cytotoxicities of oxysterols. Human dermal fibroblasts were exposed to increasing concentrations of various oxysterols in the presence of 2 mg/ml LPDS. The relative cytotoxicities were determined by comparing the concentration of oxysterols necessary to induce 50% of maximum specific ^{14}C release (horizontal dashed line). Release of ^{14}C is the average (± SD) of 4 wells per oxysterol concentration. These results are representative of several experiments performed using human dermal fibroblasts. In Fig. 1A: solid circles, 7β-OOH chol; squares, 7β-OH chol; diamonds, 7α-OH chol; upward triangles, 7-keto chol; open circles, 25-OH. In Fig. 1B: circles, 7β-OOH chol; squares, 7-keto chol; downward triangles, 7β-OH chol; diamonds, 5α,6α-epoxy chol.

11-fold more toxic than 7β-OH chol ($C_{½}$ = 16.3 ± 5.5 μM; n = 20), 15-fold more toxic than 7-keto chol ($C_{½}$ = 21.6 ± 6.5 μM; n = 17), and 17-fold more toxic than 5α,6α-epoxycholesterol ($C_{½}$ = 24.8 ± 6.2 μM; n = 4). 25-Hydroxycholesterol ($C_{½}$ = 59 ± 4.4 μM; n = 2), which is not normally associated with oxidized LDL (20, 21) but which has been studied extensively for its cytotoxic effects (34, 35), is also shown for comparison.

To determine whether the observed hierarchy of cytotoxicity among oxysterols was specific to dermal fibroblasts, we examined the relative toxicities of selected oxysterols to both rabbit and human aortic smooth muscle cells and to bovine aortic endothelial cells. We found that the relative toxicities, in order of decreasing potency, were 7β-OOH chol > 7β-OH chol > 7-keto chol in all these cell types (data not shown), similar to the order of toxicity to fibroblasts.

To examine the potential contributions of each of these oxysterols to oxidized LDL toxicity, we quantified their formation during metal ion-facilitated oxidation of LDL. Formation of the oxysterols was monitored during oxidation of LDL after incorporating [^{14}C]cholesterol, as described previously (13). **Figure 2** shows that 7β-OOH chol, a precursor of 7β-OH chol and 7-keto chol (25, 36), accumulated on oxidized LDL in amounts that rivaled the sum of the other oxysterol products, 7α-OH chol, 7β-OH chol, and 7-keto chol (representative of three experiments). These results, considered in conjunction with the relative toxicities of these compounds associated with oxidized LDL (Fig. 1), are consistent with the idea that 7β-OOH chol is the agent most likely responsible for oxidized LDL injury to cells, as previously reported in fibroblasts (13, 15).

In addition to relative toxicities on a molar basis, we also assessed the time courses for cellular injury. We found that 7β-OOH chol injured cells relatively rapidly and induced measurable ^{14}C release 7–9 h after its addition, after which specific ^{14}C release did not increase further for a given 7β-OOH chol concentration (data not shown). In contrast, the reduction product of 7β-OOH chol, 7β-OH chol, showed little toxicity at 9 h, but exhibited increasing toxicity from 24 to 72 h (data not shown). Similar extended time courses for cellular injury were observed for 7-keto chol, 5α,6α-epoxycholesterol, and 25-hydroxycholesterol (data not

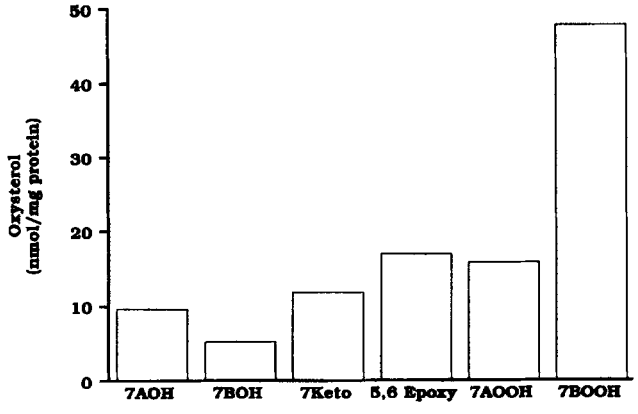

Fig. 2. Formation of various oxysterols during in vitro oxidation of LDL. Oxysterols on [^{14}C]cholesterol-labeled LDL were quantified after oxidation with 3 μM $CuSO_4$. Lipids were extracted with acetone and then developed on silica TLC plates with diethyl ether–cyclohexane 9:1. Oxysterols were quantified using a phosphorimager. This result is representative of three experiments.

shown). This result confirms that the toxicity of 7β-OOH chol is not secondary to its conversion to its degradation products 7β-OH chol and 7-keto chol.

Relative toxicities of non-sterol oxidation products

The toxicities of two non-sterol lipid products of LDL oxidation, lysoPC and 4-hydroxynonenal, were also examined. LysoPC exhibited little toxicity at concentrations up to 25 μM in the presence of 2 mg/ml LPDS (**Fig. 3A**). This was likely due to lysoPC binding to albumin present in LPDS because incubation of cells with lysoPC in the absence of serum (Fig. 3B) markedly increased its toxicity ($C_{½}$ = 6.9 ± 2.0 μM; n = 10). The absence of serum decreased the toxicity of 7β-OOH chol, perhaps due to slowed cell growth (2, 5). However, even under serum-free conditions, 7β-OOH chol was approximately 2-fold more toxic on a molar basis ($C_{½}$ = 3.4 ± 2.0 μM; n = 7) than lysoPC. Also under serum-free conditions, we found that 7β-OOH chol was around 9-fold more toxic than 4-hydroxynonenal ($C_{½}$ = 31 ± 16.6 μM; n = 6) based on a molar comparison. Similar relative toxicities were observed in the presence of LPDS.

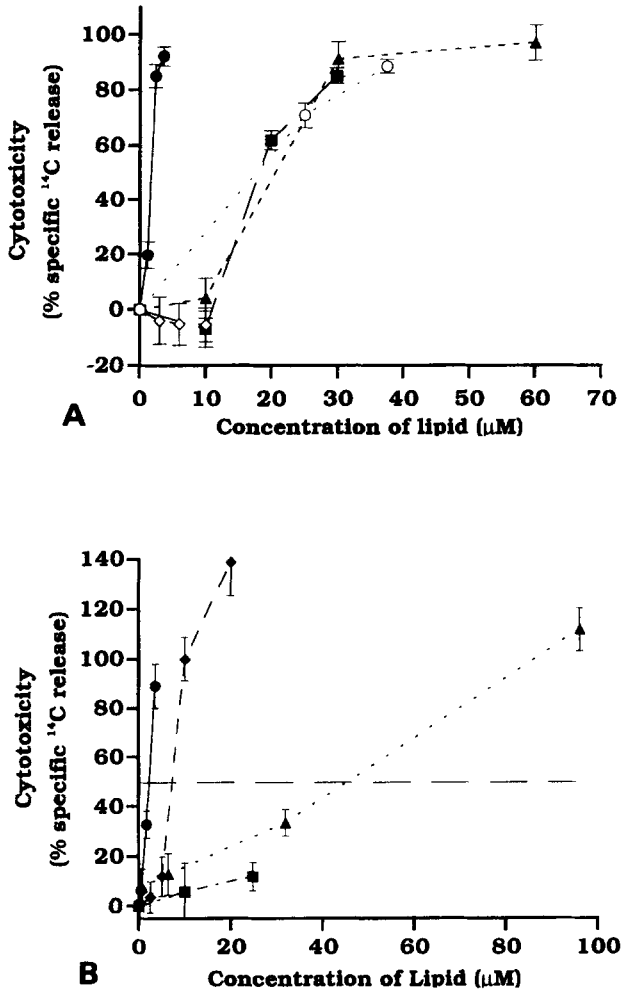

Fig. 3. Relative cytotoxicities of lysoPC, 4-hydroxynonenal, and oxysterols. Oxidized lipids were presented to cells in the presence (panel A) or absence (panel B) of 2 mg/ml LPDS. LysoPC toxicity increased significantly in the absence of serum but was still less than that of 7β-OOH chol based on the molar concentration required to achieve half-maximal toxicity. This result is representative of several experiments using dermal fibroblasts. In Fig. 3A: solid circles, 7β-OOH chol; diamonds, lysoPC; squares, 7β-OH chol; upward triangles, 4-hydroxynonenal; open circles, 7-keto chol. In Fig. 3B: solid circles, 7β-OOH chol; squares, 7β-OH chol; diamonds, lysoPC; upward triangles, 4-hydroxynonenal.

LysoPC and 4-hydroxynonenal levels in metal ion-oxidized LDL have been reported to be in the range of 200–600 nmol/mg protein (11, 37) and 5–6.5 nmol/mg protein (38), respectively. LysoPC in preparations of oxidized LDL prepared as in the present study were measured to be 390–487 nmol/mg protein (39). We found 7β-OOH chol to be present on metal ion-oxidized LDL at concentrations up to 50 nmol/mg protein (Fig. 2). Comparing 7β-OOH chol to 4-hydroxynonenal, the higher relative potency and formation of 7β-OOH chol on oxidized LDL are consistent with its being a more potent cytotoxin. In trying to assess the relative importance of lysoPC and 7β-OOH chol cytotoxicities, however, we recognized that while 7β-OOH chol is a more potent toxin, lysoPC can accumulate to a greater extent on oxidized LDL. We thus opted to compare the mechanisms of action of these two compounds, as well as the other agents under study, to elucidate further which is the dominant toxin of oxidized LDL.

Role of lipid peroxidation in cell injury

The toxicities of both oxidized LDL and 7β-OOH chol had previously been ascribed to an induction of peroxidation of cellular lipids leading to cell lysis (15). We examined whether the other lipoprotein oxidation products, including other oxysterols, injured cells through lipid peroxidation or alternate pathways.

We attempted to reduce the toxicity of submaximal concentrations of the various oxysterols by applying known inhibitors of lipid peroxidation, shown previously to inhibit both 7β-OOH chol and oxidized LDL cytotoxicities (15). **Figure 4** shows that lipid and peroxyl radical scavengers (DPPD and vitamin E) and a chelator of cellular metal ion (desferal) were able to block 7β-OOH chol toxicity, as previously reported (15); however, none of these agents inhibited cytotoxicities of 7β-OH chol or 7-keto chol. Similar findings were observed with 1 mM GSH (a participant in selenoenzyme antioxidant activity), 5–10 μM ebselen (an agent known to reduce lipid hydroperoxides), 3 mM Tiron (a chelator of cellular metal ion), and KTBA (a scavenger of alkoxyl radicals), in that these inhibitors of oxidized LDL toxicity also blunted the toxicity of 7β-OOH chol but had no

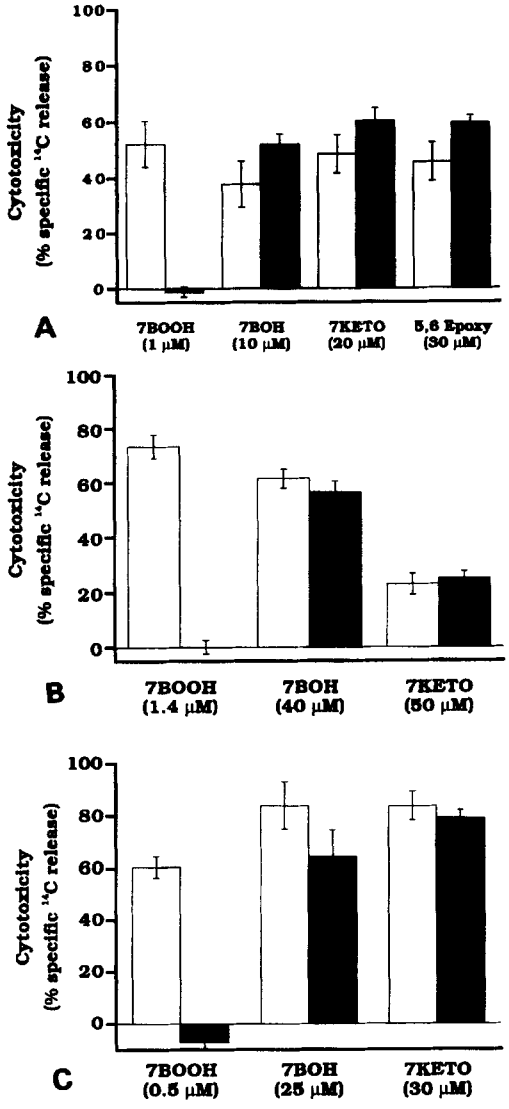

Fig. 4. Inhibition of 7β-OOH chol toxicity by antioxidants. Human dermal fibroblasts were treated with various classes of antioxidants to examine the role of lipid peroxidation in oxysterol toxicity. 7β-OOH chol toxicity was inhibited by DPPD (panel A), vitamin E (panel B), and desferal (panel C). In contrast, none of these compounds affected the toxicity of the other oxysterols, 7β-OH chol, 7-keto chol, and 5α,6α-epoxycholesterol. These results are representative of several experiments involving the various antioxidants. In Fig. 4A: open bars, control; solid bars, 0.5 μM DPPD. In Fig. 4B: open bars, control; solid bars, 25 μM vitamin E. In Fig. 4C: open bars, control; solid bars, 3 mM desferal.

effect on the other oxysterols examined (data not shown).

LysoPC toxicity and lipid peroxidation

When the effects of antioxidants on the toxicities of lysoPC and 4-hydroxynonenal were examined, we observed that an inhibitor of lipid peroxidation, vitamin E, inhibited the toxicity of lysoPC, but no such inhibition was observed for toxicity by 4-hydroxynonenal (**Fig. 5**). This result suggested that lysoPC may be injuring cells via an oxidant pathway, although the results with altered selenium (see below) would suggest that the oxidant mechanism is distinct from that induced by oxidized LDL or 7β-OOH chol.

Role of selenium in susceptibility to toxicity by oxidized LDL lipids

We further examined the role of oxidant stress by altering the selenium availability to cells. It was previously shown that cells with reduced selenium were more susceptible to injury by oxidized LDL and a mixture of cholesterol hydroperoxides (8). Consistent with these reports, we found that cells were 1.7 ± 0.4-fold (n = 4) more susceptible to injury by 7β-OOH chol when grown in a low selenium environment (**Fig. 6**). In contrast, the toxicities of other oxidized LDL-borne oxidation products, 7β-OH chol, 7-keto chol, and 4-hydroxynonenal, were not affected by these changes in selenium availability. LysoPC, which induced some characteristics of lipid peroxidation, did not show a significant response to the change in selenium (0.96 ± 0.3-fold difference in toxicity between reduced selenium and selenium supplemented cells, n = 7). These results suggest that the putative oxidant mechanism for lysoPC-induced cell injury is distinct from that observed for 7β-OOH chol and oxidized LDL.

Potential effect of exogenous cholesterol pretreatment

An alternate mechanism for the toxicity of selected oxysterols derives from their ability to interfere with

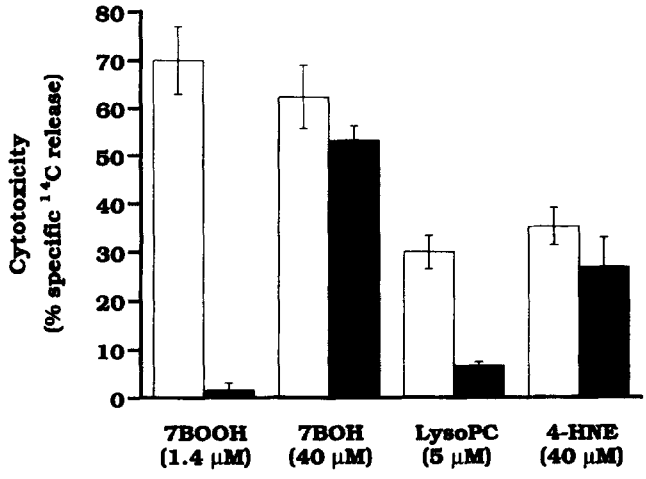

Fig. 5. LysoPC toxicity is reduced by vitamin E. Cells were incubated with vitamin E (25 μM) for 2 h prior to addition of submaximally toxic levels of lipids. LysoPC toxicity was significantly reduced in the presence of vitamin E suggesting a potential role for lipid peroxidation in lysoPC toxicity. This result is representative of several experiments. Open bars, control; solid bars, 25 μM vitamin E.

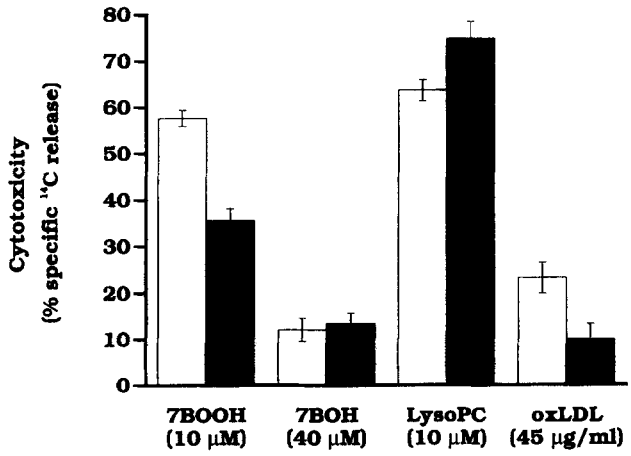

Fig. 6. Role of selenium exposure in toxicity of oxidized lipids. Human dermal fibroblasts were cultured for 4 weeks in 1% FBS ± 5 ng sodium selenite/ml. The figure shows the specific ^{14}C release evoked by submaximally toxic levels of selected oxysterols, oxidized LDL, and lysoPC. This result is representative of several experiments. Open bars, 1% FBS; solid bars, 1% FBS + 5 ng/ml Na_2SeO_3.

cholesterol metabolism through, for example, inhibition of HMG-CoA reductase (14, 34, 40). We provided cells with an exogenous source of cholesterol to test whether they could be protected against injury by oxysterols. This was examined using two protocols to provide cells with excess exogenous cholesterol. The first approach was to provide exogenous cholesterol in the form of cholesterol:albumin complexes during the time of exposure to the oxidants. Under these conditions we found that the toxicities of 7β-OH chol and 7-keto chol were significantly reduced ($P < 0.05$). No decrease in toxicity was observed for either 7β-OOH chol or oxidized LDL.

To test the possibility that this reduction was due to reduced availability of 7β-OH and 7-keto chol to cells due to their selective association with the cholesterol:albumin complexes, we exposed cells to cholesterol:albumin complexes for 24 h prior to exposure of oxysterols followed by washing cells before adding the oxysterols. As **Fig. 7A** illustrates, there was still a moderate but significant ($P < 0.05$) reduction in the toxicity of 7β-OH chol. 7-Keto chol toxicity was not consistently inhibited to a significant degree under these conditions of preloading cells with exogenous cholesterol.

The ability of exogenous cholesterol to inhibit the toxicity of non-sterol toxins was also examined. A 24-h pre-treatment with either cholesterol (50 μg/ml) or cholesterol:albumin complexes (50 μg cholesterol/ml) dramatically reduced the toxicity of lysoPC compared to solvent or albumin control (Fig. 7B). Exogenous cholesterol had no significant effect on the toxicity of 4-hydroxynonenal.

Role of protein synthesis in oxysterol toxicity

Another reported method of inhibiting toxicity by certain oxysterols, 25-hydroxycholesterol and 7-ketocholestanol, is inhibition of protein synthesis (41). In concert with these findings, **Fig. 8** shows that 1 μM cycloheximide was able to reduce the toxicity of both 7β-OH chol and 7-keto chol. There was, however, no reduction in 7β-OOH chol toxicity or the toxicity of oxidized LDL, consistent with a previous report (15).

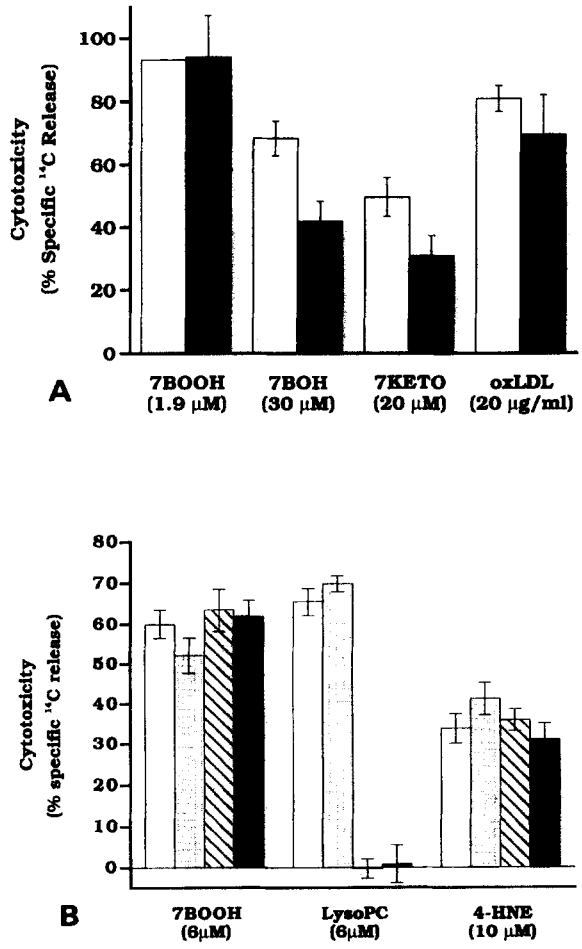

Fig. 7. Inhibition of toxicity by cholesterol loading of cells. Human dermal fibroblasts were loaded with exogenous cholesterol prior to addition of oxidized LDL lipid products. Cells were washed prior to the addition of putative toxins to remove excess cholesterol and/or albumin. In panel A cells were pre-loaded with cholesterol using cholesterol:albumin complexes. 7β-OH chol toxicity was consistently reduced by pre-loading cells with exogenous cholesterol ($P < 0.05$). There was no effect on oxidized LDL cytotoxicity. In panel B cells were pre-loaded with cholesterol using both free cholesterol (50 μg/ml) and cholesterol:albumin complexes (50 μg cholesterol/ml). The toxicity of lysoPC was dramatically inhibited by pre-loading cells with cholesterol as compared to solvent or albumin control. There was no significant effect on the toxicity of 4-hydroxynonenal (4-HNE). These results are representative of several experiments. In Fig. 7A: open bars, control; solid bars, pretreatment with 100 μg cholesterol/ml. In Fig. 7B: open bars, control; shaded bars, albumin control; diagonal lines, 50 μg cholesterol/ml; solid bars, cholesterol:albumin complexes.

We observed similar responses using emetine, another inhibitor of protein synthesis (data not shown). This result further distinguished the mechanisms of 7β-OOH chol and oxidized LDL from those of these two oxysterols.

DISCUSSION

During oxidation of LDL, the lipid moiety of the molecular complex undergoes significant changes. Numerous products of lipoprotein oxidation known to injure cells have been detected, including 7β-OOH chol (13, 25), 7-OH chol (16, 21), 7-keto chol (16, 21), epoxycholesterols (21), lysoPC (11, 37), and 4-hydroxynonenal (18). The toxicities of these compounds have been reported in various cell systems and, with the exception of 7β-OOH chol, the toxicities have been studied extensively. Not surprisingly, the cell injuring capacity of oxidized LDL has been attributed to various of these agents (7, 12, 13, 15, 16, 21). The intents of the current study were *a)* to determine the relative toxicities of these in direct comparison to one another, by quantifying relative toxicities on a molar basis; *b)* to consider these in light of the relative amounts of each formed during LDL oxidation; and *c)* to examine whether or not the mechanism of cell injury by these agents is similar to that of oxidized LDL.

The most direct approach to determine the importance of these compounds in the ability of oxidized LDL to injure cells is to isolate the lipid fractions from oxidized LDL and assess their relative toxicities and identities, but this has led to disparate results. For example, Chisolm et al. (13) reported that 7β-OOH chol was the principal cytotoxin when examined using human dermal fibroblasts as target cells; however, Hughes et al. (16) attributed oxidized LDL toxicity to 7-keto chol using porcine aortic smooth muscle cells. These two studies differed not only in the target cell type but also the lipid extraction and chromatographic techniques used to isolate lipids from oxidized LDL.

In examining the relative toxicity of these compounds, we found that 7β-OOH chol and 7α-OOH chol were significantly more toxic than the other oxysterols and lipoprotein oxidation products. 7β-OOH chol was up to 11-fold more toxic than 7β-OH chol and 15-fold more toxic than 7-keto chol. We found that the relative potencies of the various oxysterols were consistent in all of the cell types we examined, including dermal fibroblasts and smooth muscle and endothelial cells. It was also important to examine the relative accumulation levels of these oxysterols during oxidation of LDL. The oxysterol, 7β-OOH chol, has been reported previously to be present on oxidized LDL (13, 25) and it would be

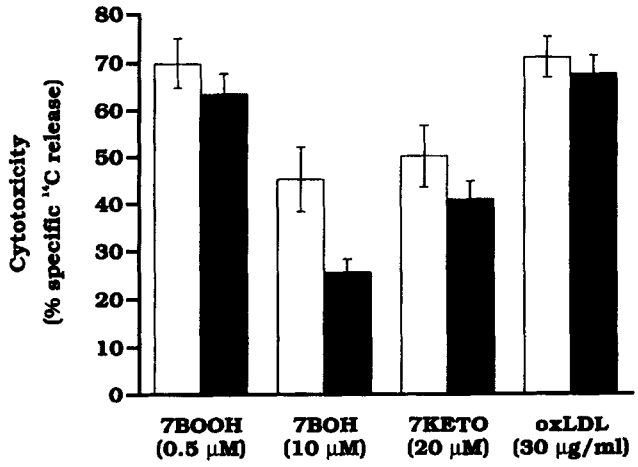

Fig. 8. Protein synthesis inhibition reduced 7β-OH chol toxicity. Cells were treated with 0.5 μM cycloheximide (solid bars) to inhibit protein synthesis during oxysterol exposure. Under these conditions only 7β-OH chol toxicity was significantly reduced ($P < 0.05$). 7β-OOH chol and oxidized LDL toxicity were not affected by inhibition of cellular protein synthesis. This result is representative of several experiments.

the likely precursor for the formation of the oxysterols 7-OH chol and 7-keto chol (25, 36). When we quantified the oxysterols formed during LDL oxidation, we found that 7β-OOH chol was present in amounts roughly equivalent to the sum of the other oxysterols (Fig. 2). We also found that 7β-OOH chol was present in molar amounts in the same range as those reported by others for other oxysterols (21) and in greater amounts than those reported for 4-hydroxynonenal (38) on various preparations of oxidized LDL. While 7β-OOH chol was more toxic than lysoPC, lysoPC accumulated to higher levels on oxidized LDL. However, it should be noted that the presence of serum proteins, in the form of LPDS, markedly reduced the toxicity of lysoPC but not that of 7β-OOH chol. Furthermore, exposure of cells to exogenous cholesterol also dramatically inhibited the toxicity of lysoPC but not that of 7β-OOH chol.

It is difficult to extrapolate from our findings to what occurs in vivo. It has not yet been proved that cell injury by oxidized LDL actually occurs in vivo, even in arterial lesions where oxidized LDL accumulates. Evidence has been reported, however, for the presence in atherosclerotic lesions of the various putative toxins we studied, including 7β-OOH chol (13, 42–45). As the cholesterol hydroperoxides are the precursors to the formation of the hydroxycholesterols and the ketocholesterol (25, 36), the presence of these more stable oxidation products can be taken as a further indication of hydroperoxide formation in vivo. The dramatic inhibition of cytotoxicity of lysoPC but not 7β-OOH chol by enhanced cellular cholesterol or the presence of serum proteins would suggest that if oxidized LDL lipids were to persist in

lesion sites in a proportion similar to that found in LDL oxidized in vitro, the toxic effects of the cholesterol hydroperoxide would be more likely to cause cell injury. The relative quantities of these lipid products present on oxidized LDL in vivo, especially within an atherosclerotic plaque, need to be assessed. The unknown influences of conditions that pertain in a lesion site may markedly alter the accumulation of these lipids.

As a second approach to determining the importance of these compounds in the toxicity of oxidized LDL, we sought to examine which of these compounds worked through a mechanism similar to that of oxidized LDL. Coffey et al. (15) reported that oxidized LDL and 7β-OOH chol killed fibroblasts via peroxidation of cellular lipids. We confirmed that the toxicities of oxidized LDL and 7β-OOH chol were inhibited by a variety of antioxidants known to blunt cellular lipid peroxidation; however, in the present study none of the toxicities of the other oxysterols or 4-hydroxynonenal was affected by exposure to these antioxidants. This suggests that these compounds are injuring cells via mechanisms that do not involve lipid peroxidation or the generation of lipid radicals. LysoPC toxicity was blunted by the presence of vitamin E, suggesting that lysoPC may be able to trigger lipid peroxidation by an unknown mechanism.

Thomas et al. (8) reported that oxidized LDL and lipid hydroperoxide toxicity was dependent upon the selenium environment of the cells. They proposed that selenium-containing antioxidant enzymes were decreased when cells were grown in a selenium-poor environment. We also examined, indirectly, the role of selenium-containing antioxidant enzymes in oxysterol- and lysoPC-induced injury by reducing the availability to cells of exogenous selenium. Cells grown in a selenium-deficient environment were significantly more susceptible to injury by oxidized LDL and 7β-OOH chol than their selenium-supplemented counterparts, consistent with these previous findings (8). In contrast, these alterations in selenium availability had no effect on the toxicities of 7β-OH or 7-keto chol. Furthermore, the ability of lysoPC to injure cells was not dependent upon the selenium environment of the cells. This strengthens further the indication that the lysoPC-induced oxidant mechanism of cell injury differs from that of 7β-OOH chol and oxidized LDL.

To distinguish further among the various mechanisms of cellular injury induced by these lipid products of LDL oxidation, we sought means of inhibiting the toxicity of oxysterols, 7β-OH chol and 7-keto chol. It has been reported that providing exogenous cholesterol or inhibiting protein synthesis reduced the susceptibility of cells to injury by various oxysterols (41, 46). The mechanisms for protection by these treatments are unclear but have been proposed to involve maintaining cholesterol homeostasis. In our system we found that protein synthesis inhibition or pre-loading cells with exogenous cholesterol provided some protection against 7β-OH chol but not oxidized LDL or 7β-OOH chol. Whether these effects are related to inhibition of HMG-CoA reductase is unclear as exogenous cholesterol also inhibited lysoPC toxicity. This may be an indication that providing cells with exogenous cholesterol may be protecting cells in part through stabilizing the plasma membrane.

In summary, our results are consistent with the concept that 7β-OOH chol is the most potent toxin of the oxidized lipids formed during LDL oxidation and that, with the exception of lysoPC, it is formed in amounts comparable to or greater than that of other putatively toxic lipoprotein oxidation products. The relative potencies of the toxicities of these agents appear to be similar for fibroblasts, vascular smooth muscle cells, and endothelial cells. In addition, 7β-OOH chol, but not 7β-OH chol, 7-keto chol, 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol or 4-hydroxynonenal, exhibits a mechanism of cell injury that closely resembles that observed for oxidized LDL. Surprisingly, lysoPC injury to cells was inhibitable by an antioxidant, but was distinct from the toxicity by oxidized LDL or 7β-OOH chol, as selenium depletion enhanced susceptibility to 7β-OOH chol and oxidized LDL but had no effect on susceptibility of cells to injury by lysoPC. The relevance of these findings to the composition of the oxidized LDL that is known to accumulate in atherosclerotic lesions, or the roles of these oxidized LDL associated lipids in altering cell function in lesion sites in vivo, remains the subject of hypothesis; however, the further elucidation of the composition of LDL as it oxidizes and the mechanisms of injury by oxidized LDL components will likely suggest ways to test these hypotheses.

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