Arachidonate metabolism and the signaling pathway of induction of apoptosis by oxidized LDL/oxysterol

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Abstract Owing at least in part to oxysterol components that can induce apoptosis, oxidized LDL (oxLDL) is cytotoxic to mammalian cells with receptors that can internalize it. Vascular cells possess such receptors, and it appears that the apoptotic response of vascular cells to the oxysterols borne by oxLDL is an important part of the atherogenic effects of oxLDL. Thus, an analysis of the signaling pathway of apoptotic induction by oxysterols is of value in understanding the development of atherosclerotic plaque. In a prior study, we demonstrated an induction of calcium ion flux into cells treated with 25-hydroxycholesterol (25-OHC) and showed that this response is essential for 25-OHC-induced apoptosis. One possible signal transduction pathway initiated by calcium ion fluxes is the activation of cytosolic phospholipase A_2 ($cPLA_2$). In the current study, we demonstrate that activation of $cPLA_2$ does occur in both macrophages and fibroblasts treated with 25-OHC or oxLDL. Activation is evidenced by 25-OHC-induced relocalization of $cPLA_2$ to the nuclear envelope and arachidonic acid release. Loss of $cPLA_2$ activity, either through genetic knockout in mice, or by treatment with a $cPLA_2$ inhibitor, results in an attenuation of arachidonic acid release as well as of the apoptotic response to oxLDL in peritoneal macrophages or to 25-OHC in cultured fibroblast and macrophage cell lines.—Panini, S. R., L. Yang, A. E. Rusinol, M. S. Sinensky, J. V. Bonventre, and C. C. Leslie. **Arachidonate metabolism and the signaling pathway of induction of apoptosis by oxidized LDL/oxysterol.** ***J. Lipid Res.*** **2001.** 42: **1678–1686.**

Supplementary key words arachidonic acid • calcium • $cPLA_2$ • eicosanoids

Oxidation of LDL, produced by a variety of chemical and biochemical processes, is believed to be the major basis of the atherogenicity of LDL (1, 2). The resultant oxidized LDL (oxLDL) produces its proatherogenic effects in several ways, including, for example, inflammatory responses (3, 4), foam cell formation in smooth muscle cells and macrophages (5), and cytotoxic effects on vascular smooth muscle cells (6), macrophages (7), and endothelial cells (8). These cytotoxic effects appear to be due, at least in part, to the induction of apoptosis (9, 10).

OxLDL contains several lipid-derived bioactive molecules such as oxysterols, lysophospholipids, and fatty acid peroxides (11, 12). Of these, the cholesterol oxidation products, referred to as oxysterols, can largely account for the cytotoxicity (13, 14) of oxLDL, and more particularly, its apoptotic activity (15). A model compound for such oxysterols is 25-hydroxycholesterol (25-OHC), which has been shown to induce apoptosis in monocyte-macrophage (16, 17) and lymphoid cell lines (18, 19) in the range of 1–10 μM. Another model oxysterol, 7-ketocholesterol, has also been shown to induce apoptosis in vascular endothelial and smooth muscle cells (20). We have presented evidence further supporting the hypothesis that the cytotoxic agent in oxLDL is an oxysterol and that induction of apoptosis by 25-OHC can be observed in CHO-K1 fibroblasts (21). In particular, we have shown that apoptotic induction by oxLDL will occur in CHO-K1 cells expressing the CD36 oxLDL receptor and that a somatic cell mutant resistant to apoptotic induction by 25-OHC, in the signaling pathway, is cross-resistant to apoptotic induction by oxLDL.

We also showed (21) that a critical early second message of oxysterol-induced apoptosis was an enhanced influx of calcium. Apoptosis was blocked when calcium influx was prevented either by treating cells with 25-OHC in calcium-free medium, or in the presence of nifedipine or by somatic cell mutation. Increases in cytosolic calcium concentration can initiate several signal transduction pathways in-

Abbreviations: 25-OHC, 25-hydroxycholesterol; $AACOCF_3$, arachidonyl trifluoromethyl ketone; BEL, bromoenol lactone; $cPLA_2$, cytosolic phospholipase A_2; ETYA, 5,8,11,14-eicosatetraynoic acid; F12FC5, Ham's F-12 medium supplemented with 5% fetal bovine serum; GFP, green fluorescent protein; oxLDL, copper-oxidized low density lipoprotein; PMA, phorbol 12-myristate 13-acetate.

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cluding activation of the calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$), which has previously been shown to be involved in some apoptotic pathways—a widely reported example being the induction of apoptosis by tumor necrosis factor α (TNF-α) (22, 23). In the current study, we examine the hypothesis that oxysterol stimulation of calcium uptake activates $cPLA_2$, which plays a critical role in oxLDL/oxysterol induction of apoptosis of susceptible cells.

MATERIALS AND METHODS

Reagents

25-OHC was from Steraloids (Wilton, NH). 5,8,11,14-Eicosatetraynoic acid (ETYA), bromoenol lactone (BEL), and nifedipine were from Biomol (Plymouth Meeting, PA). Arachidonyl trifluoromethyl ketone ($AACOCF_3$) was from Alexis (San Diego, CA). [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (200 Ci/mmol) was from American Radiolabeled Chemical (St. Louis, MO). Ac-DEVD-AMC and Ac-DEVD-CHO were from BD PharMingen (San Diego, CA). OxLDL was prepared from human LDL as previously described (21).

Medium and cells

CHO-K1 cells were purchased from the American Type Culture Collection (Manassas, VA) and were grown in Ham's F-12 medium containing 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 units/ml) (F12FC5) at 37°C and 5% CO_2. All cell culture reagents were obtained from Life Technologies (Rockville, MD). Macrophages were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES buffer (pH 7.4), 2 mM glutamine, 1 mM pyruvate, penicillin (100 units/ml), streptomycin (100 μg/ml), and 50 μM 2-mercaptoethanol. Resident mouse peritoneal macrophages for arachidonate release studies were isolated by peritoneal lavage with the culture medium. Isolated macrophages were plated at a density of 2–3 × 10^5 cells per well in 24-well culture dishes (2 cm^2; Costar Corning, Cambridge, MA) and allowed to attach overnight in a humidified 5% CO_2 incubator at 37°C. Unattached cells (~50%) were removed by washing twice with PBS. THP-1 cells (TIB-202) were obtained from the American Type Culture Collection and were grown at 37°C in a humidified CO_2 atmosphere. Cells were plated at 1 × 10^5 per well in 24-well culture dishes and induced to differentiate into macrophages by incubation for 72 h in the presence of 100 nM phorbol 12-myristate 13-acetate (PMA; Alexis).

TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) assay of $cPLA_2(-/-)$ macrophages

Resident peritoneal macrophages (5 × 10^4 cells) from $cPLA_2(-/-)$ mice (24) or their wild-type littermates were plated on glass coverslips coated with mouse collagen type IV (12.5 μg/cm^2) and incubated for 48 h with or without oxLDL (50 μg/ml). After washing with PBS, the cells were fixed in 4% buffered paraformaldehyde for 30 min at room temperature. Coverslips were rinsed, and the cells were permeabilized with 1% Triton X-100 in 100 mM sodium citrate and incubated for 1 h at 37°C with terminal deoxynucleotidyltransferase and fluorescein isothiocyanate-dUTP to end label the fragmented DNA. Washed coverslips were then mounted in antifade mounting solution (Prolong; Molecular Probes, Eugene, OR) and observed under a Nikon Diaphot-200.

Arachidonate release

CHO-K1 cells, plated at 5 × 10^4 cells per 60-mm dish and grown for 24 h, were labeled by incubation for 24 h in 1 ml of serum-free medium containing [^{3}H]arachidonate (1 μCi/ml). After labeling, cells were washed three time with PBS, refed with F12FC5, and incubated for 1 h. In experiments utilizing metabolic inhibitors (15 μM $AACOCF_3$, 20 μM BEL, or 100 μM nifedipine), they were added to the culture medium at this point. These inhibitor concentrations were determined to produce optimal effects in preliminary experiments. Cells were then refed fresh medium containing either 0.03% ethanol (vehicle control) or 25-OHC (3 μg/ml in ethanol) and, when present, the various inhibitors. Radioactivity in supernatant fractions and cell lysates (in 1% Triton X-100) was measured by liquid scintillation counting. The percent release of arachidonate was calculated as the [medium dpm/(cells + medium) dpm] × 100 and was then normalized to the value of unstimulated controls.

Macrophages (THP-1 or mouse peritoneal) were labeled overnight in 0.5 ml of culture medium containing [^{3}H]arachidonic acid (0.5 μCi/ml). Monolayers were gently washed twice with PBS and preincubated for 60 min in 0.5 ml of culture medium alone or in medium supplemented with either 15 μM $AACOCF_3$ or 20 μM BEL. The cells were then refed 0.5 ml of fresh culture medium containing appropriate inhibitors and either 25-OHC (5 μg/ml) or oxLDL (50–75 μg/ml) and returned to the incubator. The medium was collected at the end of 1–5 h of incubation and centrifuged in an Eppendorf 5715 microcentrifuge for 5 min, and the radioactivity in the supernatant fraction was determined in a Wallac (Turku, Finland) 1414 scintillation counter. The cells were lysed in 0.5 ml of 1% Triton X-100 before the measurement of incorporated radioactivity.

Release of labeled arachidonate, as the free fatty acid (rather than as an acyl glyceride), was confirmed by comigration of radioactivity with the authentic standard on silicic acid thin-layer chromatography, using a mobile phase of hexane–diethyl ether–acetic acid 80:20:1 (v/v/v).

Caspase 3 activity assay

CHO-K1 cells (2 × 10^6 per 100-mm plate) were treated with 25-OHC (3 μg/ml) for 24 h. THP-1 cells (5 × 10^5 per 35-mm well) were differentiated for 72 h in the presence of 100 nM PMA and then treated with 25-OHC (5 μg/ml) for 24 h. The cells released from the dish during the treatment and those harvested from the plate by trypsinization or scraping were collected by centrifugation (800 *g* for 10 min) and pooled. Cells were washed twice with ice-cold PBS and lysed in 200 μl of lysis buffer [10 mM Tris (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaP_i, and 10 mM $NaPP_i$]. Samples were incubated on ice for 10 min, passed through a 21-gauge syringe needle several times, and then centrifuged at 15,000 *g* for 20 min at 4°C. Protein concentration in the supernatant fraction (total cell lysate) was measured with a micro-BCA kit (Pierce, Rockford, IL). Aliquots of lysate protein (~100 μg) were incubated with the fluorescent caspase 3 substrate Ac-DEVD-AMC (20 μM) in the presence or absence of the specific inhibitor Ac-DEVD-CHO (100 nM) for 1.5 h at 37°C in protease assay buffer [20 mM HEPES (pH 7.5), 10% glycerol, 2 mM dithiothreitol]. Liberated AMC from Ac-DEVD-AMC was measured with a Spex (Edison, NJ) FluoroMax-3 spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

Transient transfections with enhanced green fluorescent protein (EGFP)-$cPLA_2$

For preparation of the EGFP-$cPLA_2$ fusion protein construct, human $cPLA_2$ cDNA was obtained and modified by PCR as previously described (25). The modified $cPLA_2$ cDNA sequence in

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the pCR 2.1 vector was cut with *Kpn*I and *Pst*I and cloned into pGEM-4Z (Promega, Madison, WI). It was then excised with *Sac*I and *Pst*I and cloned into the pEGFP-C3 mammalian expression vector (Clontech, Palo Alto, CA). CHO-K1 cells were seeded on ethanol-washed 18-mm glass coverslips at a density of 1×10^5 per 60-mm plate and transiently transfected with 6 μg of the expression vector encoding the EGFP-cPLA$_2$ fusion protein (pEGFP-cPLA$_2$) or a control vector, pEGFP-N3, using the Stratagene (La Jolla, CA) MBS mammalian transfection kit according to the instruction manual. Cells were incubated for 48 h before a 4-h treatment with 25-OHC (5 μg/ml) and EGFP expression was examined by fluorescence microscopy with a Nikon Diaphot-200. Digital deconvolution of the images was performed with Image Pro software (Oncor, Gaithersburg, MD).

Gel shift analysis of cPLA$_2$ phosphorylation

After treatment with 25-OHC, CHO-K1 cells (5×10^6 per 100-mm dish) were harvested by scraping into 100 μl of lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 100 μM sodium orthovanadate, 10 mM NaPP$_i$, 100 mM sodium fluoride, 300 nM *p*-nitrophenyl phosphate, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 1 mM phenylmethylsulfonyl fluoride]. After incubation on ice for 30 min, the lysates were centrifuged at 15,000 *g* for 20 min at 4°C. The supernatant fraction was boiled for 5 min in SDS sample buffer. Samples (~75 μg of protein) were resolved on 20-cm 10% SDS-polyacrylamide gels (1% bisacrylamide, pH 8.3) and then transferred to a nitrocellulose membrane. After blocking with 5% nonfat dry milk for 1–2 h, the membrane was incubated overnight at 4°C with anti-cPLA$_2$ polyclonal antibody at a 1:5,000 dilution in 20 mM Tris (pH 7.6), 137 mM NaCl, and 0.05% Tween (TTBS buffer) containing 5% milk, followed by incubation with anti-rabbit IgG-horseradish peroxidase conjugate (1:5,000 dilution in TTBS) for 1 h at 25°C. The immunoreactive protein was detected with the Amersham (Arlington Heights, IL) ECL system.

25-OHC cytotoxicity assays

CHO-K1 cells were seeded at a density of 500 cells per 35-mm dish in F12FC5 on day 0. On day 1, the cells were rinsed twice with PBS and then fed with fresh F12FC5 containing various inhibitors, with or without 25-OHC (3 μg/ml), as indicated. After the treatment, cells were refed fresh F12FC5 and allowed to grow for 5 days. The surviving colonies were then fixed and stained with crystal violet.

RESULTS

Effect of oxLDL on cPLA$_2$ in mouse peritoneal macrophages

We examined the activation of cPLA$_2$ by oxLDL in resident mouse peritoneal macrophages. These cells have been shown (26) to have a calcium flux-activated cPLA$_2$. Furthermore, by using mouse peritoneal macrophages from cPLA$_2$-deficient mice, it has been confirmed that cPLA$_2$ mediates arachidonate release in response to diverse agonists in primary macrophages (27). As shown in **Fig. 1**, oxLDL induced significant release of arachidonic acid above control values in peritoneal macrophages. The arachidonic acid release was quantitatively suppressed by the cPLA$_2$ inhibitor AACOCF$_3$ (28). Because AACOCF$_3$ may also inhibit group VI calcium-independent phospholipase A$_2$ (iPLA$_2$), the effect of BEL was also determined because it inhibits iPLA$_2$ but not cPLA$_2$ (29–31). BEL had no effect on oxLDL-induced arachidonic acid release, consistent with the conclusion that oxLDL stimulates arachidonic acid release through activation of cPLA$_2$.

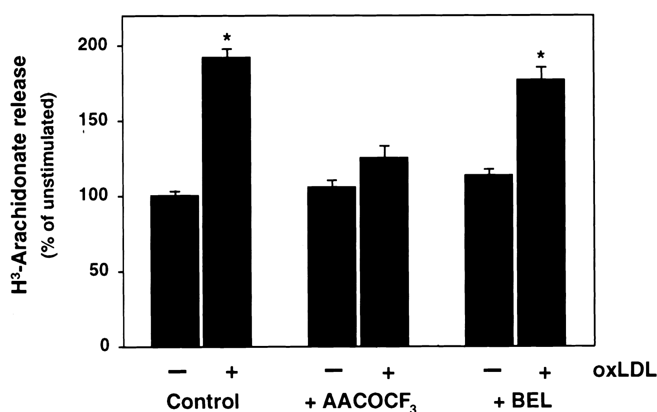

Fig. 1. Stimulation of arachidonate release by oxLDL in mouse peritoneal macrophages. Resident peritoneal macrophages were loaded with [^{3}H]arachidonate by incubation overnight (0.5 μCi/ml) and then treated with fresh culture medium with or without either 15 μM AACOCF$_3$ or 20 μM BEL for 1 h. Triplicate wells were then incubated for 2 h with 0.5 ml of medium supplemented with appropriate inhibitors as indicated and oxLDL (50 μg of protein per ml). The percentage of [^{3}H]arachidonate released in each case is normalized to the value obtained with unstimulated controls (7–8% released per h). A statistically significant ($P <$ 0.01) stimulation of arachidonate release by oxLDL is indicated by an asterisk.

If cPLA$_2$ is involved in the induction of apoptosis by oxLDL, it would be expected that macrophages from cPLA$_2$-deficient mice would be resistant to induction of apoptosis by oxLDL. We compared the susceptibility to apoptosis of mouse peritoneal macrophages prepared from wild-type and cPLA$_2$ knockout littermates. Apoptosis was assayed by the TUNEL method. The results (**Fig. 2A**) show that induction of apoptosis by oxLDL is significantly attenuated in macrophages prepared from cPLA$_2$ knockout mice. In three separate determinations, oxLDL treatment increased apoptosis by an average of 6.2-fold in macrophages from wild-type mice compared with 2.4-fold in macrophages prepared from the cPLA$_2$(−/−) littermates, a 73% reduction in the degree of apoptosis. The difference was statistically significant, using a paired *t*-test ($P = 0.03$). As expected, stimulation of arachidonate release by oxLDL was attenuated in the macrophages from the cPLA$_2$ knockout mice (Fig. 2B).

Effect of 25-OHC on arachidonate release and cPLA$_2$ activation in CHO-K1 cells

As documented above, it appears that, at least in part, the cytotoxic effects of oxLDL are mediated by an oxysterol component of the oxLDL. Previously, we have shown in both CHO-K1 cells and the THP-1 human macrophage cell line that an enhanced calcium flux is produced by treatment with 25-OHC and that this increase in calcium flux is required for the induction of apoptosis. Because calcium flux can activate cPLA$_2$, we determined whether 25-OHC stimulated arachidonate release in CHO-K1 cells

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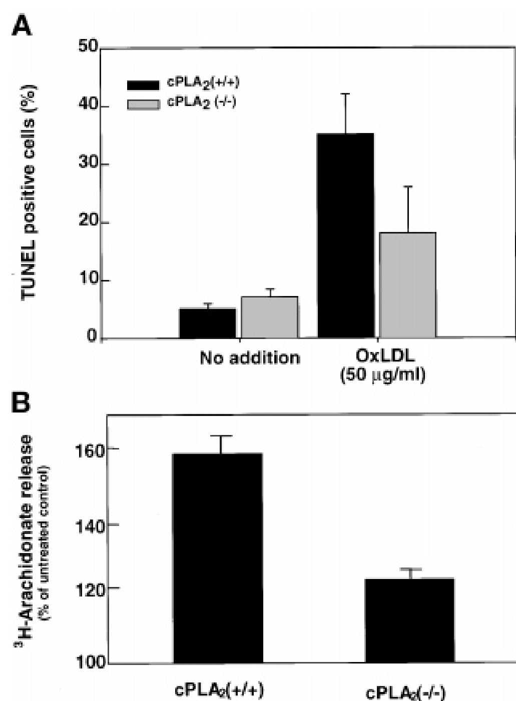

Fig. 2. Resistance to induction of apoptosis (A) and of arachidonate release (B) by oxLDL in peritoneal macrophages from $cPLA_2(-/-)$ mice. Apoptosis was measured by TUNEL assay after 48 h of treatment with oxLDL (50 μg/ml) or the arachidonate release was measured after 1 h of treatment of prelabeled macrophages with oxLDL (50 μg/ml) as described in Materials and Methods.

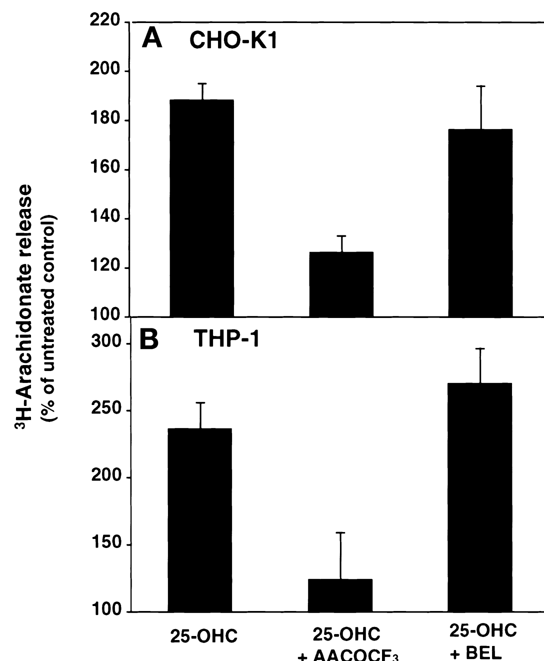

Fig. 3. Stimulation of arachidonate release by 25-OHC in CHO-K1 cells (A) and in THP-1 macrophages (B). Cells were labeled with [^{3}H]arachidonate for 24 h. After labeling, cells were washed three times with PBS, and incubated for 1 h with or without either $AACOCF_3$ (15 μM) or BEL (20 μM) as indicated. The cells were then incubated in fresh medium supplemented with appropriate inhibitor and with 25-OHC or vehicle only (control) for 5 h. The percentage of total labeled arachidonate released into the culture medium was determined as described in Materials and Methods. Treatment with neither BEL nor $AACOCF_3$ had a significant effect on the basal level of arachidonate release. In both cell types inhibition of 25-OHC stimulated arachidonate release by $AACOCF_3$ was statistically significant ($P < 0.01$).

and in THP-1 cells. In both cell lines (**Fig. 3**) arachidonate release was significantly increased by treatment with 25-OHC and the increase was blocked by $AACOCF_3$ but was not by the selective $iPLA_2$ inhibitor BEL. These results are consistent with $cPLA_2$ activation by 25-OHC treatment and with the concept that oxLDL exerts at least some of its biological effects through an oxysterol component. In response to 25-OHC treatment, arachidonate accumulation in the culture medium increased linearly for up to 8 h in these cell lines and could be observed to be elevated over untreated controls in as little as 1 h (data not shown).

We examined further the mechanism of activation of $cPLA_2$ by 25-OHC. In most cell lines studied, $cPLA_2$ is activated by an increase in cellular Ca^{2+} concentration, which results in a relocalization of the enzyme from the cytosol to the nuclear envelope/endoplasmic reticulum (32). To determine whether such relocalization of $cPLA_2$ occurs in CHO-K1 cells treated with 25-OHC, we utilized an EGFP-$cPLA_2$ fusion protein. We transiently transfected cells with the fusion construct and examined them by fluorescence microscopy 4 h after treatment with 25-OHC (5 μg/ml). Under these conditions, a dramatic relocalization of $cPLA_2$ was observed, consistent with the usual mechanism of Ca^{2+}-dependent activation. The appearance of typical transfected cells treated without (**Fig. 4A**) or with (Fig. 4B) 25-OHC is shown.

The other mechanism by which $cPLA_2$ is activated is through the phosphorylation of Ser-505 by mitogen-activated protein kinases (26, 32–34). Phosphorylation of $cPLA_2$ on Ser-505 induces a characteristic retardation in electrophoretic mobility (gel shift), which was evaluated by immunoblot (**Fig. 5**). As controls, lysates from unstimulated and okadaic acid-treated mouse peritoneal macrophages were also analyzed. Okadaic acid treatment results in stoichiometric phosphorylation of $cPLA_2$ on Ser-505 as evidenced by a complete gel shift (Fig. 5, lane 7). In contrast, $cPLA_2$ from unstimulated macrophages (Fig. 5, lane 1) is seen to be in the more rapidly migrating unphosphorylated form. Most of the $cPLA_2$ detected in untreated CHO-K1 cells was gel shifted, indicating that it is constitutively phosphorylated on Ser-505. Treatment of CHO-K1 cells with 25-OHC did not increase the proportion of $cPLA_2$ phosphorylated on Ser-505 (Fig. 5, lanes 2–6). With regard to the apparent dephosphorylation of $cPLA_2$ at the 48-h time point (Fig. 5, lane 6), we previously showed (21) that entrainment to apoptosis is complete at the end of 12 h of exposure to 25-OHC. Thus, we would expect that the signaling phase of apoptosis is over by then. The execution phase of apoptosis involves activation of many degradative enzymes. We would not expect that the dephosphorylation of $cPLA_2$ that occurs between 24 and 48 h is relevant to the signaling pathway of apoptosis, which is the subject of the current study. In unstimulated

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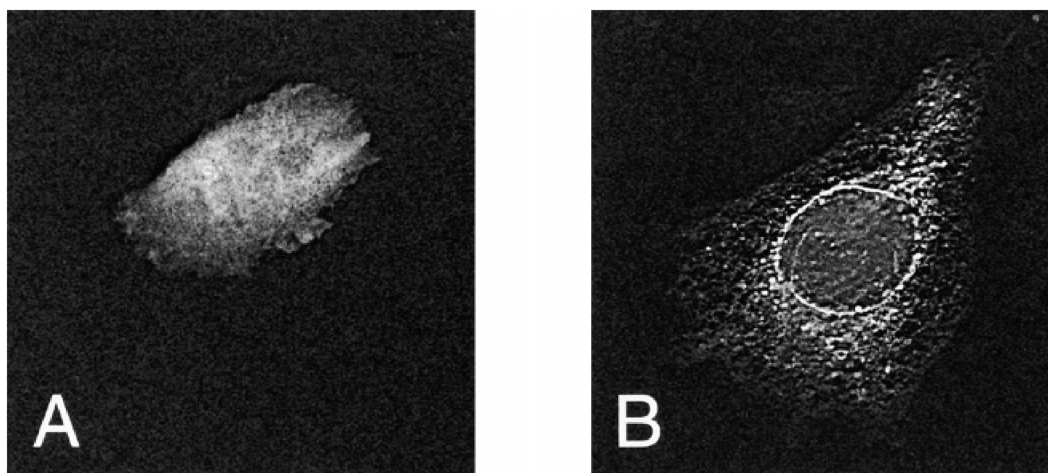

Fig. 4. Relocalization of an EGFP-$cPLA_2$ fusion protein after treatment of CHO-K1 cells with 25-OHC. Cells, on coverslips, were examined by fluorescence microscopy and digital deconvolution after transient expression of pEGFP-$cPLA_2$ for 48 h, followed by an additional 4-h incubation in the absence (A) or presence (B) of 25-OHC (5 μg/ml).

THP-1 macrophages, $cPLA_2$ was also found to be constitutively phosphorylated on Ser-505 (data not shown). This result is, again, consistent with the notion that the observed activation is mediated by Ca^{2+}-dependent relocalization of $cPLA_2$.

To test further the hypothesis that 25-OHC mediates arachidonate release through a Ca^{2+}-dependent process, we examined the effect of blockage of 25-OHC-stimulated Ca^{2+} uptake on arachidonate release. We have previously demonstrated (21) that the stimulation of Ca^{2+} uptake by 25-OHC is blocked by nifedipine (100 μM) in CHO-K1 cells and is constitutively defective in a 25-OHC-resistant CHO-K1 cell mutant, OX^R. We, therefore, examined the effect of 25-OHC treatment on arachidonate release in OX^R cells and in nifedipine-treated, wild-type, CHO-K1 cells. We found (**Fig. 6**) that in both models of inhibition of 25-OHC-stimulated Ca^{2+} uptake, arachidonate release was also inhibited.

Arachidonate metabolism is involved in 25-OHC-induced cytotoxicity in CHO-K1 cells

We have previously established that 25-OHC, at concentrations of 3 μg/ml or above, is cytotoxic to CHO-K1 cells in standard culture medium through an induction of apoptosis. To ascertain whether 25-OHC stimulation of arachidonate release signals the induction of apoptosis, we examined the effect of inhibition of arachidonate release by $AACOCF_3$ on 25-OHC-induced cytotoxicity in CHO-K1 cells by means of a single-cell plating assay. The results (**Fig. 7**) show that $AACOCF_3$ can block the cytotoxic response of CHO-K1 cells to treatment with 25-OHC.

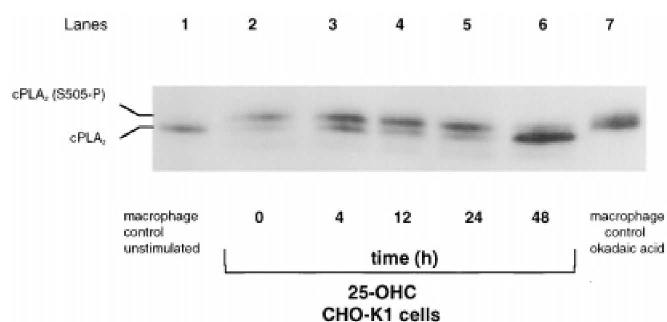

Fig. 5. 25-OHC treatment does not produce a change in phosphorylation status of $cPLA_2$ in CHO-K1 cells. Phosphorylation of $cPLA_2$ on Ser-505 was evaluated by Western blots of cell lysates (75 μg of protein) of CHO-K1 cells treated with 25-OHC (3 μg/ml) for 0, 4, 12, 24, or 48 h (lanes 2–6). Phosphorylation of $cPLA_2$ on Ser-505 retards its rate of migration. As controls, lysates (10 μg of protein) from unstimulated peritoneal macrophages (nonphosphorylated $cPLA_2$, lane 1) and macrophages treated for 90 min with 1 μM okadaic acid (phosphorylated $cPLA_2$, lane 7) are shown for comparison.

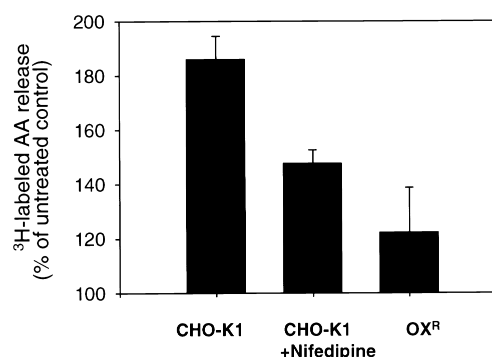

Fig. 6. Stimulation of arachidonate release is dependent on calcium uptake. CHO-K1 cells or 25-OHC-resistant mutant OX^R cells, which are defective in 25-OHC-stimulated Ca^{2+} influx, were labeled with [^{3}H]arachidonate (1 μCi/ml) and the percent release was determined as described in Materials and Methods. After labeling, cells were washed with PBS, refed with culture medium (F12FC5) supplemented with 100 μM nifedipine, where indicated, and incubated for 1 h. Cells were then treated with or without 25-OHC (3 μg/ml) and, where indicated, nifedipine. The results are expressed relative to controls not treated with 25-OHC. AA, arachidonic acid.

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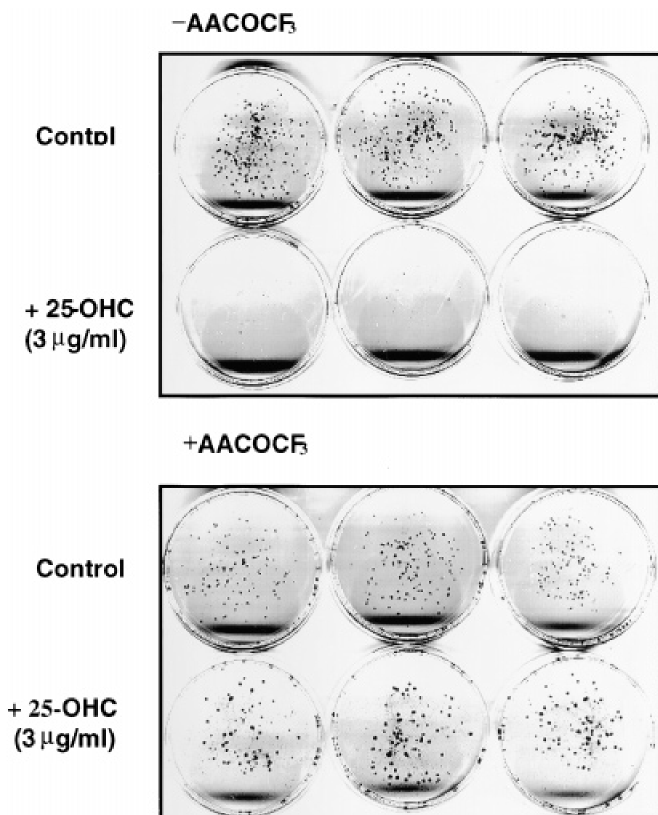

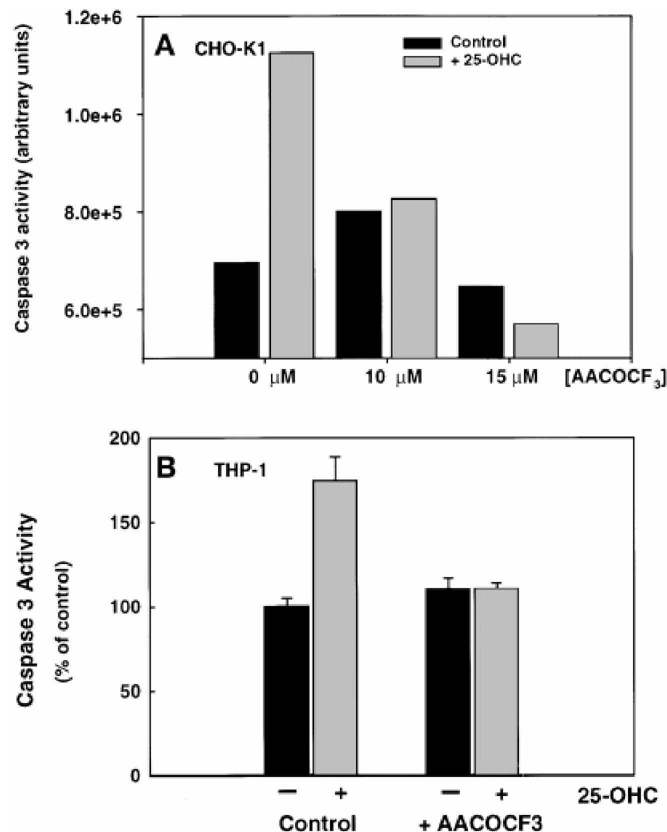

Fig. 7. The $cPLA_2$ inhibitor $AACOCF_3$ blocks the cytotoxic effect of 25-OHC. CHO-K1 cells were seeded at 500 cells per 35-mm dish and incubated overnight. The culture medium was then supplemented with $AACOCF_3$ (10 μM) and either 0.03% (v/v) ethanol or 25-OHC (3 μg/ml) in ethanol and incubated for 36 h. The medium was removed and the cells were washed with PBS and incubated for 5 days in standard culture medium (F12FC5) before being fixed and stained.

Fig. 8. The $cPLA_2$ inhibitor $AACOCF_3$ blocks the stimulation of caspase 3 activity by 25-OHC. CHO-K1 cells (A) or THP-1 macrophages (B) were treated with 25-OHC (3 and 5 μg/ml, respectively) in culture medium for 24 h before enzyme assay. The CHO-K1 results were determined in duplicate for 10 and 15 μM $AACOCF_3$, as shown. For the THP-1 cells, the values are expressed relative to untreated control (100%) and represent means ± SEM of enzyme activity of cells from three independent wells.

We have reported (35) that 25-OHC activates a caspase cascade in CHO-K1 cells, resulting in the canonical activation of the death protease caspase 3. Consistent with the results of Fig. 7, treatment with $AACOCF_3$ blocked 25-OHC activation of caspase 3 in CHO-K1 cells (**Fig. 8A**). We also examined the effects of inhibition of arachidonate release in nonproliferating THP-1 macrophages by assay of activation of caspase 3. The results (Fig. 8B) demonstrate that inhibition of arachidonate release with $AACOCF_3$ blocks caspase 3 activation by 25-OHC in the THP-1 macrophages as well.

The arachidonate released may act as a signal through oxidative conversion to other metabolic products. We, therefore, examined the effect of ETYA, which inhibits multiple pathways of arachidonate metabolism, on 25-OHC cytotoxicity. ETYA could be demonstrated to block the cytotoxic effects of 25-OHC in CHO-K1 cells (**Fig. 9**). This result is in agreement with the hypothesis that an oxygenated metabolite of arachidonate is involved in the signaling of the induction of apoptosis by 25-OHC. This result argues against the possibility that a lysophospholipid generated by $cPLA_2$ is involved in the induction of apoptosis by 25-OHC.

Prostaglandins, which arise from arachidonate through the action of a cyclo-oxygenase, are candidate products that could function in signaling pathways. In a preliminary experiment to test the possibility that a prostaglandin is the arachidonate product that signals apoptosis, we examined the effect of the cyclo-oxygenase inhibitor indomethacin on 25-OHC cytotoxicity. As was the case with ETYA, indomethacin blocked 25-OHC cytotoxicity (**Fig. 10**), consistent with the hypothesis that a prostaglandin could be in the signal transduction pathway of oxysterol-induced apoptosis.

DISCUSSION

In a prior report, we demonstrated that an early step in the apoptotic response to oxysterol was an influx of extracellular Ca^{2+} (35). Apoptosis has been reported to be signaled by sustained increases in intracellular Ca^{2+} (36). The signal transduction pathway, distal to sustained increases in intracellular Ca^{2+}, proceeds through the activation of the 85-kDa $cPLA_2$. Activation of $cPLA_2$ by increased intracellular Ca^{2+} occurs, at least in part, through

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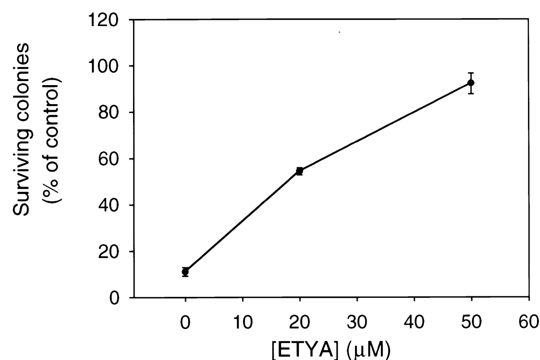

Fig. 9. The inhibitor of arachidonate metabolism, ETYA, blocks the cytotoxic effects of 25-OHC. CHO-K1 cells were seeded at 500 cells per 35-mm dish and incubated overnight. The cells were then refed with medium supplemented with various concentrations of ETYA, as shown, with or without 25-OHC (3 μg/ml), and incubated for an additional 36 h. The medium was then changed to standard culture medium and the cells were incubated for 5 days. The colonies derived from surviving cells were fixed, stained, and counted. The data are expressed relative to controls not treated with 25-OHC.

binding of Ca^{2+} to the C2 domain on $cPLA_2$ and its subsequent translocation to the nuclear envelope and endoplasmic reticulum (32). Activation of $cPLA_2$ is also partially mediated by mitogen-activated protein kinase cascades (33). Activation of $cPLA_2$, in turn, results in the formation of the biologically active eicosanoid products of arachidonate. Experiments with mice in which the $cPLA_2$ gene has been disrupted clearly demonstrate an obligatory role of $cPLA_2$ in eicosanoid production (37, 38).

In the current report we have examined these general concepts of Ca^{2+}-dependent $cPLA_2$-mediated apoptosis and have found results consistent with the induction of apoptosis by oxLDL and 25-OHC through such processes. OxLDL or 25-OHC could be shown to stimulate arachidonate release in primary peritoneal mouse macrophages or the human THP-1 macrophage cell line, respectively. The susceptibility of the release, in both systems, to inhibition by $AACOCF_3$ but not by BEL is consistent with the release occurring through activation of $cPLA_2$. Increasing the concentration of either compound, above the level used, did not increase the degree of inhibition of arachidonate release. Also consistent with this hypothesis is the observation that susceptibility of peritoneal macrophages from $cPLA_2(-/-)$ mice to stimulate arachidonate release in response to oxLDL treatment is attenuated.

That the arachidonate release is calcium dependent could be demonstrated in CHO-K1 cells, where we have previously demonstrated a requirement for calcium influx in the induction of apoptosis by 25-OHC (21). This conclusion is supported by the loss of arachidonate release in response to 25-OHC after treatment with nifedipine, which we have previously shown blocks calcium influx and apoptosis in response to 25-OHC treatment in CHO-K1 cells and in THP-1 macrophages (21). This conclusion is also supported by the loss of stimulation of arachidonate release in 25-OHC-resistant OX^R cells, which do not increase calcium uptake in response to 25-OHC treatment (21). The calcium dependence of the stimulation of arachidonate release is also characteristic of $cPLA_2$.

Furthermore, by genetic knockout in primary peritoneal macrophages and by pharmacological blockade in THP-1 macrophages, loss of $cPLA_2$ prevented the induction of apoptosis by oxLDL and 25-OHC, respectively. Blockage of arachidonate release also eliminated the cytotoxic effects of 25-OHC in CHO-K1 cells. These observations are consistent, with oxLDL and 25-OHC activating $cPLA_2$ and also suggest that the activation of this enzyme is in the apoptotic signal transduction pathway triggered by these agents. The lack of complete prevention of induction of either the arachidonate release or apoptosis in $cPLA_2(-/-)$ macrophages, however, points to the existence of alternative mechanisms for these phenomena.

PLA_2 enzymes have been proposed to play a role in mediating apoptosis in various models, although the specific PLA_2 enzyme involved appears to depend on the cell model and the agents used to induce cell death (39). $cPLA_2$ has been shown to be involved in cell death induced by TNF in cells sensitized to killing with either ade-

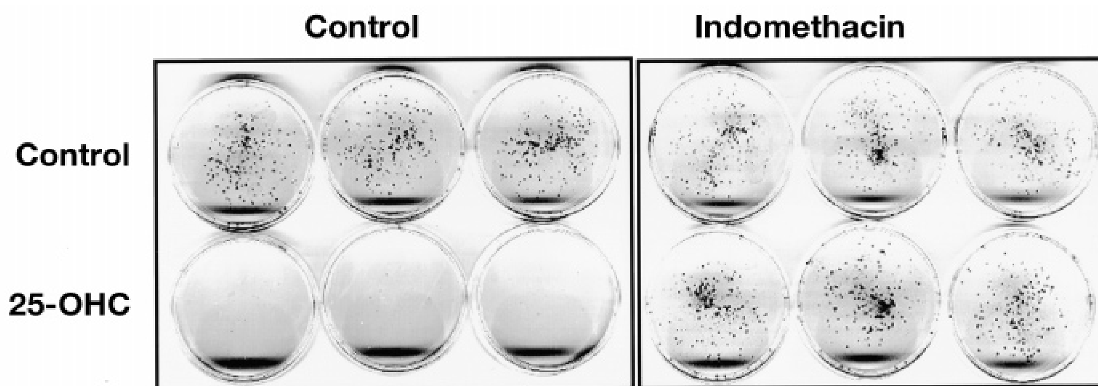

Fig. 10. The cyclo-oxygenase inhibitor, indomethacin, blocks the cytotoxic effects of 25-OHC. CHO-K1 cells were seeded at 500 cells per 35-mm dish and incubated overnight. The medium was then refreshed with medium supplemented with indomethacin (50 μM), with or without 25-OHC (3 μg/ml), and incubated for an additional 36 h. The medium was then changed to standard culture medium and the cells were incubated for 5 days. The colonies derived from surviving cells were fixed and stained.

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novirus infection or inhibitors of transcription/translation (40, 41). $cPLA_2$ has also been shown to be crucial for TNF-induced cytotoxicity in a TNF-sensitive cell line, L929, but not to be involved in Fas-mediated apoptosis (22, 42). It has been shown that $cPLA_2$ is cleaved and inactivated by caspases in HeLa cells undergoing apoptosis and during Fas-mediated cell death in U937 cells (43, 44). $cPLA_2$ has cleavage sites for caspases 1 and 3 and proteolysis at these sites disrupts the catalytic diad and inactivates the enzyme (44, 45). Rather than $cPLA_2$, which is inactivated, a role for the group VI $iPLA_2$ in Fas-mediated apoptosis has been proposed on the basis of the observation that $iPLA_2$ inhibitors suppress induction of arachidonic release and apoptosis by Fas. $iPLA_2$ also contains a caspase cleavage site but this cleavage enhances its catalytic activity (46). The enhanced $iPLA_2$ activity is proposed to accelerate turnover of phospholipids that may influence membrane changes that occur during apoptosis.

Our results clearly demonstrate that oxysterol-induced apoptosis is a calcium-dependent process that involves activation of $cPLA_2$. On the basis of the Western blot of $cPLA_2$ (Fig. 5), 25-OHC does not appear to induce a degradation of $cPLA_2$ in CHO cells, unlike the finding in certain other models of apoptosis (44, 45). It is notable that even after 24 h of 25-OHC exposure, a period in which we have shown 25-OHC activation of caspase 3 to be maximal (35), there is no loss of signal intensity of intact $cPLA_2$ band(s).

Arachidonate can be oxidatively metabolized to diverse biologically active products. ETYA blocks all of the known oxidative metabolic pathways including cyclo-oxygenase and all lipoxygenases (47–49) as well as cytochrome P-450-dependent arachidonate metabolism (50). Rescue of CHO-K1 cells from killing by 25-OHC by ETYA is, therefore, consistent with a metabolite of arachidonate being a second message of oxLDL/oxysterol-induced apoptosis but does not indicate which arachidonate oxidative pathway is involved. It has been reported that 25-OHC treatment of arterial endothelial and smooth muscle cells produces a dramatic conversion of labeled arachidonate into total prostaglandins (51) and this is due, at least in part, to upregulation of prostaglandin G/H synthase 2 (51, 52). These reports fit with our finding that 25-OHC upregulates $cPLA_2$ and suggest that a prostaglandin may be the arachidonate-derived second messenger of apoptosis. Our observation of the inhibition of 25-OHC cytotoxicity by the cyclo-oxygenase inhibitor indomethacin is also consistent with this hypothesis.

We have previously demonstrated that the killing of CHO cells by 25-OHC, seen in single-cell plating assays described in Figs. 7 and 10, is due to apoptosis (21, 35). When the initiation of the signaling pathway of apoptosis induced by 25-OHC is blocked by either $AACOCF_3$ or indomethacin, CHO fibroblasts are completely rescued from the killing effect of oxysterol. This finding suggests that apoptosis is the major cytotoxic mechanism by which 25-OHC kills CHO cells at the concentration and culture conditions used in our experiments. We had observed similar total rescue of the plating efficiency of CHO cells treated with either oxLDL or 25-OHC when the Ca^{2+} influx is blocked (21). The fact that cells that are resistant to induction of apoptosis by 25-OHC (such as wild-type CHO cells incubated in Ca^{2+}-deficient media or in the presence of nifedipine, or OX^R cells that are defective in Ca^{2+} uptake) are cross-resistant to killing by both 25-OHC and oxLDL is, in our opinion, strong evidence supporting the notion not only that oxysterols are the component in oxLDL that induces apoptosis in CHO cells but also that killing occurs entirely through apoptosis at appropriate concentrations of oxysterol and incubation periods in these cells. We hasten to add that these conclusions do not rule out the possibility that the response of other cell types to oxLDL and its various lipid components could be quite different. Nevertheless, CHO fibroblasts appear to constitute a valuable model with which to explore the detailed mechanisms of oxLDL- and oxysterol-induced cell death.

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