Akt/PKB regulation of Bcl family members during oxysterol induced apoptosis.

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Running Title: Oxysterols increases Akt degradation.
**Abbreviations:** 25-hydroxycholesterol (25-OHC), arachidonyltrifluoromethyl ketone (AACOCF₃), 5,8,11,14-eicosatetraynoic acid (ETYA), oxidized low density lipoprotein (oxLDL), proteasome inhibitor I (PI-I).
Summary: Cells of the vasculature, including macrophages, smooth muscle cells and endothelial cells, exhibit apoptosis in culture upon treatment with oxLDL, as do vascular cells of atherosclerotic plaque. Several lines of evidence support the hypothesis that the apoptogenic component of oxLDL is one or more oxysterols which have been shown to induce apoptosis through the mitochondrial pathway. Activation of the mitochondrial pathway of apoptosis is regulated by members of the Bcl family of proteins. In this report, we demonstrate that in a murine macrophage-like cell line, P388D1, oxysterols (25-hydroxycholesterol and 7-ketocholesterol) induce the degradation of the pro-survival protein kinase, Akt (PKB). This, in turn, leads to the activation of the BH3 only domain proteins Bim and Bad and down-regulation of the anti-apoptotic multi-Bcl homology domain protein Bcl-XL. These responses would be expected to activate the pro-apoptotic multi-Bcl homology domain proteins Bax and Bak leading to the previously reported release of cytochrome c observed during oxysterol induced apoptosis. Somewhat surprisingly, si-RNA knockdown of Bax resulted in a complete block to the induction of apoptosis by 25-hydroxycholesterol.
**Introduction:** Many of the pathological events associated with the development of atherosclerosis are believed (1) to be mediated by oxidized low density lipoprotein (oxLDL). The constitutive uptake by macrophage of oxLDL is through specialized scavenger receptors which results in these cells becoming lipid-laden, foam cells (2). The formation of such cells is the hallmark of atherosclerosis. Furthermore, oxLDL has been shown to be cytotoxic to macrophage (3) through a process requiring such receptors (4,5). This cytotoxicity could be very important in the atherogenencity of oxLDL through the lysis of foam cells and concommitant deposition of lipids in the coronary vasculature. The cytotoxic effects of oxLDL have been shown to proceed, at least in part, through apoptotic pathways, in general, (reviewed in 6 and 7) as well as in macrophages, in particular (3, 8,9).

The cholesterol oxidation products (oxysterols) found in oxLDL (10) have been recognized as a probable basis for its cytotoxicity (11,12), at least in part, via apoptotic mechanisms (7,13,14). A model compound, for such oxysterols, is 25-hydroxycholesterol (25OHC), which has been shown to induce apoptosis in monocyte-macrophage(9,15,16) and lymphoid cell lines(17,18) in the range of 1-10 μM. Prior studies have been consistent with the activation of the mitochondrial death pathway by oxysterols with its canonical cytochrome c release (19,20). Cytochrome c release from mitochondria is regulated, in turn, through the activation of pro-apoptotic Bcl family members, along with possible inactivation of anti-apoptotic Bcl family members (21). In this manuscript we describe the role of
the Akt regulated Bax/Bad pathway in oxysterol induced apoptosis of murine macrophage cell lines.
Experimental Procedures:

Materials - RAW 264.7 was purchased from ATCC (Rockville, MD). P388D1 cells (MAB variant- ref.22) were provided by Dr. Edward Dennis, University of California, San Diego. RPMI-1640 and DMEM were from GIBCO, BRL (Rhode Island, NY), NovaCell I fetal bovine serum (FBS) was from Nova-Tech (Grand Island, NE). All other cell culture reagents were obtained from GIBCO BRL. 5,8,11,14-Eicosatetraynoic acid (ETYA), Ac-DEVD-CHO, AC-DEVD-AFC and AACOCF₃ were purchased from BIOMOL (Plymouth Meeting, PA). Oxysterols were purchased from STERALOIDS, Inc. (Wilton, NH). All antibodies were purchased from Cell Signaling Technology, Inc (Beverly, MA) with the exception of anti-Akt1/2 from Santa Cruz Biotechnology (Santa Cruz, CA), anti-myc from Upstate (Charlottesville, VA) and anti-HSP70 from StressGen (Victoria BC, Canada). Peroxidase-conjugated secondary antibodies were from Pierce (Rockford, IL). pEGFP-C3 was from Clontech. Proteasome Inhibitor I was from Calbiochem (San Diego, CA). Mitotracker™ was from Molecular Probes.

Cell Culture - All cell lines were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Raw 264.7 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 10mM Hepes buffer (pH 7.4), 2mM glutamine, 1mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol. P388D1 cells were cultured in DMEM medium
supplemented with 10% FBS, 2mM glutamine, 1mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Caspase 3 Assay- P388D1 cells were seeded at 2 x10^6/well in12-well culture plates and RAW 264.7 cells at 2.5x10^6/well in 6-well culture plates. The media was supplemented with 25OHC dissolved in ethanol or an equivalent volume of ethanol alone (control treatments). Inhibitors (ETYA or AACOCF₃) were added to the media 2h prior to the addition of 25OHC. Following an 18h incubation, the adherent and nonadherent cells were collected by scraping and centrifugation at 1,000 x g for 5 min. The cells were washed with ice-cold PBS, resuspended in lysis buffer (10mM Tris (pH 7.5), 130mM NaCl, 1% Triton X-100, 10mM NaPi, and 10mM NaPPi), incubated on ice for 10 min and centrifuged at 12,000 x g for 20 min at 4°C. The supernatant or extract was assayed for protein using the micro-BCA kit (Pierce, Rockford, IL) and for caspase 3 activity as follows. Equivalent amounts of each sample were incubated for 2.5 h at 37° C in caspase assay buffer (20 mM Hepes, pH 7.5, 10% glycerol, 2mM dithiothreitol) containing 5 µM caspase-3 substrate (Ac-DEVD-AFC) in the presence and absence of a caspase 3 specific inhibitor, Ac-DEVD-CHO, added at 100 nM 30 minutes prior to the addition of the substrate. Liberated AFC was measured using a spectrofluorometer (FluroMax 3 equipped with microplate reader, Jobin Yvon Inc.) with an excitation wavelength of 400 nm and an emission wavelength of 505 nm. For each sample, the net Caspase 3 activity was determined by subtracting the relative fluorescent light units (RFLUs) obtained in the presence of the
inhibitor from the RFLUs obtained in the absence of the inhibitor and normalizing to the protein content of the sample. Each treatment was done in triplicate and the data is presented as the mean ± S.D.

**Bad-GFP fusion protein** - Bad open reading frame was excised from pEBG-mBad (Cell Signaling Technology) with HindIII and Not I and ligated in frame into pEGFP-C3 after the creation of an appropriate Not I site by using the Quick Change Kit from Stratagene. The finished construct was confirmed by sequencing and transfected into RAW 264.7 cells using GeneJammer from Stratagene.

**Detection of Bad-GFP in RAW 264.7 cells** - Transiently transfected RAW 264.7 cells were incubated overnight on glass chambered coverslips. Cells were then washed and incubated with 25OHC (10 µg/ml) for 6 h. After washing with PBS, cells were observed live under fluorescence microscope. In co-localization experiments, mitochondria were also stained with MitotrackTM red (Molecular Probes) following manufacturer’s instructions. Confocal images were obtained by digital deconvolution of 10-slice stacks acquired on a Nikon Diaphot 200 equipped with a Photometrics Sensys cooled CCD digital camera or Nikon D100, Oncor Z-drive and Oncor Image software.

**Bax gene suppression** - Bax gene suppression was achieved by stably transfecting cells with pSi-Bax, a plasmid that generates small interfering RNAs (si-RNAs) that target Bax mRNA for degradation. To produce pSi-Bax, the
following complementary oligonucleotides were annealed and cloned into Apa I/EcoR I digested pSilencer (Ambion, Austin, TX). Bax 1: ACTGGTGCTCAGGCCC TGTTCAAGAGACAGGGCCTTGAGCACCAGTTTTTTT and Bax 2: AATTAAAAAACGCTGATCAAGGCCCTGTCTCTTGAACAGGGCCTTGAGCACCAGTGGCC. The negative control vector, pSi-RandomBax, was constructed by ligating annealed, custom synthesized oligonucleotides of randomized Bax target sequence (RandomBax 1: ACCGCTCGAGCGTGCTAGTTTCAAGGAAA CTCAGCACTCGAGCGTCTCTCTTGAAAACGCGTCTGAGCGGCTTGAGC and RandomBax 2: AATTAAAAAACGCTCGAGCGTGCTAGCTCTCTTGAAAACGCGTCTGAGCGGCTTGAGC) into Apa I/EcoR I digested pSilencer.

P388D1 cells (1 X 10⁶) were co-transfected with a plasmid carrying the neomycin gene, pEGFP (Clontech, Palo Alto, CA) and either pSi-Bax or pSi-RandomBax using Lipofectamine PLUS (InVitrogen, Carlsbad, CA) according to the manufacturer’s directions. Stable clones were first selected by infinite dilution in media containing 1 mg/ml G418 for 7 days followed by 14 days in 0.25 mg/ml G418. G418 resistant clones were screened by PCR for integration of the pSilencer vector. The PCR positive-G418 resistant clones were then subjected to selection in media containing 10 µg/ml 25OHC for 68 h. The surviving cells were expanded and maintained in media containing 500 µg/ml G418. No 25OHC resistant clones were obtained from PCR positive-G418 resistant pRandomBax clones. The suppression of Bax expression was determined by immunoblotting whole cell lysates prepared from the isolated clones, wild type cells and G418 resistant pRandomBax clones using a Bax specific antibody (Cell Signaling
Expression of constitutively active Akt- P388D1 cells were stably transfected with a vector expressing a Myc-His tagged mouse Akt1 (activated) under the control of the CMV promoter (Upstate, Charlottesville, VA) using Lipofectamine PLUS according to the manufacturer’s directions. Stable clones were isolated by selection in media containing 1 mg/ml G418 for 6 days followed by 10 days in 250 µg/ml G418. The G418 resistant clones isolated, expanded and screened for expression of the Myc-Akt fusion protein by immunoblotting with a total Akt1/2 antibody (Cell Signaling Technology) and a Myc specific antibody (Upstate). Myc-Akt positive clones were maintained in media containing 250 µg/ml G418.

For transient transfection experiments, P388D1 cells were seeded at 2 X 10⁶ cells/well in 6-well tissue culture plates. Twenty-four hours later the cells were rinsed with PBS, refed standard growth media and transfected with pUSEamp or pUSEamp containing myr-Akt cDNA using Tojene™ transfection reagent (Avanti Polar Lipids, Inc) according to the manufacturer’s directions. Following transfection, expression of the transfected construct was allowed to proceed for 24 h prior to the addition of oxysterol and analysis of Caspase 3 activity.

Cell lysis and Immunoblotting- P388D1 cells were grown to a density of 2x10⁶/ml in regular growth medium supplemented with either vehicle (ethanol) or increasing amounts of 25OHC. After different periods of time, cells were spun and treated with lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-
Glycerolphosphate, 1 mM Na₃VO₄, 1 µg/ml Leupeptin) and incubated on ice for 30 min. Insoluble debris was removed from the extracts by centrifugation for 10 min at 10,000xg and the protein concentration in the supernatants determined by micro BCA assay (Pierce). Proteins were resolved by SDS-PAGE on 4-12% NuPAGE gels (InVitrogen) and transferred to PVDF membranes (Immobilon-P, Millipore). The membranes were stained with SYPRO® Ruby protein stain (Molecular Probes, Inc, Eugene, OR) to ascertain equivalent loading of the gel and efficient transfer of proteins to the membrane before immunoblotting. The blots were then processed using antibodies specific for the protein of interest and the appropriate peroxidase-conjugated secondary antibodies per manufacturer’s directions. The proteins of interest were visualized by enhanced chemiluminescence using SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) as directed.

**Pulse-chase experiments**- P388-D1 cells were grown in methionine deficient medium for 2 h and then pulsed with Tran³⁵Slabel (100 µCi/ml) (ICN) for three hours. Cells were then washed and chased for different time periods in regular growth medium containing 1mM cold methionine and either vehicle (ethanol) or 10 µg/ml 25OHC. Cells were then lysed as described above and subjected to immunoprecipitation with anti-Akt antibodies using assays were performed using Seize™ Coated Plate Immunoprecipitation Kits from Pierce (Rockford, IL) according to manufacturer’s instructions. After binding, washing, and elution, presence of radiolabeled Akt in the precipitates was examined by SDS-PAGE and phosphorimaging.
Akt Kinase assay- The active form of Akt was measured using a non-radioactive Akt Kinase assay kit from Cell Signaling Technology following the manufacturer’s instructions. Essentially, an antibody to Akt was used to selectively immunoprecipitate Akt from cell lysates. The immunoprecipitate was then incubated with GSK-3β fusion protein in the presence of ATP and Kinase Buffer, which allows immunoprecipitated Akt to phosphorylate GSK-3β. Phosphorylation of GSK-3β was then measured by immunoblotting using a phospho-GSK-3α/β (Ser21/9) antibody and quantitated by phosphorimaging.
Results:

*25OHC induces apoptosis in P388D1 cells through a process dependent on arachidonate metabolism* - We have previously characterized oxysterol induction of apoptosis in both a fibroblast cell line, CHO-K1 and a monocyte-macrophage cell-line, THP-1, as being dependent on arachidonate release and metabolism (9). Because of difficulties we encountered in transfection of THP-1 cells, and the well-established use of the murine macrophage P388D1 cells in studies of arachidonate metabolism (22), we determined whether P388D1 cells undergo apoptosis in response to oxysterols in a similar fashion. We confirmed a prior report (23) that 25-OHC induces apoptosis in P388D1 cells and also demonstrated, as was observed for the other cell lines, that the induction of apoptosis is blocked both by the cPLA$_2$ inhibitor, arachidonyltrifluoromethyl ketone (AACOCF$_3$) and by ETYA, an inhibitor of arachidonate metabolism (Figure 1).

*25OHC downregulates Akt in P388D1 cells* - Akt (PKB) has been well characterized as an anti-apoptotic kinase (24) which transduces cellular survival signals in many cell-types (25). In particular, a critical role for Akt has been ascribed to the survival of macrophages (26). We, therefore, examined the effect of overnight treatment with 25OHC on the activity of Akt in the murine macrophage cell line, P388D1. Activity was assayed with glycogen synthase kinase-3β (GSK-3) as substrate. The results (Figure 2A) clearly indicate that there is a down-regulation of Akt activity in P388D1 cells in response to treatment with 25OHC.
The mechanism of down-regulation of Akt was explored by immunoblot and radio-immunoprecipitation experiments. Immunoblot analysis (Figure 2B) demonstrated that oxysterol treatment produced a reduction in the level of total Akt in P388D1 cells. Pulse-chase radio-immunoprecipitation studies revealed that 25OHC treatment greatly enhanced the rate of degradation of Akt (Figure 2C). Furthermore, the effect is observed with no time lag after 25OHC addition suggesting that this is an early signaling event. We also examined the effect of treatment with a proteosome inhibitor (PI-I) on the cellular levels and activity of Akt in 25OHC treated cells. This was done to determine whether this enhanced degradation rate was responsible for the decrease in its activity, as well as to gain insight into the mechanism of regulated degradation. The results (Figure 2A, 2D) demonstrate that inhibition of Akt degradation significantly attenuates the loss in its activity in response to 25-OHC treatment. Therefore, the primary mechanism by which 25-OHC down-regulates Akt activity appears to be through a stimulation of its rate of degradation. This mechanism of degradation is also consistent with an early, regulated event.

We wanted to confirm this putative critical role for Akt signaling in 25-OHC induced apoptosis. Therefore, we examined the effect of expression of a constitutively active myc-tagged form of Akt (myr-Akt, ref.27) on the activation of caspase 3 by 25OHC treatment. We isolated a clone, clone B, stably expressing transfected myr-Akt (Figure 3A). Akt activity in clone B was shown to be elevated approximately 2-fold relative to wild-type P338D1, as could be demonstrated in vitro by enzyme assay (Figure 3B) both in untreated and 25-OHC treated cells.
Clone B was also seen to be relatively resistant to induction of apoptosis by 25-OHC or 7-ketocholesterol, as measured by caspase-3 activity (Figures 3B, 3C). To guard against the possibility that this result was due to another genetic variation in clone B, the ability of myr-Akt to protect cells from oxysterol induced caspase-3 activation was also demonstrated by transient transfection with the same construct (Figure 3D).

**Effect of 25-OHC treatment on BH3-only domain proteins: activation of Bad and increased cellular levels of Bim-** A common mechanism by which Akt inactivation is coupled to apoptosis is through the regulation of Bad by Akt (25). Akt phosphorylates Bad on two serine residues resulting in its sequestration in cytosol, bound to 14-3-3 (28-30). In the absence of ongoing Akt catalyzed phosphorylation, Bad becomes dephosphorylated by any of several phosphatases (31). This results in its relocalization to the mitochondria (32-34) where it heterodimerizes via its BH3 domain (35) with the anti-apoptotic Bcl family members Bcl-2 and Bcl-X\textsubscript{l} (36). These are critical signaling events in the mitochondrial apoptotic pathway which result in the canonical cytochrome c release (37). Since we have previously demonstrated that oxysterols mediate cytochrome c release from mitochondria during apoptotic induction in other cell-types(19), we anticipated that a consequence of the observed Akt degradation would be an activation of Bad.

Bad activation can be detected either through determination of its dephosphorylation or by its relocalization. We examined both processes. Immunoblot of Bad from 25OHC treated P388D1 cells with a phospho-Bad
specific antibody is consistent with a loss of phosphorylation (Figure 4). In clone B, BAD was observed to be hyperphosphorylated both in untreated and 25OHC treated cells (Figure 4) which demonstrates the activity, and, in particular, the anti-apoptotic activity, of the myr-Akt construct in whole cells. Immunoblots of total mitochondrial Bad are consistent with an increase in the mitochondrial levels of Bad after 25-OHC treatment (data not shown). Relocalization of Bad was also demonstrated by transfection of RAW 264.7 murine macrophage with a GFP-Bad fusion protein. Such a reporter has previously been utilized to demonstrate Bad activation in another system (33). RAW 264.7 cells were used for this experiment because they grow attached and spread out on the surface of the plastic petri dish allowing better resolution of the cellular distribution of the fluorescent fusion protein. The results (Figure 5) indicate a redistribution of Bad from a diffuse cytosolic localization to a punctate distribution largely co-localized with the mitochondrial marker Mitotracker. This is precisely the result to be expected for the relocalization of Bad associated with dephosphorylation (33).

Korsmeyer and colleagues (38) have presented evidence for two classes of BH3 only domain proteins. The first category is exemplified by Bad which functions through “sensitization” towards apoptosis by heterodimerization, and inactivation, of antiapoptotic multidomain Bcl family members. The second category of BH3-only domain proteins activate the multidomain proapoptotic Bcl family members by inducing their oligomerization to release cytochrome c. Expression of one of these proteins, Bim, has been shown to be down-regulated by Akt (39). We, therefore, examined P3888D1 cells for increased expression of
Bim, by immunoblot, after 25-OHC treatment. The results (Figure 6) are consistent with an increase in Bim levels in cells and in mitochondria.

*Role of multi- Bcl homology domain proteins in induction of apoptosis by 25OHC*-

Release of cytochrome c from mitochondria in response to signaling by the BH3-only domain proteins would be expected to involve two downstream events: the inactivation (sensitization) of the antiapoptotic Bcl multidomain proteins and the activation of the proapoptotic Bcl multidomain proteins. As mentioned above, the potential targets of Bad are Bcl-2 and Bcl-XL. However, it has been reported that Bad can only reverse the death repressor activity of Bcl-XL (36). Furthermore, immunoblot analysis indicated that P388D1 cells do not express Bcl-2 (data not shown). This suggests that the target of Bad during 25OHC-induction of apoptosis is Bcl-XL. It has been reported (39) that Akt up-regulates the expression of Bcl-XL suggesting the possibility that 25OHC treatment might down-regulate its expression. Immunoblots of Bcl-XL, after 25OHC treatment, demonstrated that this did occur (Figure 7A). Down regulation of Bcl-XL mRNA levels by 25OHC, as determined from Northern blots, was also observed (data not shown).

As described above, current thinking (38,40) on the mechanism of Bad signaling in mitochondria is that its binding to Bcl-XL would in turn activate the multidomain pro-apoptotic Bcl family members Bax and Bak to release cytochrome c (36,41). Immunoblot analysis demonstrated that P388D1 cells express both Bak and Bax, with Bax having the usual (41) dual cytoplasmic and mitochondrial localization and Bak being found only in mitochondria (data not
shown). It has been shown that Bax and Bak have over-lapping functionality but that suppression of Bax attenuates the apoptotic response to a variety of stimuli that act through the intrinsic pathway (42). To confirm that the Bax/Bak pathway operates as expected in the induction of apoptosis by oxysterols we examined the effect of si-RNA knockdown of Bax on the apoptotic response to 25-OHC. After selection (see Experimental Procedures), we were able to isolate a clone of P388D1 cells (clone #1) which appeared to be highly reduced in Bax expression as determined by immunoblot (Figure 7B). This clone also exhibited no detectable apoptotic response to 25-OHC (Figure 7C).

In contrast, prolonged exposure of clone B to 25-OHC still results in cell death and caspase-3 activation (Figure 8A). This is apparently due to eventual degradation of the over-expressed, constitutively active Akt resulting in a significant decrease in its cellular levels, as measured by immunoblot (Figure 8B). This result is also consistent with the expression of constitutively active Akt being the cause of the oxysterol resistance in clone B rather than an artifact of clone selection.

**Discussion:**

The results presented in this report are consistent with the conclusion that an important regulatory event in the induction of apoptosis by oxysterols is accelerated degradation of Akt. The activity of Akt has most commonly been described to be regulated by phosphorylation (24). In one well characterized apoptotic signal transduction pathway, that induced by ceramide, activation of
PP2A, leading to dephosphorylation of Akt, plays a critical role (43). However, there have been other reports of regulated Akt degradation playing a role in apoptotic signal transduction pathways. For example, H$_2$O$_2$ induced apoptosis is accompanied by degradation of Akt (44) as is the induction of apoptosis by the prostaglandin 15dPGJ$_2$ (45) both probable examples of apoptosis with reactive oxygen species (ROS) second messengers (44,46). It is worth noting, in this context, that, ROS have been suggested to play a role in oxysterol induced apoptosis (14,20).

An important consideration is whether the activation of Akt degradation is upstream or downstream of the many caspases activated during apoptosis. Our data are consistent with activation of Akt degradation being an early event. First, the degradatory response is observed in as little as 30 minutes after 25-OHC addition (Figure 2C), whereas we have previously observed caspase-3 activation to be undetectable in response to 25-OHC treatment in as much as 5h (19). Second, the inhibition of degradation by a proteasome inhibitor (Figure 2D) is consistent with a non-capsase mediated degradatory process. Finally, and most tellingly, over-expression of a constitutively active Akt (myr-Akt) in clone B delays the activation of caspase-3 by 25-OHC, consistent with caspase activation being downstream of Akt degradation rather than the other way around. It is also noteworthy, that the eventual degradation of myr-Akt produced by 25-OHC treatment can still result in capsase-3 activation, consistent with a temporal pattern of Akt degradation preceeding capsase-3 activation.
The responses of the various Bcl family studied in this report are predictable from the loss in Akt activity. The role of Akt in phosphorylation and inactivation of Bad is well documented (25, 28-36) and, therefore, it is not surprising that loss of Akt results in dephosphorylation of Bad (Figure 4A) and its relocalization to the mitochondria (Figures 4B, 5) where it would be expected to heterodimerize with the multidomain anti-apoptotic Bcl family members. Further sensitization to apoptotic induction is achieved through loss of Akt because of its role in signaling transcriptional up-regulation, through the NF-κB pathway (47-49), of the antiapototic multidomain Bcl family member, Bcl-X$_l$ (39, 50).

Transcriptional down-regulation of the BH3-only domain protein, Bim, by Akt has also been reported (39) and our observation of increased Bim expression in whole cells and mitochondria (Figure 6), in response to 25-OHC, is consistent with this prior work. In the mitochondrial apoptosis pathway, Bim functions to stimulate the oligomerization of the proapoptotic multidomain Bcl family members, Bax and Bak, thereby producing the release of cytochrome c (38).

Sensitization to apoptotic induction by downregulation of Bcl-X$_l$ and relocalization of Bad to mitochondria and expression of Bim should lead to activation of Bax/Bak. Somewhat surprisingly, rather than attenuation of apoptosis in response to 25-OHC treatment after siRNA knockodown of Bax, we were able to isolate a clone with no detectable Bax by immunoblot which exhibited no detectable apoptotic response (Figure 8). It has been reported that some stimuli, notably staurosporine, which operate through the intrinsic apoptotic
pathway are more responsive to Bax knockout than Bak knockout (42) and we may be observing such a differential response to 25-OHC.

It has been reported (51) that another oxysterol, 7-ketocholesterol, activates apoptosis in fibroblasts through a signal transduction pathway that requires phosphorylation of Stat1 on Ser-727. This is noteworthy since some signal transduction pathways to Stat1 serine phosphorylation proceed through Akt (52). However, reports have appeared that have implicated other kinases in Stat1 serine phosphorylation including p38 MAP kinase and PKC (53,54). Ser(727) phosphorylation in macrophages, in particular (54), seems to be more dependent on these other kinases.

While this manuscript was under review, a report appeared (55) describing the Ca++-release mediated activation of the endoplasmic reticulum unfolded protein response (UPR) pathway during the induction of apoptosis by free cholesterol loading of macrophage. The ER was interpreted to be the site of cholesterol damage leading to the calcium signal. The oxysterol induction of apoptosis appears to be different, at least in some respects, from this system since oxysterol apoptotic signaling appears to proceed through a signal transduction pathway requiring arachidonic acid release (9), and possibly ROS formation (14,20), both of which might be expected to be upstream of the Akt degradation reported here. Whether cholesterol loading can activate Akt degradation or whether oxysterols can activate one or more elements of the UPR remain to be determined.
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**Figure Legends**

**FIG. 1.** A specific inhibitor of cPLA₂ (AACOCF₃) as well as a general inhibitor of arachidonate metabolism (ETYA) prevent induction of Caspase 3 by 25-hydroxycholesterol in P388D1 cells. P388D1 cells were cultured in media supplemented with or without 20 μM AACOCF₃ or 10 μM ETYA for 2 h prior to the addition of 25-hydroxycholesterol (25OHC) or vehicle (ethanol). Following an 18 h incubation the cells were harvested and Caspase 3 and “Caspase 3 like” activity was measured as described in Materials and Methods. The results are expressed as the fold increases in fluorescence relative to the mean fluorescence determined for control treatments (vehicle only). The data represent the mean ± S.D. of triplicate treatments.

**FIG. 2.** 25-Hydroxycholesterol induces proteasome dependent Akt degradation. **A)** P388-D1 cells were treated with 10 µg/ml 25OHC for 12 h then Akt kinase activity was measured as described in Materials and Methods using GSK-3 fusion protein as a substrate. The data represent the mean ± S.D. of triplicate measurements. **B)** P388-D1 cells were treated with 10 µg/ml 25OHC for different times and then the levels of total Akt were measured by immunoblotting as described in Materials and Methods. **C)** P388-D1 cells were pulse-labeled with 100µCi/ml of Tran³⁵Slabel and chased in the absence (empty circles) or presence (filled circles) of 10 µg/ml 25OHC as described in Materials and Methods. At different times, total Akt was immunoprecipitated and radioactivity
was measured by SDS-PAGE and phosphorimaging. The extrapolated half-life for Akt was \(~8\)h and \(~100\) min in the controls and 25OHC-treated cells respectively. **D)** Total Akt levels were determined by immunoblotting after treatment with 10 µg/ml 25OHC for 12 h in the presence or absence of 15 µM Proteasome Inhibitor I (PI-I).

**FIG. 3.** *Ectopic expression of constitutively active Akt reduces 25-hydroxycholesterol induction of caspase 3 activity in P388D1 cells.*

**A)** Whole cell lysates obtained from P388D1 cells stably transfected with a myr-Akt expression vector (Clone B) or the empty vector (Control) were subjected to SDS-PAGE, transferred to PVDF membranes and immunoblotted using a total Akt antibody and a myc antibody as indicated. Clone B and Control cells were incubated with media containing either 10 µg/ml 25OHC or 10 µg/ml 7-ketocholesterol (**Panels B and C, respectively**) for various times. The media was then replaced with media without oxysterol and the incubation continued for 16h. The fold induction of Caspase 3 activity was then determined as described for figure 1. **D)** P388D1 cells transiently transfected with either no DNA (Mock), empty vector (pUSEamp) or vector containing Akt cDNA (myr-Akt) were incubated in media supplemented with or without 10 µg/ml 25OHC for 6h as indicated. The media was refreshed without oxysterol supplementation and, following a 16h incubation, the fold induction of Caspase 3 activity was determined.
FIG. 4. **Effect of 25-hydroxycholesterol on phosphorylation of Bad.** Equal amounts of whole cell lysates from P388D1-Akt clone B and P388-Control cells cultured in media supplemented with 10 µg/ml 25OHC for various periods were subjected to immunoblotting using a phospho-Bad (ser 136) specific antibody as described in Materials and Methods. The membranes were then stripped and immunoblotted with anti-HSP-70 to control for equivalent loading of the gel and efficient transfer of proteins to the membrane.

FIG 5: **25-hydroxycholesterol induces Bad translocation to the mitochondria.** RAW 264.7 were transiently transfected with Bad-GFP fusion protein. After 16 h, cells were treated with 10µg/ml 25OHC and incubated for 6h. The localization of the Bad-GFP chimera was determined by fluorescence microscopy as described in Materials and Methods. The figure shows the same cell before and after treatment. Mitotracker is a fluorescent probe that specifically stains mitochondria.

FIG. 6. **25-hydroxycholesterol treatment increases total and mitochondrial Bim levels.** P388-D1 cells were treated with 10 µg/ml 25OHC for 12h and then mitochondrial and total cell levels of Bim were measured by immunoblotting. PVDF membranes were then stripped and reprobed with anti-HSP70 as a control for loading and transfer.
FIG. 7. **Suppression of Bax expression protects P388D1 cells from 25OHC induced apoptosis.**  A) P388D1 cells were treated with different amounts of 25OHC for 12h and then mitochondrial and total cell levels of Bcl-X\textsubscript{L} were measured by immunoblotting. PVDF membranes were subsequently stripped and reprobed for HSP70 as a control for loading and transfer. B) Immunoblot analysis of Bax and Bak protein levels in whole cell lysates from P388D1 cells stably transfected with pSi-Bax, an siRNA expression vector targeting Bax mRNA (SiBax #1), or pSi-RandomBax, a control vector expressing randomized Bax target sequence (R-Bax). C) The P388D1-SiBax (SiBax #1) and RandomBax (R-Bax) clones were cultured for 16h in media containing increasing amounts of 25OHC as indicated. Control cells received an equal amount of vehicle only (ethanol). Caspase 3 activity was then determined as described in figure 1. Each treatment was done in triplicate and the data is presented as the mean ± S.D.

FIG. 8. **Caspase 3 activation after degradation of constitutively active myr-Akt in P388D1 clone B cells following prolonged treatment with 25OHC.**  A) Immunoblot analysis of total Akt in whole cell lysates prepared from P388D1 clone B and P388D1 control. Cells were incubated with or without 10 µg/ml 25OHC for 16h and 20 h as indicated. B) Caspase 3 activity was assayed from cells treated in parallel with those used for the Akt immunoblot shown in panel A.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6
**A)**

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-X&lt;sub&gt;L&lt;/sub&gt;</td>
<td>HSP70</td>
</tr>
</tbody>
</table>

25OHC (μg/ml) 0 5 10 25OHC (μg/ml) 0 5 10

**B)**

<table>
<thead>
<tr>
<th>Si-Bax #1</th>
<th>R-Bax</th>
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<tbody>
<tr>
<td>Bax</td>
<td>Bak</td>
</tr>
</tbody>
</table>

**C)**

![Graph showing fold induction vs. μg/ml 25-OHC](image)

Figure 7
Akt/PKB regulation of Bcl family members during oxysterol induced apoptosis
Antonio R. Rusinol, Douglas Thewke, June Liu, Natalie Freeman, Sankhavaram R. Panini
and Michael S. Sinensky

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