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REVIEWS: CURRENT TOPICS

Oxysterols and mechanisms of apoptotic signaling: implications in the pathology of degenerative diseases☆

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Abstract

Oxysterols, or cholesterol oxidation products, are oxygenated derivatives of cholesterol which are formed endogenously during the biosynthesis of bile acids and steroid hormones. In addition, oxysterols may also be absorbed from the diet as they are found in many commonly consumed foods. Oxysterols have been shown to possess many potent and diverse biological activities, and the study of the effects of these oxidation products on the human body forms a wide field of research. The results of most research efforts support the conclusion that certain oxysterols, predominantly those found in oxidized low-density lipoprotein, exert pathological effects such as the induction of apoptotic cell death. Moreover, apoptosis induced by oxysterols has been strongly implicated in the pathogenesis of atherosclerosis as well as a variety of other diseases. The study of oxysterol-induced apoptosis is an emerging area, and the following review aims to provide a detailed account on the chronology of events involved. Current evidence of the involvement of the death receptor pathway and protein kinases is examined as well as important apoptosis regulators such as the mitochondria, B-cell lymphoma-2 proteins and caspases. The effect of oxysterols on gene expression, protein interactions and membrane properties are also discussed.

Keywords: Apoptosis; Cholesterol; Oxides; Oxysterols

1. Introduction

Oxysterols are derivatives of cholesterol that contain a second oxygen atom as a carbonyl, hydroxyl or epoxide group. In vivo, oxysterols act as intermediates in cholesterol catabolism, regulators of lipid metabolism and as toxic sterols with proatherogenic effects [1]. For instance, oxysterols have been ascribed a number of important roles in connection with cholesterol turnover, atherosclerosis, apoptosis, necrosis, inflammation, immunosuppression and the development of gall stones [2–5]. Oxysterols usually occur at low levels accompanied by high concentrations of the parent cholesterol. Accumulation of oxysterols in the body can occur in several different ways, the major ones being dietary intake (nutritional oxysterols) and internal chemical and enzymatic oxidation [6]. The abbreviations and nomenclature of some commonly encountered oxysterols are presented in Table 1, while Fig. 1 illustrates the basic structures of some of these oxysterols.

Moderate levels of oxysterols can occur in foods, most notably milk powders, meat and meat products (including

Abbreviations: ABCA1, ATP-binding cassette A1; ABCG1, ATP-binding cassette G1; Ca^{2+}, calcium; AD, Alzheimer's disease; AIF, apoptosis-inducing factor; AMD, age-related macular degeneration; CAD, caspase-activated deoxyribonuclease; cIAP-1/2, cellular IAP-1/2; DIABLO, direct IAP binding protein with low pI; ER, endoplasmic reticulum; ERKs, extracellular signal-regulated kinases; IAPs, inhibitor of apoptosis proteins; insig 1/2, insulin-inducible genes 1/2; JNK, c-Jun N-terminal protein kinase; LXRs, liver X receptors; MAPKs, mitogen-activated protein kinases; MMP, mitochondrial membrane permeabilization; ORPs, OSBP-related proteins; OSBP, oxysterol-binding protein; oxLDL, oxidized low-density lipoprotein; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; PTP, permeability transition pore; ROS, reactive oxygen species; RXR, retinoid X receptor; SCAP, SREBP cleavage-activating protein; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; Smac, second mitochondria-derived activator of caspase; SMCs, smooth muscle cells; SREBP, sterol regulatory element binding protein; TNF, tumor necrosis factor; TNFR, TNF receptor; XIAP, X-chromosome-linked IAP.

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Table 1
Nomenclature of commonly encountered oxysterols

Abbreviation	Trivial name	Systematic name
Triol	Cholestanetriol	5α-Cholestane-3β,5,6β-triol
4α-OH	4α-Hydroxycholesterol	Cholest-5-ene-3β,4α-diol
4β-OH	4β-Hydroxycholesterol	Cholest-5-ene-3β,4β-diol
6β-OH	6β-Hydroxycholesterol	Cholest-4-ene-3β,6β-diol
7α-OH	7α-Hydroxycholesterol	Cholest-5-ene-3β,7α-diol
7β-OH	7β-Hydroxycholesterol	Cholest-5-ene-3β,7β-diol
24-OH	24-Hydroxycholesterol	Cholest-5-en-3β,24(S)-diol
25-OH	25-Hydroxycholesterol	Cholest-5-en-3β,25-diol
26-OH/27-OH	26-Hydroxycholesterol/27-Hydroxycholesterol	25(*R*)-Cholest-5-en-3β,26-diol
7α-OOH	7α-Hydroperoxycholesterol	3β-Hydroxycholest-5-ene-7α-hydroperoxide
7β-OOH	7β-Hydroperoxycholesterol	3β-Hydroxycholest-5-ene-7β-hydroperoxide
7-keto	7-Ketocholesterol	3β-Hydroxycholest-5-en-7-one
7-keto-diene	7-Ketocholestadiene	Cholesta-3,5-diene-7-one
α-epoxide	Cholesterol-5α,6α-epoxide	5,6α-Epoxy-5α-cholestan-3β-ol
β-epoxide	Cholesterol-5β,6β-epoxide	5,6β-Epoxy-5β-cholestan-3β-ol

Adapted from Brown and Jessup [3] and Morrissey and Kiely [15].

fish), cheese, eggs and egg products [7–13]. During processing, preparation and storage, these foods can be exposed to heat, air, light or radiation [14]. These events produce fatty acid radicals and hydroperoxides which lead to the formation of oxysterols within the food product [15]. While the actual intake of dietary oxysterols is not known, various studies have shown that the amount of oxysterols in foods can frequently reach 1% of total cholesterol and occasionally 10% or more [16]. Moreover, numerous animal studies have demonstrated that cholesterol feeding leads to increased levels of oxysterols in vivo [17–23]. The most commonly detected oxysterols in food are 7-keto, 7α-OH, 7β-OH, α-epoxide, β-epoxide and triol [3]. The degree of oxide formation in foods is related to the composition of the food matrix, processing methods, processing times and temperatures, pH, storage conditions, pro- and antioxidants and water activity [14,24]. Hence, formation of oxysterols in animal products can be minimized by application of low processing temperatures, use of oxygen-proof packaging and a protective atmosphere, by low-temperature and light-free storage, and/or the dietary addition of antioxidants to animal feed or antioxidant addition to foods [14].

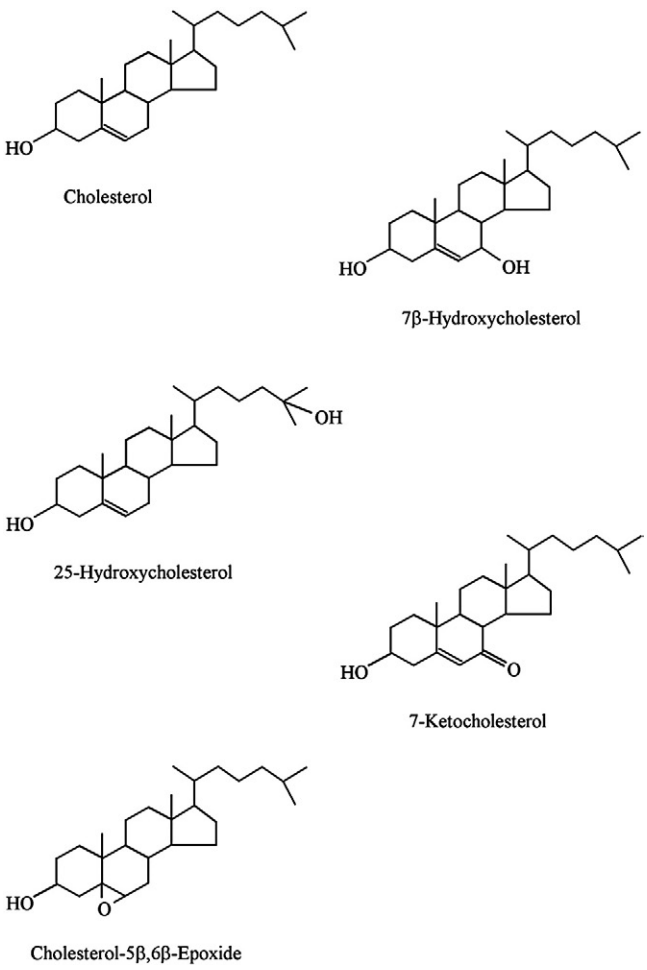

Fig. 1. Chemical structures of some commonly occurring oxysterols.

The introduction of an oxygen atom into cholesterol (formation of oxysterols) drastically reduces its half-life and is a mechanism by which some cells eliminate excess cholesterol [15]. In addition, oxysterols are often synthesized by particular cellular enzymes in response to metabolic needs and they regulate enzymes of cholesterol metabolism through highly specific interactions with nuclear receptors [4,25,26].

Oxysterols may be generated in the arterial wall after nonenzymatic oxidation of lipoproteins by smooth muscle cells, endothelial cells and macrophages [27] or alternatively may be derived from circulating lipoproteins. Oxidized low-density lipoprotein (oxLDL) is rich in oxysterols and shows unabated uptake by macrophages and smooth muscle cells derived from the arterial wall, resulting in significant sterol loading in vitro [21]. 7-Keto, 7β-OH, α-epoxide, β-epoxide and α-triol are the main oxysterols found in plasma and aortic tissue [6]. Patients with familial combined hyperlipidemia, the most commonly inherited disorder of lipid metabolism, display markedly increased plasma levels of

oxysterols [28]. Iuliano et al. [29] revealed that atherosclerotic plaques contained 45 times greater amounts of 7β-OH and 7-keto compared to normal arteries. Studies such as these have implicated oxysterols in the development of atherosclerosis.

From a quantitative point of view, the most important oxysterols found in vivo are enzymatic products of cholesterol metabolism that are involved in the early steps of cholesterol conversion to bile acids and steroid hormones [4]. While all cells have the potential to oxygenate cholesterol, the liver and the endocrine organs have the highest capacity for such reactions [30]. Products of cytochrome P450-mediated oxygenations are found in the circulation as dominating sterols and are involved in the early stages of bile acid formation. These enzymatically derived oxysterols include 27-OH, 24-OH, 25-OH and 7α-OH [6]. Several oxysterol-binding protein (OSBP)-related proteins (ORPs) bind these oxysterols, initiating intracellular events, as described below.

2. Oxysterol signal transduction

Oxysterols display many biological activities in both normal and diseased states. Cells respond to these compounds via diverse mechanisms (Fig. 2), possessing distinct selectivities for different oxysterols. Liver X receptors (LXRs) are nuclear receptors that are either ubiquitous, in the case of LXRβ, or which are only expressed at high levels in the liver, adipose, intestine, kidney and macrophages (LXRα). LXRs operate as heterodimers with the retinoid X receptor (RXR) and, upon binding of certain oxysterols, promote expression of target genes. These targets include the ATP-binding cassette A1 (ABCA1) and G1 (ABCG1) genes that encode proteins facilitating efflux of sterols (cholesterol or oxysterols) from cells [31]. By contrast, sterols inhibit expression of genes promoted by the sterol regulatory element binding protein (SREBP) family. SREBP is an endoplasmic reticulum (ER) membrane protein that can exist as a complex with two additional membrane-spanning proteins, the SREBP cleavage-activating protein (SCAP) and insulin-inducible genes 1 or 2 (insig 1/2). Under high sterol conditions, cholesterol binds to the sterol-sensing domain of SCAP; and certain oxysterols, such as 25-OH, associate with insig 1/2. Decreased sterol levels lead to dissociation of insig 1/2 from SCAP, and an association of coatomer proteins with this protein, resulting in ER to Golgi transport of the SCAP–SREBP complex. Site 1 and Site 2 proteases within the Golgi cleave SREBP, releasing its N-terminal region which translocates to the nucleus and acts as basic-helix-loop-helix-leucine zipper transcription factor, promoting expression of genes involved in enhancing sterol accumulation, such as the LDL receptor [32].

Oxysterols can also elicit rapid, nongenomic effects, via modification of interactions between oxysterol-sensing proteins and other protein or lipid macromolecules. In addition to causing slow (minutes to hours) increases in the expression of the ABCA1 gene, oxysterols also promote rapid (seconds) dissociation of LXRβ/RXR heterodimers bound to the ABCA1 protein, thereby removing a tonic inhibitory effect and driving sterol efflux [33].

Oxysterols elicit many of their biological effects via interactions with OSBP/ORP family members. The human genome contains at least 12 OSBP/ORP genes, whose structural diversity is increased by mRNA splicing events. Conserved features of this protein family include a C-terminal oxysterol-binding domain and an N-terminal pleckstrin-homology domain that facilitates interaction with biological membranes. Different ORP members display distinct oxysterol selectivity profiles and promote diverse cellular events [34]. For example, ORP8 suppresses expression of the ABCA1 gene in THP-1 cells [35], whereas OSBP increases the turnover of this sterol-transporting protein in J774 macrophages [36]. The OSBP/ORP family also influences cellular trafficking: OSBP, ORP3 and ORP9 all interact with vesicle-associated membrane protein-associated protein A, leading to their translocation from the ER to the Golgi or plasma membrane [37–39]. ORP1L (long splice form) is a component of multiprotein complex that includes the small GTPase Rab7 and which modulates endosomal trafficking [40]. ORPs potentially regulate the cytoskeleton since an N-terminally truncated form of ORP4 promotes aggregation of the intermediate filament protein vimentin [41]. In addition, ORP3 associates with the small G-protein R-Ras to promote cell–cell adhesion [42]. Given the diverse, and in some cases unexplored, roles of the OSBP/ORP family, it is likely that these proteins contribute to many of the cellular effects of oxysterols, including regulation of cell death. Furthermore, expression profiles of the different members of this family might explain some of the discrepancies reported between distinct cell types for the effects of individual oxysterols on such processes.

The final reported mechanism via which oxysterols can regulate cell functions is by modification of the biophysical properties of cell membranes. Cholesterol promotes the formation of ordered structures within lipid bilayers, particularly promoting generation of lipid rafts, which are biologically important membrane subdomains, abundant in specific signaling proteins. Distinct oxysterols can either enhance or inhibit lipid order, depending on their conformation, which in turn depends on the position and type of second oxygen group present [43]. In THP-1 macrophages, 7-keto promotes nifedipine-sensitive calcium (Ca^{2+}) influx, Ca^{2+}-dependent Bad dephosphorylation and apoptosis, as well as redistribution of trpc1 Ca^{2+} channels into lipid rafts [44]. However, it is debatable whether trpc1 plays a role in the Ca^{2+} influx elicited by 7-keto since there are no published reports of these channels being sensitive to nifedipine and since death of PC12 phaeocytochroma cells triggered by 7-keto is not blocked by ruthenium red, a trp channel inhibitor [45].

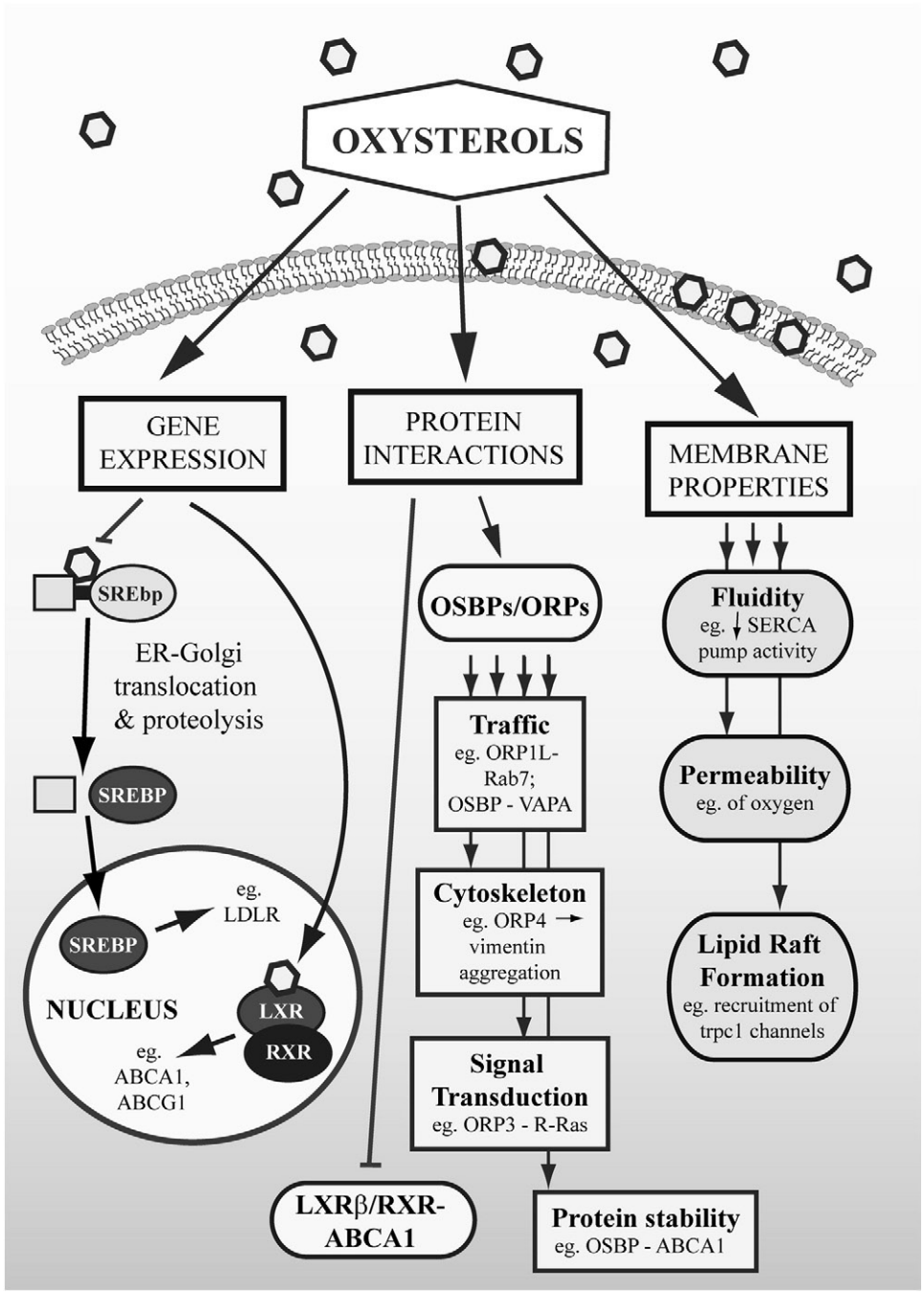

Fig. 2. Oxysterol signal transduction. →, activation; ⊣, inhibition. LDLR, low-density lipoprotein receptor.

Decreased ER membrane fluidity induced by cholesterol loading of macrophages inhibits the activity of the sarcoplasmic/ER Ca^{2+} ATPase (SERCA) 2b, resulting in depletion of this intracellular Ca^{2+} pool and triggering of the ER stress response [46]. It is anticipated that oxysterols might enhance or inhibit SERCA activity, depending on their individual biophysical properties. Cholesterol also decreases oxygen permeability through phospholipid bilayers [47], and consequently, oxysterols may modulate the diffusion of solutes across biological membranes. Such mechanisms could result in cell injury, for example, by restricting the supply of oxygen [48]. These candidate biophysical actions of oxysterols and their roles in apoptosis await further investigation.

3. Oxysterol-induced apoptosis

Concerns about the accumulation of oxysterols arise from their potential cytotoxic, mutagenic, atherogenic and possible carcinogenic effects [49]. The relationship between oxysterols and atherosclerosis has been widely studied in

both in vitro and in vivo models. However, recent research suggests that oxysterols may also be involved in the pathogenesis of other degenerative diseases such as Alzheimer's disease (AD) [50–52] and age-related macular degeneration (AMD) [53,54].

Much of the cytotoxicity attributable to oxysterols is derived from their ability to induce apoptosis. Apoptosis is a genetically controlled and evolutionary conserved form of programmed cell death which is of critical importance for normal embryonic development and for the maintenance of tissue homeostasis in the adult organism [55]. It is a coordinated and often energy-dependent process characterized by distinct morphological features including chromatin condensation, nuclear fragmentation, membrane blebbing and cell shrinkage. The apoptotic network involves the activation of a group of cysteine proteases called "caspases" and a complex cascade of events that link the initiating stimuli to the final demise of the cell [56]. However, abnormal rates of programmed cell death are believed to play a primary or secondary role in a number of pathologies, with essentially too little or too much apoptosis leading to proliferative or degenerative diseases, respectively [55].

A large number of in vitro studies have described the potential proapoptotic effect of oxysterols in various cell systems. Oxysterols may increase intracellular levels of reactive oxygen species (ROS), induce modification of cell proteins and alter various signaling pathways and gene expression [15]. However, structure–activity studies have demonstrated that oxysterols vary greatly in their ability to induce apoptosis and so a universal mechanism of oxysterol-induced apoptosis remains to be clarified.

The induction of apoptosis occurs via two major pathways: the death receptor-dependent (extrinsic) pathway (Fig. 3) or the mitochondrial (intrinsic) pathway (Fig. 4). Oxysterols can induce apoptosis through both pathways; however, evidence relating oxysterol-induced apoptosis to the death receptor pathway is limited.

3.1. The death receptor pathway

The death receptor pathway (Fig. 3) is activated through the binding of cytokine ligands to receptors of the tumor necrosis factor (TNF) superfamily such as Fas, TNF receptor (TNFR) or TNF-related apoptosis-inducing ligand. Upon ligand binding, the receptors aggregate and form membrane-bound signaling complexes. These are responsible for the recruitment of several molecules of the initiator pro-caspase-8. This event results in the formation of the death-inducing signaling complex, activation of caspase-8 and triggering of the proteolytic caspase cascade. Alternatively, caspase-8 can cleave the protein Bid into the active form, truncated Bid, which provides a cross-talk between the extrinsic and intrinsic pathways [57].

3.1.1. FasL/Fas

The ubiquitous death receptor Fas is expressed on most tissues and plays an important role in regulating the immune response [58,59]. Preliminary data implicate that the Fas-mediated pathway is involved, at least in part, in oxysterol-induced apoptosis. Lee and Chau [60] showed that 7β-OH and 25-OH up-regulated expression of both Fas and Fas ligand (FasL) and induced apoptosis in vascular smooth muscle cells (SMCs) to various degrees. Lordan et al. [61] found that Fas inhibition substantially reduced apoptosis in 7β-OH-treated cells but had no effect on β-epoxide-induced apoptosis. Moreover, treatment with 7-keto predisposed human aortic SMCs to undergo Fas-mediated death. These findings indicate the active participation of mitochondria in sensitization of the cells to the Fas-mediated pathway [62].

3.1.2. TNF-α/TNFR

TNF-α is a proapoptotic ligand which exerts its biological activity by signaling via its two receptors, TNFR-1 and TNFR-2 [63]. In the presence of 7-keto, Lee et al. [64] demonstrated the activation of TNFR-mediated death pathway in human aortic SMCs. TNF-α was found to be up-regulated by 22-OH in human peripheral monocytes as well as in the human monocytic cell line THP-1 [65]. However, in human endothelial cells, TNF-α secretion was not detected during 7β-OH- and 7-keto-induced apoptosis [66]. Treatment with 7β-OH for 4 h did not induce TNF-α secretion from THP-1 cells [67]; however, in a separate study, significant levels were observed following 24 h incubation [68]. These results suggest a slow but gradual increase in TNF-α levels following exposure to oxysterols.

3.2. Protein kinases

Apoptosis is a multistep process, and protein kinases have been implicated in both the upstream induction phase of apoptosis and in the downstream execution stage, as direct targets for caspases [69]. While information on apoptosis has increased greatly, it is only in recent years that the function of protein kinases in the apoptotic program has been elucidated, with some being suggested to play a role in oxysterol-induced apoptosis. These include the mitogen-activated protein kinase (MAPK) family, Akt (also called protein kinase B) and protein kinase C (PKC).

3.2.1. Mitogen-activated protein kinases

To date, several distinct MAPKs have been identified, including extracellular signal-regulated kinases 1 and 2 (ERKs-1/2), c-Jun N-terminal protein kinase (JNK) and p38 MAPK. These kinases represent the terminal stages of signaling cascades, initiated by the ligation of growth/survival factor or death receptors. Activation of JNK and p38 is generally associated with the promotion of apoptosis, while ERK activity inhibits apoptosis [69]. Numerous in vitro studies have demonstrated modifications in ERK-1/2 activity following exposure to oxysterols [68,70–73]. In C6 glioma cells, an early decrease in ERK-1/2 activity was found to correlate with the toxic effects of 7β-OH [74]. Following treatment with a different oxysterol, 7-keto, ERK-1/2 phosphorylation in THP-1 cells peaked at 2–3 h then declined toward basal levels after 12 h, while apoptosis increased significantly [75].

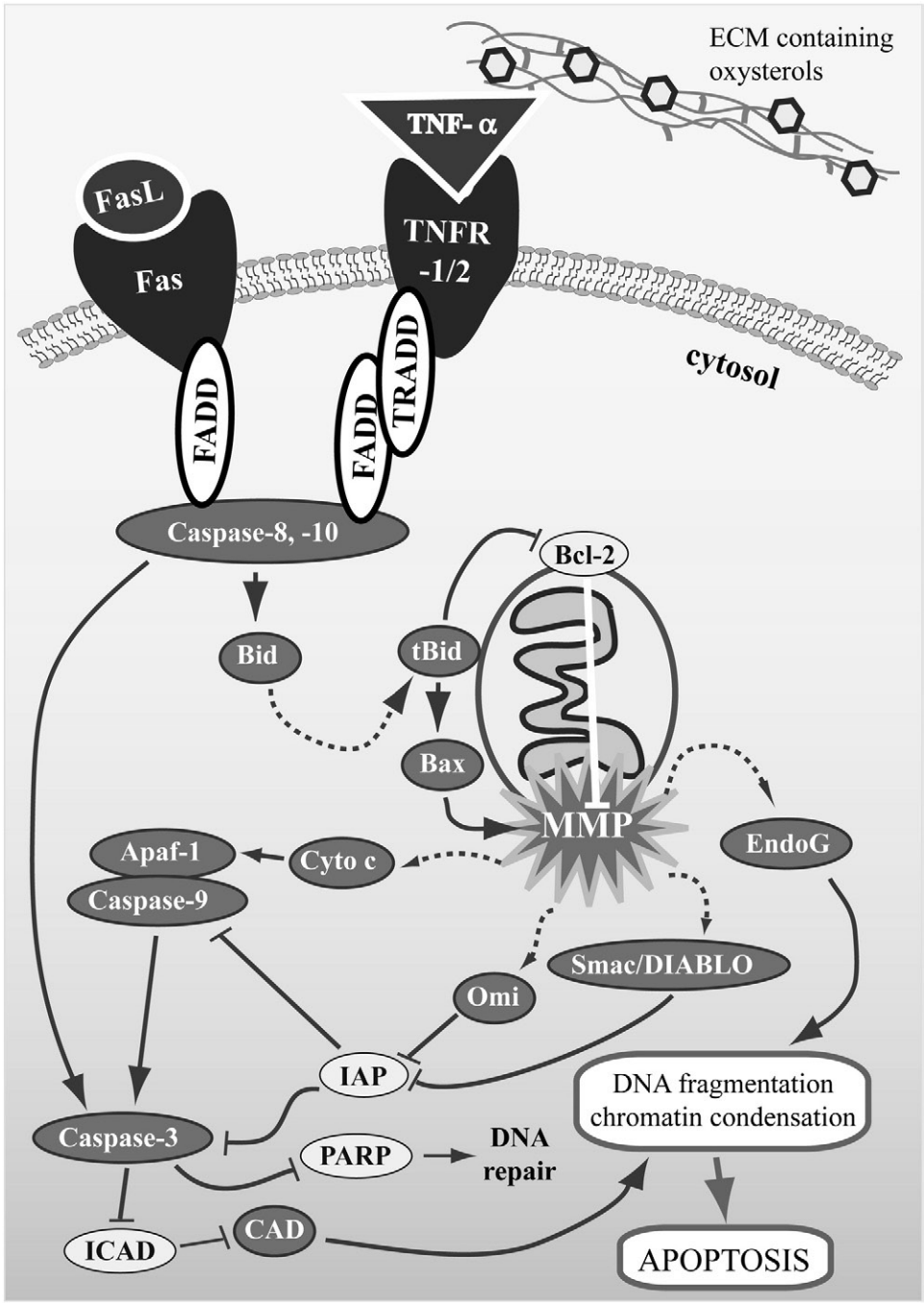

Fig. 3. The death receptor (extrinsic) pathway. →, activation; - - -▶, translocation; ⊣, inhibition. Apaf-1, apoptosis-protease activating factor-1; Cyto c, cytochrome *c*; FADD, Fas-associated death domain; ICAD, inhibitor of CAD; tBid, truncated Bid; TRADD, TNFR-associated death domain.

Other studies involving various cell lines have observed increased ERK-1/2 activity immediately after cell challenge with oxysterols with no activation of JNK or p38 [70,72,75]. However, this pattern of activity has not been consistently detected. Exposure to 7-keto for 24 h induced significant levels of apoptosis in THP-1 cells along with a remarkable increase in the level of the phosphorylated forms of ERK-1/2, JNK and p38 [73]. Conversely, Yoon et al. [71] showed that 22-OH activates ERK-1/2 and p38, but not JNK, in human cholangiocytes. Taken together, these results suggest that the involvement and function of MAPKs may be determined by a number of factors, including the type of oxysterol, the cell type being examined and the abundance of kinases relative to that of other apoptotic proteins.

3.2.2. Akt

An important function of active Akt in cells is maintaining cell survival via inhibition of apoptosis. The downstream

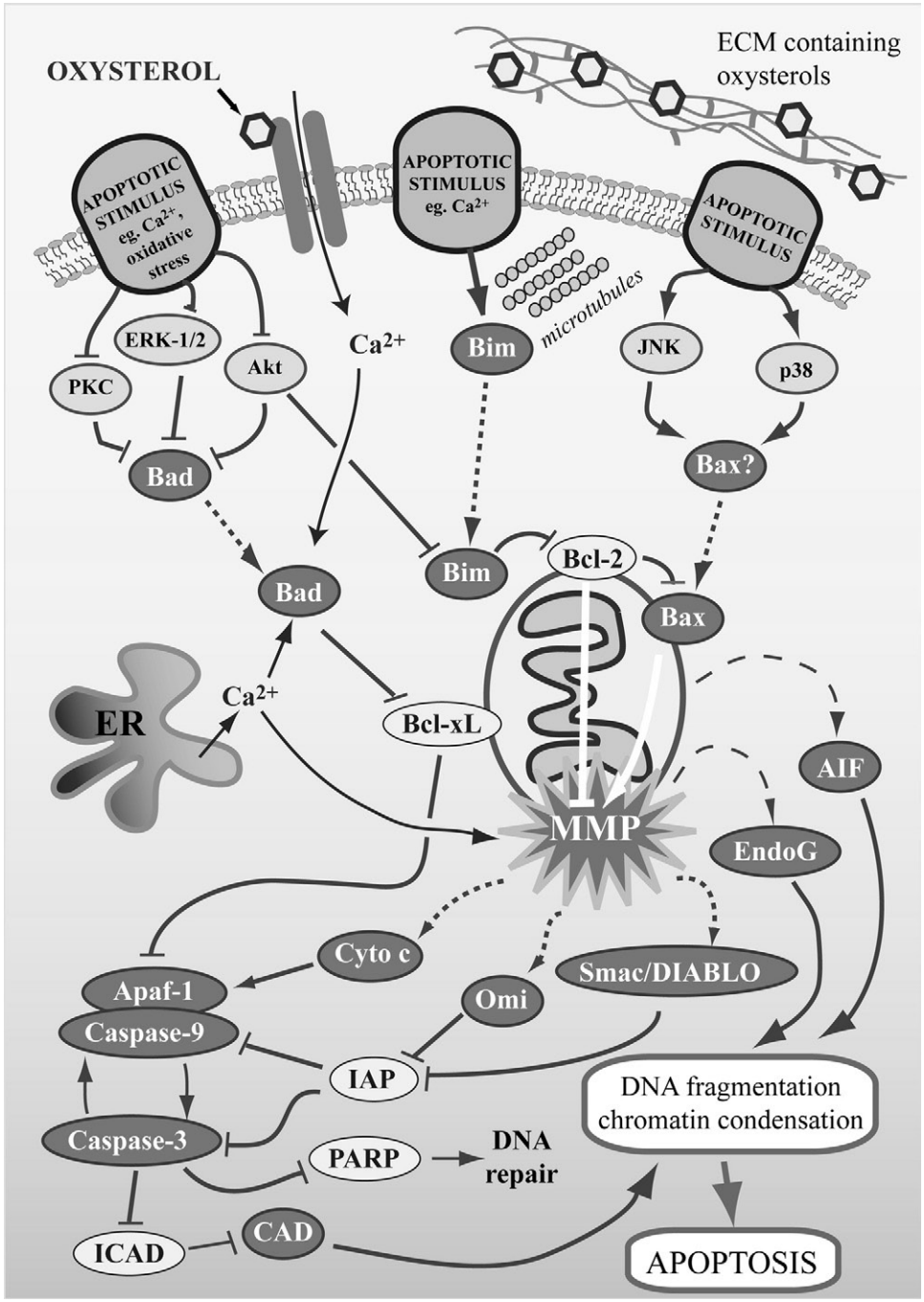

Fig. 4. The mitochondrial (intrinsic) pathway. →, activation; - - -▶, translocation; ⊣, inhibition. Apaf-1, apoptosis-protease activating factor-1; Cyto c, cytochrome *c*; ICAD, inhibitor of CAD.

actions of Akt appear to include phosphorylation of proteins involved in the apoptotic cascade and regulation of the expression of apoptotic proteins [57,69]. Therefore, for apoptosis to occur, Akt must be inactivated early in the apoptotic process. In a murine macrophage-like cell line, 25-OH was found to induce apoptosis through degradation of Akt [76]. Berthier et al. [75], observed Akt inactivation during the initial steps of 7-keto-induced apoptosis in THP-1 cells. In support of these findings, a recent study revealed that treatment of THP-1 macrophages with 7-keto coincides with decreased levels of Akt [73]. Moreover, 7β-OH-induced apoptosis caused a decrease in Akt activation compared to untreated U937 cells [77]. Taken together, these results suggest that the apoptotic processes induced by certain oxysterols include a reduction in Akt activity.

3.2.3. Protein kinase C

Like many other signaling effectors, PKC is not a single entity but a multigene family consisting of numerous isoenzymes. PKC is ubiquitously expressed and has long

been considered as having a key "housekeeping" function [69]. While the function of PKC in the regulation of oxysterol-induced apoptosis remains to be identified, some preliminary studies indicate a link. For instance, lymphocytes treated with 7β,25-dihydroxycholesterol (7,25-OH) showed a reduction in PKC activity [78], and the proapoptotic oxysterol 7β-OH was a potent inhibitor of α-PKC in H19-7/IGF-IR neuronal cells [79]. In contrast, treatment of murine peritoneal macrophages with 7-keto, β-epoxide and 7β-OH resulted in a dose-dependent increase in PKC activity [80]. Although these findings are conflicting, the research may still implicate PKC activation as part of the signaling process of oxysterol-induced apoptosis.

3.3. Mitochondrial control

It is widely accepted that mitochondria play a key role in the regulation of apoptosis. Alterations in their transmembrane potential in response to various triggers lead to the production of ROS or mitochondrial membrane permeabilization (MMP). MMP can be induced by the proapoptotic Bax/Bak, which interacts with the voltage-dependent anion channel/adenine nucleotide transporter. This interaction results in the release of a number of small molecules, including cytochrome *c*, second mitochondria-derived activator of caspase (Smac)/direct IAP binding protein with low pI (DIABLO), Omi/HtrA2, apoptosis-inducing factor (AIF) and endonuclease G (Endo G), which activate caspase-dependent and caspase-independent apoptotic cell death pathways [81]. With regard to oxysterol-induced apoptosis, loss of mitochondrial transmembrane potential, cytosolic release of cytochrome *c* and activation of apoptotic proteins have all been reported (Fig. 4).

3.3.1. Cytochrome c

Cytochrome *c* is located in the mitochondrial intermembrane/intercristae spaces; however, upon apoptotic stimuli, this protein is released into the cytosol where it interacts with its adaptor molecule apoptosis-protease activating factor-1. This results in the recruitment, processing and activation of various proteins, leading to biochemical and morphological features characteristic of apoptosis [82]. There are several reports that describe a loss in mitochondrial transmembrane potential accompanied by cytochrome *c* release during oxysterol-induced apoptosis [62,83–91]. Apoptosis induced by 7β-OH was associated with a loss in transmembrane potential after 2 h, followed by cytochrome *c* release from the mitochondria into the cytosol after 16 h [92]. Nevertheless, Seye et al. [93] demonstrated that 7-keto-induced loss in mitochondrial transmembrane potential and cytochrome *c* content can be normalized upon removal of the oxysterol after 16 h.

An in vivo study involving hamsters has also demonstrated a connection between cytochrome *c* and oxysterol-induced apoptosis. After a 15-day diet supplemented with oxysterols, a release of cytochrome *c* into the cytosolic fraction of the whole heart occurred, indicating apoptosis of one or several types of cardiac cells [94]. Prunet et al. [95] revealed that caspase-3 is essential to trigger cytochrome *c* release during 7-keto- and 7β-OH-induced apoptosis. This study employed three cell lines: U937 cells, MCF-7 cells, in which the caspase-3 gene is deleted, and MCF-7/c3 cells, which were stably transfected with caspase-3. Following exposure to the oxysterols, mitochondrial release of cytochrome *c* was observed in the U937 and MCF-7/c3 cells. However, in the caspase-3-deficient MCF-7 cell line, less than 1% of cells were found to be apoptotic with no release of cytochrome *c*.

As previously stated, different pathways of apoptosis can be induced depending on the oxysterol or the cell type, and this is also true with regard to cytochrome *c*. In U937 cells, inhibition of cytochrome *c* release prevented cell death induced by 7β-OH but it did not protect against β-epoxide-induced apoptosis [96]. While Ghelli et al. [97] showed that incubation of ECV304 cells with 7-keto caused a decrease in cell viability, no release of cytochrome *c* from the mitochondria into the cytoplasm was observed in the oxysterol-treated cells. Overall, however, these data are consistent with the induction of apoptosis by oxysterols proceeding, at least in part, through the mitochondrial cytochrome *c* pathway.

3.3.2. Smac/DIABLO and Omi

In healthy cells, Smac/DIABLO resides within the mitochondria, and upon cellular stress, it is released into the cytosol where it is capable of promoting cell death [98]. Smac/DIABLO can compete with caspase-9 for binding to inhibitor of apoptosis proteins (IAPs), resulting in caspase activation and apoptosis [81,99]. The serine protease Omi, also known as HtrA2, has many similarities to Smac/DIABLO. It is localized in the mitochondria and is released into the cytoplasm during apoptosis. However, Omi seems to have a dual function: (i) as an inhibitor of IAPs, propagating caspase cascades, and (ii) as a protease, propagating atypical caspase-independent cell death. Omi and Smac/DIABLO also display a different tissue distribution pattern. While Omi is expressed ubiquitously, Smac/DIABLO is most abundant in heart, liver, kidney and testis with little or no expression detected in skeletal muscle, lung, thymus and the brain [99].

So far, little is known about the participation of these proteins in oxysterol-induced apoptosis. An extensive study by Berthier et al. [75] examined the involvement of several transduction pathways in THP-1 cells during 7-keto-induced apoptosis. Smac/DIABLO was released into the cytosol following 30 h treatment with the oxysterol and was found to succeed depolarization of the mitochondria and cytochrome *c* release. In addition, inhibition of ERK-1/2 increased release of both cytochrome *c* and Smac/DIABLO. These findings suggest that the proapoptotic effects of cytochrome *c* and Smac/DIABLO are modulated by MAPK signaling. As oxysterols are probably the components of oxLDL which have the strongest involvement in the induction of apoptosis, results from research on oxLDL may provide data pertaining

to oxysterol-induced apoptotic signaling. Similar to the previous study, exposure to oxLDL for 24 h induced an increase in the release of cytochrome *c* and Smac/DIABLO in human endothelial cells [100]. Moreover, disruption of mitochondrial membrane function by oxLDL treatment resulted in the specific release of the proapoptotic proteins Smac/DIABLO and Omi from isolated mitochondria [101]. Hence, it appears that oxysterols have the potential to stimulate the release of Smac/DIABLO and Omi from the mitochondria to the cytosol. However, considerable research needs to be carried out to determine the overall effect that this has on the apoptotic pathway.

3.3.3. Apoptosis-inducing factor

Upon an apoptotic stimulus, AIF translocates, in a B-cell lymphoma-2 (Bcl-2)-controlled fashion, from mitochondria to the cytosol and further to the nucleus where it triggers chromatin condensation as well as large-scale DNA fragmentation. AIF has a direct effect on both isolated nuclei and purified mitochondria where it provokes the release of caspase-9. In addition, AIF can indirectly elicit cell death through ROS production [102].

Research carried out by Prunet et al. [95] presents evidence of involvement of AIF during oxysterol-induced apoptosis. Exposure to two oxysterols, 7-keto and 7β-OH, for 24 h induced mitochondrial release of AIF into the cytosolic fraction of both U937 and MCF-7 cells. Moreover, incubation with a specific caspase-3/7 inhibitor had no effect on AIF release. This suggests that mitochondrial release of AIF into the cytosol precedes caspase-3 and/or caspase-7 activation. Further work allowed the investigators to rule out AIF as a contributor to cytochrome *c* release triggered by 7-keto and 7β-OH. As a result, the authors favor the hypothesis that AIF translocates into the cytosol through a mechanism independent of mitochondrial permeability transition pore (PTP). The study also implicates AIF as a caspase-independent mitochondrial death effector which translocates into the cytosol regardless of the mode of cell death and the cells used. Similar results were found in a study investigating oxLDL-induced AIF release. OxLDL induced an increase in AIF in the cytosolic fraction in a time- and dose-dependent manner. Interestingly, oxLDL-induced release of AIF from mitochondria was inhibited by EGTA but not by calpeptin, cyclosporine A or a multicaspase inhibitor. These data suggest that oxLDL-induced AIF release is Ca^{2+}-dependent but independent of calpain activation, mitochondrial PTP opening or caspase activation [103]. Overall, these studies link oxysterol-induced AIF release with a mechanism independent of caspases and PTP opening.

3.3.4. Endonuclease G

Endonuclease G is a nonspecific nuclease which resides in the mitochondrial intermembrane space [99]. During apoptosis in mammalian cells, Endo G translocates from mitochondria to the nucleus, where it extensively degrades nuclear DNA into oligonucleosomal fragments [102]. Mitochondrial outer membrane permeabilization induced by proapoptotic Bcl-2 family members such as Bid and Bim play a pivotal role in the release of Endo G [104]. Moreover, Endo G digests nuclear DNA in the absence of caspase activity or the caspase-activated deoxyribonuclease (CAD). It is therefore believed that AIF and Endo G, whether or not synergistically, may be important for the execution of apoptosis in situations where caspase activation is limited or compromised [99].

Recently, Roussi et al. [91] analyzed cytosolic extracts of Caco-2 cells following exposure to 7β-OH and found that the level of Endo G expression was enhanced after 12 h. The authors also reported a loss in mitochondrial membrane potential at an early stage of the apoptotic process which was associated with cytochrome *c* release into the cytosol. In addition, 7β-OH did not activate caspase-3, indicating that Endo G was related to caspase-independent apoptosis. In MCF-7 cells, a marked translocation of Endo G and AIF from mitochondria into the cytoplasm was observed following treatment with 7-keto and 7β-OH. As internucleosomal DNA fragmentation was not inhibited by a broad-spectrum caspase inhibitor, the data suggest that AIF and Endo G contribute to nuclear DNA damage via a caspase-independent pathway. In contrast, in U937 cells, 7-keto- and 7β-OH-induced cell death was associated with mitochondrial release of cytochrome *c* and AIF but not Endo G. Even in the presence of a specific caspase-3/7 inhibitor, Endo G was not released. The differences observed are in agreement with previous investigations reporting that the mode of cell death induced by oxysterols may depend on the cells used. It was suggested that 7-keto and 7β-OH probably simultaneously stimulate various signaling pathways leading to cell demise [95].

3.4. The Bcl-2 family

In mammalian cells, Bcl-2 family members are one of the main "apoptotic sensors" which act primarily on the mitochondria, where they regulate the survival or death signals in a preventive or provocative fashion [105]. Consequently, these Bcl-2 proteins have classically been categorized into three groups. One group inhibits apoptosis (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bcl-B and A1), whereas a second group promotes apoptosis (Bax, Bak and Bok). A third divergent class of BH3-only proteins (Bad, Bik, Bid, Hrk, Bim, Bmf, Noxa and Puma) have a conserved BH3 (Bcl-2 homology-3) domain that can bind and regulate the antiapoptotic Bcl-2 proteins to promote apoptosis [106]. When activated by cytotoxic signals, the BH3-only proteins engage prosurvival relatives by inserting the BH3 domain into a hydrophobic groove on their surface. This coupling primes the cell for apoptosis, but commitment also requires activation of Bax and/or Bak. Once activated, Bax and Bak form oligomers in intracellular membranes, including the mitochondrial outer membrane. The resulting membrane permeabilization releases proapoptotic proteins, such as cytochrome *c* and Smac/DIABLO, that provokes activation of the caspases mediating apoptosis [107]. In addition, Bcl-2

family proteins can influence the levels of releasable Ca^{2+} in the ER and thus determine whether the released Ca^{2+} is sufficient to overload mitochondria and induce cell death [108]. Overall, direct involvement of Bcl-2 proteins enables integration and interpretation of apoptotic or survival signals originating either from extracellular or from intracellular stimuli [105]. Hence, as a result of their unequivocal significance, a number of reports implicating regulation of the Bcl-2 family members in oxysterol-induced apoptosis have been documented.

3.4.1. BH3-only proteins: Bad, Bid and Bim

BH3-only proteins act as monitors of cell death and they coordinate the fine-tuning of apoptotic response through their interactions with pro- and antiapoptotic Bcl-2 members [105]. In THP-1 cells, 7-keto-induced apoptosis revealed a significant dephosphorylation/activation of Bad, which first appeared no later than 6 h after exposure. This dephosphorylation resulted mainly from activation of the Ca^{2+}-dependent phosphatase, calcineurin, via oxysterol-induced Ca^{2+} increase. Moreover, the correlation between the inhibition of Bad activation and apoptotic features (mitochondrial depolarization and cytochrome *c* release) by a calcineurin inhibitor strongly argues that Bad is one of the main components in the signaling pathway of 7-keto-induced apoptosis [44]. Further investigations found that, during the initial steps of 7-keto-induced apoptosis, Bad was phosphorylated after ERK activation, and this phosphorylation delayed apoptosis by preventing mitochondrial damage [75]. Taken together, these results suggest that, as well as being involved in the promotion of apoptosis, Bad is a target in an early survival pathway which attempts to delay 7-keto-induced apoptosis.

Besides Bad, Bim functions also as a sensor for apoptotic stimuli by heterodimerization and inactivation of antiapoptotic Bcl-2 proteins. Berthier et al. [75] reported that 7-keto-induced Ca^{2+} influx activated the disassociation of Bim from microtubules and consequently induced its translocation to Bcl-2 at the mitochondrial level, leading to mitochondrial damage and apoptosis. Additionally, apoptosis induced by 25-OH resulted in the degradation of the protein kinase Akt which, in turn, led to the activation of Bim and Bad and the down-regulation of the antiapoptotic Bcl-xL. The role of Akt in phosphorylation and inactivation of Bad is well documented and, therefore, it is not surprising that loss of Akt resulted in dephosphorylation of Bad [76].

Bid has also been shown to participate in oxysterol-induced apoptosis. A study by Prunet et al. [95] demonstrated that, while Bid was cleaved during both 7-keto- and 7β-OH-mediated U937 apoptosis, Bid degradation was more pronounced in the presence of 7β-OH. The requirement of an active caspase-3 to trigger Bid cleavage was confirmed by the use of an inhibitor, caspase-3-deficient MCF-7 cells and MCF-7/c3 cells (MCF-7 cells stably transfected with pro-caspase-3).

3.4.2. Antiapoptotic Bcl-2 and Bcl-xL

Alteration of the expression levels of prosurvival Bcl-2 family proteins is another way in which apoptosis can be regulated [106]. As regard to oxysterols, down-regulation of antiapoptotic Bcl-2 proteins has been observed in a number of studies. For instance, the apoptotic processes induced by 7β-OH, β-epoxide and 25-OH in U937 or THP-1 cells have been associated with decreased expression of Bcl-2 [109,110]. Also, treatment of vascular SMCs revealed that both 7-keto and 25-OH led to a dramatic decrease in Bcl-2 protein [111], while apoptosis induced by 7β-OH caused Bcl-xL degradation after 18 h [112]. Moreover, overexpression of Bcl-2 was found to be protective against oxysterol-induced apoptotic cell death [113,114].

3.4.3. Proapoptotic Bax and its interactions with Bcl-2 and Bcl-xL

Either directly or indirectly, Bax induces the release of proteins from the space between the inner and outer mitochondrial membranes. This process of mitochondrial outer membrane permeabilization results in the release of cytochrome *c* and other soluble proteins into the cytosol, where they promote caspase activation [106]. A study by Rho et al. [62] involving treatment with 7-keto is in accordance with this hypothesis. In dying human vascular SMCs, Bax was translocated from the cytosol to mitochondria and cytochrome *c* was shown to be released from the mitochondria into the cytosol. In another study, Bax decreased in the cytosol as early as 4 h after treatment with 7-keto and became strongly associated with the mitochondria-enriched fraction of vascular SMCs [93].

Initiation of the mitochondrial pathway can result in the activation of proapoptotic proteins such as Bax or Bak and inactivation of antiapoptotic Bcl-2 family members such as Bcl-2 or Bcl-xL [57]. This type of coordination is evident in apoptotic processes induced by oxysterols. In THP-1 cells, Bax expression was significantly increased, whereas Bcl-2 and Bcl-xL levels were remarkably decreased following 24 h exposure to 7-keto [73]. Subsequent to treatment with 25-OH, Bcl-2 mRNA level in Sertoli cells decreased by 60% which coincided with a 40% increase in Bax mRNA [115]. On the other hand, in Caco-2 cells exposed to 7β-OH, no changes in Bcl-2 and Bax expression were detected at 8, 12 or 24 h indicating a Bcl-2/Bax-independent cell death pathway [91].

3.5. Caspases

Apoptosis is, minimally, a two-step proteolytic pathway, and its function is to activate proteases that cleave a number of cellular proteins to drive forward the biochemical events that culminate in death and the dismantling of the cell. The cytosolic proteases that transmit the apoptotic pathway are referred to as caspases [116]. There are 14 members of the caspase family which constitute two general classes: the initiator (or apical) caspases and the effector (or executioner) caspases [117,118]. Caspases are signaling proteases that are intended for specific protein cleavage and not for protein degradation. They make a limited number of cuts in cellular proteins and therefore modify the protein structure. Apoptosis proceeds by the subsequent action of one caspase on the

latent pro-caspase form of the next one in line. These sequential acts either amplify the original signal in a cascade-like manner or allow for additional regulatory points in the pathway [116].

One of the earliest and most consistently observed features of oxysterol-induced apoptosis is the induction of caspase activity. For example, a study by Larsson et al. [119] demonstrated that 7β-OH-induced apoptosis is associated with a significant increase in caspase activation. Moreover, a mixture of 7β-OH and 25-OH, which is representative of the oxysterol composition reported in human atheromatic lesions, also induced caspase activation. In human vascular SMCs, treatment with caspase inhibitors significantly reduced 7-keto-induced TNFR-mediated apoptosis [64]. In U937 cells, caspase inhibition resulted in a marked decrease in apoptosis induced by 7-keto, 7β-OH and β-epoxide [96,120]. Furthermore, the caspase inhibitor, zVAD-fmk, prevented 7-keto-induced DNA fragmentation and inhibited SMC death by 75%. However, cytochrome *c* release was not influenced by caspase inhibition, indicating that caspase-independent intrinsic mechanisms were involved in the 7-keto-induced cytochrome *c* release [93].

3.5.1. Initiator caspases

Caspases 2, 8, 9, 10, and 12 trigger apoptosis and are known as upstream or initiator caspases. They activate the effector or downstream caspases which ultimately execute apoptotic cell death [118]. In U937 cells, caspase-2 and caspase-8 activation were associated with β-epoxide-induced apoptosis; however, these initiator caspases were not involved in the apoptotic processes induced by 7β-OH [92,110]. Luthra et al. [121] demonstrated that treatment of human microvascular endothelial cells with 7-keto activated caspase-8 and caspase-12 without affecting caspase-9. Cells were then pretreated with various caspase inhibitors, and 7-keto-induced caspase-8 activity was measured. Inhibition of caspase-12 increased caspase-8 activity while caspase-9 and caspase-3/7 inhibitors had no effect. These results suggest that 7-keto-induced caspase-8 and caspase-12 pathways have unique inhibition patterns. In addition, the authors suggested that caspase-12 is not upstream or feeding into caspase-8, and so these pathways may function in parallel to each other. In a conflicting study, when Caco-2 cells were treated with 7β-OH, caspase-9 was activated after 56 h while caspase-8 activity remained at basal levels [122].

Apoptosis induced by the oxysterol 25-OH also involves the activation of the initiator caspases. An increase in caspase-2 activity in CEM-C7 cells reached a seven-fold increase 48 h after addition of 25-OH [123], while activation of caspase-8 and caspase-9 occurred in CHO-K1 cells [124]. In general, these studies support the concept that early steps in apoptosis can differ among oxysterols as well as among cell types [125].

A study by Lizard et al. [83] revealed that GSH substantially decreased 7-keto-induced apoptosis, and this was associated with reduced pro-caspase-8 degradation. Therefore, it appears that the production of ROS is a potential trigger for activation of caspase-8 during 7-keto-induced apoptosis.

3.5.2. Effector caspases

The extrinsic and intrinsic pathways both finish at the point of the execution phase, which is considered the final pathway of apoptosis. It is the activation of the effector caspases that begins this stage of apoptosis. Effector caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that break down the nuclear and cytoskeletal proteins. Caspase-3, caspase-6 and caspase-7 function as effector or executioner caspases, cleaving various substrates such as poly(ADP-ribose) polymerase (PARP) that ultimately cause the morphological and biochemical changes seen in apoptotic cells [56].

Caspase-3 is considered to be the most important of the effector caspases and, as regard to oxysterol-induced apoptosis, it is the most widely studied. In numerous cell lines, the apoptotic processes induced by 7-keto, 7β-OH, 24-OH, 25-OH and β-epoxide have all been shown to involve caspase-3 activation [62,73,76,86,87,89,95,96,109,115,119,121,126–129]. Furthermore, cells with activated caspase-3 have been detected in atherosclerotic lesions [130]. As a result, activation of caspase-3 is often used as an indicator of an apoptotic event. For instance, treatment of U937 cells with 7-keto and 7β-OH was associated with cleavage of pro-caspase-3 and increased caspase-3 activity, while in the presence of 7α-OH no cleavage or activity was detected. When examined, the morphological aspects of 7α-OH-treated cells and their nuclei were the same as those of untreated cells. Together, these observations confirmed that 7-keto and 7β-OH, but not 7α-OH, are major inducers of apoptosis in the U937 cell line [85,131].

Caspase-3 is activated by any of the initiator caspases. In apoptotic cells, activated caspase-3 cleaves inhibitor of CAD to release CAD which then degrades chromosomal DNA within the nuclei and causes chromatin condensation. In addition, caspase-3 induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies [56]. Research by Ryan et al. [132] revealed that, in U937 cells, 7β-OH stimulated cleavage of pro-caspase-9 at 9, 12 and 24 h, pro-caspase-3 at 12 h, with subsequent degradation of PARP at the 24- and 48-h time points. The authors suggested that, in the 7β-OH-induced apoptotic pathway, caspase-9 acts as the initiator caspase responsible for activating caspase-3, while caspase-9 may also be cleaved by caspase-3 in a feedback loop.

As previously addressed, Ca^{2+} appears to play a role in apoptosis induced by certain oxysterols and, therefore, it can also affect activation of caspase-3. When oxysterol-induced Ca^{2+} influx was blocked by Ca^{2+} channel inhibitors such as nifedipine and verapamil, caspase-3 activity decreased significantly [44,133]. In addition, recent evidence has emerged of a potential link between the Ca^{2+}-binding protein calmodulin and oxysterol-induced apoptosis. Han et al. [90]

and Lee et al. [45] reported that calmodulin antagonists attenuated 7-keto-induced cell death via reduced caspase-3 activation. These studies strengthen the view that Ca^{2+} is an influential component in the apoptotic processes induced by oxysterols.

Overall, the data indicate that the caspase cascade is a crucial event in oxysterol-induced apoptosis. While components of the initiation phase may differ, the requirement of an active effector caspase, like caspase-3, appears to be more universal.

3.6. Inhibitor of apoptosis proteins

The IAPs are a family of antiapoptotic proteins that can specifically bind and inhibit caspase-3, caspase-7 and caspase-9 and thereby prevent apoptosis. Growing evidence indicates that IAPs also modulate cell division, cell cycle progression and signal transduction pathways. Human IAPs include X-chromosome-linked IAP (XIAP), cellular IAP-1/2 (cIAP-1/2), survivin and Apollon [134]. IAPs inhibit at least two of the major pathways involved with initiation of caspase activation: the mitochondrial pathway with cytochrome *c* and the death receptor pathway with the TNF family of death receptors [135].

Like Smac/DIABLO, findings from oxLDL studies are used to advocate involvement of antiapoptotic IAPs in oxysterol-induced apoptosis. In human coronary artery endothelial cells and murine macrophages, 24-h treatment with oxLDL resulted in the down-regulation of IAP expression, which corresponded to a significant cytosolic accumulation of Smac/DIABLO [100,101]. These findings comply with the literature as Smac/DIABLO is a well-known IAP antagonist. Blanc-Brude et al. [136] revealed discrete IAP survivin expression in human aorta lipid streaks but virtually none in advanced atherosclerotic plaques. The authors deduced that dysregulation of survivin expression caused by re-current oxLDL exposure may favor apoptosis in advanced atherosclerotic plaques despite up-regulated cIAP-2 and XIAP. As previously discussed, oxLDL and atherosclerotic plaques contain substantial levels of oxysterols, and it is the apoptosis-inducing abilities of these oxysterols which are believed to have dramatic consequences on the development of atherosclerosis. Hence, it is probable that oxysterols have an effect on IAP levels; however, further research involving both individual oxysterols and oxysterol mixtures is needed before definitive conclusions can be made.

4. Conclusions

Cell culture systems and ex vivo studies are commonly used in an attempt to accurately define the signal transduction pathways induced by oxysterols. While this may not be ideal, numerous in vitro studies have concluded that apoptotic processes induced by oxysterols depend largely on the cell type and the oxysterol in question. The current weight of evidence strongly suggests that the majority of oxysterols induce apoptosis through the intrinsic pathway. Nonetheless, while there is little to suggest induction of apoptosis through the death receptor pathway, involvement of Fas and TNFR in apoptosis induced by 7-keto, 7β-OH and 25-OH has been described. Moreover, a variety of studies have illustrated that the generation of an oxidative stress and perturbations in intracellular Ca^{2+} by oxysterols may be initiating factors of apoptotic transduction within the cell. Thus far, oxysterol-induced cell death is associated with the following hallmarks of apoptosis: activation of the Bcl-2 protein Bax, loss of mitochondrial transmembrane potential, release of cytochrome *c*, Smac/DIABLO and Endo G into the cytosol, followed by activation of caspase-3 with subsequent degradation of internucleosomal DNA. While the intrinsic and extrinsic apoptotic pathways are presented as separate entities, it is important to note, however, that cross-talk exists between them. Therefore, the concept of multiple signal transduction pathways is useful in helping to realize that different effects may occur in different systems, which is true of oxysterol-induced apoptosis.

To date, much research has focused on the role of oxysterols in the development of atherosclerosis. Oxysterols appear to be concentrated in foam cells and early lesions, and it is now emerging that high plasma levels of certain oxysterols may be risk factors in cardiovascular disease [130,137–139]. However, apart from the proatherogenic properties displayed by oxysterols, several new lines of evidence support the view that oxysterols may also be involved in the pathogenesis of degenerative diseases such as AD and AMD. More extensive studies on the diverse apoptotic pathways evoked by different oxysterols may help to discriminate among the apoptotic effectors that exist and allow for development of therapies for these degenerative conditions.

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