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Oxidised Products of Cholesterol: Their Role in Apoptosis

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Abstract: Oxysterols are oxygenated derivatives of cholesterol that may be formed endogenously or absorbed from the diet and are particularly found in highly processed foods of animal origin. These compounds elicit a range of biological effects such as inhibition of HMG-CoA reductase, alteration in cellular membrane properties and the induction of cell death in a number of *in vitro* models. Much research, to date, has focused on oxysterols and their potential role in the development of atherosclerosis due to the ability of certain oxysterols to induce apoptosis in cells of the arterial wall, a primary process in the development of atheroma. Apoptotic cell death is the culmination of a series of events centring on the cascade of caspases, which triggers downstream effects leading to DNA cleavage. Though the induction of apoptosis by oxysterols has been the topic of intense research over the last few years, the exact mechanism through which certain oxysterols induce apoptotic cell death remains to be clarified. This review focuses on the biological effects of oxysterols, with particular emphasis on oxysterol-induced apoptosis.

Keywords: Apoptosis, atherosclerosis, caspases, oxLDL, oxysterols, signal transduction.

INTRODUCTION

Cholesterol (cholest-5-en-3 -ol) is an unsaturated neutral lipid found ubiquitously in mammalian tissue. Oxysterols are oxygenated derivatives of cholesterol, (Fig. **1**), that may be formed exogenously by autoxidation of cholesterol and endogenously by free-radical attack upon cholesterol or by enzymatic processes, mainly in biosynthesis of bile acids and steroid hormones [1]. The biological effects attributable to oxysterols include inhibition of the enzyme hydroxymethyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) leading to reduced endogenous cholesterol synthesis, alteration in cellular membrane properties and the induction of cell death in a number of *in vitro* models [2]. Studies suggest the cytotoxicity of low-density lipoprotein (LDL) in the development of atherosclerotic plaque may be due to the presence of oxysterols. Oxysterols, apoptotic and/or necrotic cells have been identified in these plaques. While the induction of apoptosis by oxysterols has been the topic of intense research over the last few years, the exact mechanism through which certain oxysterols induce apoptotic cell death remains to be clarified.

SOURCES OF OXYSTEROLS

Dietary Oxysterols

Oxysterols may be absorbed from the diet and are particularly found in highly processed foods of animal origin. Various studies have shown that the amount of oxysterols in food can reach between 1-10% of total cholesterol [3]. Absorption of dietary oxysterols occurs in the upper intestinal tract and transportation into the plasma occurs within chylomicrons [4].

Certain variables are important in relation to oxysterol formation in foods including: composition of the food matrix, polyunsaturated fatty acid content and oxidizability, cholesterol levels, processing methods, processing times and temperatures, pH, packaging conditions, pro- and antioxidant content and water activity. Dietary sources of oxysterols are limited to foods of animal origin, since only these contain cholesterol in appreciable amounts. Among foods and food products rich in cholesterol are: eggs and egg products, meat, meat products and animal fats, fish and fish oil, milk and milk products and deep fried foods [3,5,6]. The most common oxysterols present in foodstuff include 7-ketocholesterol (7-keto), 7 -hydroxycholesterol (7 -OH), 7 -hydroxycholesterol (7 -OH), cholesterol 5 ,6 -epoxide (-epoxide), cholesterol 5 ,6 -epoxide (-epoxide), 25-hydroxycholesterol (25-OH), 19-hydroxycholesterol (19-OH), 20-hydroxycholesterol (20-OH) and cholestanetriol (triol) [6,7].

Oxysterols Formed Endogenously

Oxysterols may be generated within mammalian tissue by enzymatic and non-enzymatic oxidation of cholesterol. They may be derived from enzymatic oxidation of cholesterol during the synthesis of bile acids and steroid hormones or *via* the biosynthesis of cholesterol from lanosterol [8]. The principal enzymes involved in bile acid biosynthesis are the microsomal enzyme 7 -hydroxylase and the mitochondrial sterol 27-hydroxylase [6]. 7 -Hydroxylase, present in the liver, is the rate-limiting enzyme of the classic bile acid biosynthetic pathway responsible for converting cholesterol to 7 -OH. Cholesterol may also be oxidized by sterol 27-hydroxylase, in an alternative pathway, to 27-OH. Cholesterol is also oxidized to 25-OH (by 25-hydroxylase), 27-OH and 24-OH (by 24-hydroxylase) in the liver, lung and brain, respectively [9].

Endogenous non-enzymatic oxidation or autoxidation of cholesterol may be initiated by a variety of oxygen species including reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH·). Hodis *et al.* [10]

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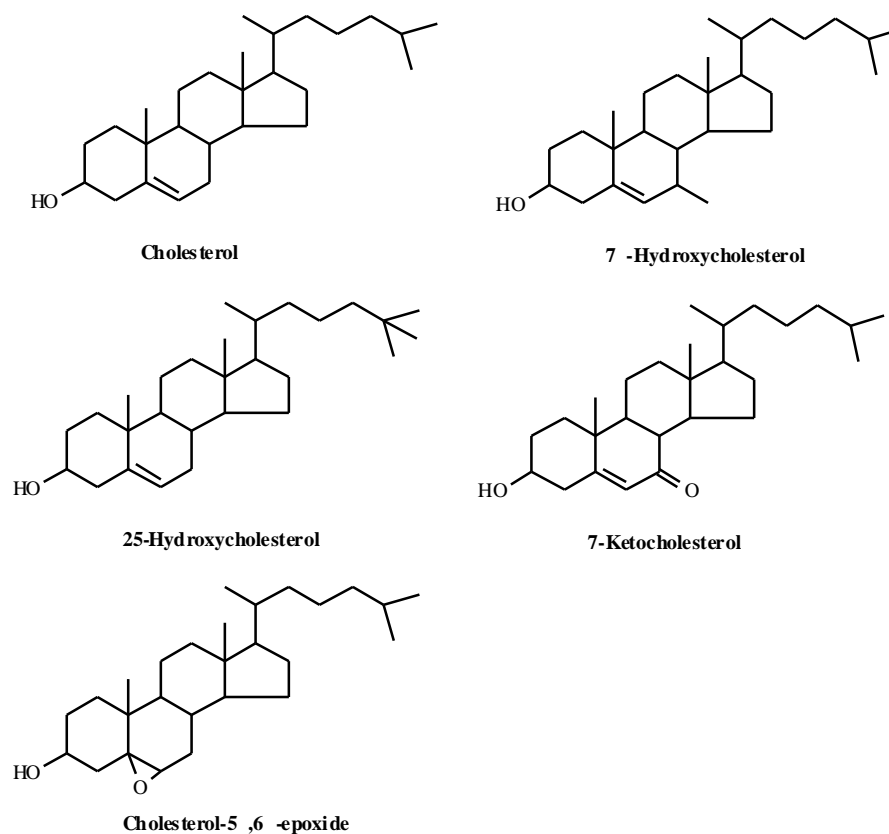
Cholesterol

7 -Hydroxycholesterol

25-Hydroxycholesterol

7-Ketocholesterol

Cholesterol-5 ,6 -epoxide

Fig. (1). Structures of some commonly occurring oxysterols.

found that plasma levels of -epoxide, -epoxide and 7-keto oxysterols, formed through non-enzymatic processes, were increased in cholesterol-fed rabbits even though oxysterols were not detected in the feed, providing evidence for the non-enzymatic oxidation of cholesterol *in vivo*. Further study by Hodis *et al.* [11] demonstrated that addition of the antioxidant probucol to cholesterol-fed rabbits significantly reduced plasma oxysterol levels, implicating a role for the non-enzymatic route in the formation of oxysterols.

Oxysterols in Plasma

The levels of oxysterols present in plasma are difficult to determine accurately due to complications in the separation and identification of various oxysterols and the generation of certain oxysterols from cholesterol autoxidation during sample storage, processing and analysis [8]. The concentration of oxysterols in human plasma may vary from 1µM in healthy subjects to almost 40µM in individuals suffering from hypercholesterolaemia [8]. Emanuel *et al.* [4] examined the concentration of oxysterols in the plasma following the administration of a cholesterol-rich meal. Their study illustrated that oxysterols in food caused an increase in plasma oxysterols levels. A study by Mafouz and Kummerow [12] showed that, in rabbits, cholesterol feeding induced severe hypercholesterolaemia and increased plasma levels of certain oxysterols.

The most abundant oxysterols in plasma are those that are generated *in vivo* through enzymatic oxidation of cholesterol, 27-OH, 24-OH and 7 -OH. Other oxysterols

such as 7 -OH, 7-keto, -epoxide, -epoxide and triol have also been found in human plasma and aortic tissue [13]. Oxysterols in plasma may be esterified to long-chain fatty acyl esters by intracellular acylCoA:cholesterol acyltransferase (ACAT) or by extracellular lecithin:cholesterol acyltransferase (LCAT). Esterified oxysterols may be transferred between lipoproteins through the action of cholesteryl ester transfer protein. Macrophages may accumulate both esterified and non-esterified oxysterols and, consequently, induce foam cell formation [2].

BIOLOGICAL EFFECTS OF OXYSTEROLS

Oxysterols are thought to be involved in many biological processes such as regulation of cholesterol homeostasis, altering membrane function, synthesis of steroid hormones and bile acids and cell growth and proliferation. Cholesterol is ubiquitously present in mammalian tissue and is essential for the formation and function of cellular membranes. Oxysterols may be incorporated into membranes in place of cholesterol [2]. This ability of oxysterols to insert themselves into plasma membranes, displacing membrane cholesterol, may greatly affect membrane stability and may alter cell morphology, survival, growth and function.

Cells may form oxysterols in order to facilitate the elimination of excess cholesterol. Oxysterols can cross cell membranes and equilibrate with lipoproteins at a much faster rate than cholesterol. Side-chain oxidised oxysterols, such as 24-OH and 27-OH in particular, are involved in the transport of cholesterol. Upon entering the circulatory system,

oxysterols are rapidly taken up by the liver where they are further oxidised to bile acids and other water-soluble metabolites [14].

Oxysterols can modify the activity of different enzymes related to sterol metabolism. Several oxysterols, especially those hydroxylated on the side-chain, such as 25-OH, have been shown to exert inhibitory effects on the activity of HMG-CoA reductase, an enzyme which converts HMG-CoA to mevalonate, the rate limiting step in the cholesterol biosynthetic pathway [15]. Inhibition of HMG-CoA reductase in actively dividing cells could result in deficient cholesterol synthesis and impaired membrane function. Studies have suggested that this inhibitory effect is due to a reduction in de novo synthesis of HMG-CoA reductase as well as stimulation of its degradation [16]. Other enzyme activities that may be altered by oxysterols include acetoacetyl CoA thiolase (ACAT), cholesterol 7 -hydroxylase, cholesterol-5,6-epoxide hydrolase, HMG-CoA synthase, methylsterol oxidases and mevalonate kinase [2].

OXYSTEROLS AND APOPTOSIS

Of the biological effects attributable to oxysterols, the one that has received most attention, over the last couple of decades, is their ability to induce apoptosis in a variety of cell lines *in vitro* (Tables **1** and **2**). There are two fundamental types of cell death, apoptosis and necrosis. Apoptosis, or programmed cell death, is an active process with unique morphological and biochemical features including cell shrinkage, membrane blebbing, chromatin margination and condensation with subsequent internucleosomal fragmentation of DNA and finally formation of discrete membrane enclosed vesicles known as apoptotic bodies [17]. In contrast, necrosis, or accidental cell death, is a passive process characterised by an increase in cell volume, swelling of the mitochondria and vacuolisation in the cytoplasm. The necrotic process culminates in membrane rupture and cell lysis [18].

Certain oxysterols have been shown to be cytotoxic *in vitro* and the mode of toxicity has been identified as apoptosis in certain cells lines [19-24]. Christ *et al.* [25] showed that 25-OH and 7 ,25-dihydroxycholesterol (7,25-OH) induced cell death in murine lymphoma cells *in vitro* and in mouse thymocytes. Cell death induced by these oxysterols exhibited many characteristics of apoptosis such as DNA fragmentation, considered to be the hallmark of apoptotic cell death. O'Callaghan *et al.* [26] investigated the cytotoxic effects of 7 -OH in a human monocytic blood cell line, U937 and a human hepatoma cell line, HepG2. 7 -OH induced apoptosis in the U937 blood cell line but necrosis in the HepG2 liver cell line. An earlier study by O'Callaghan *et al.* [27] found that while 7 -OH induced apoptosis in U937 cells, 25-OH was cytostatic only. Lizard *et al.* [28] examined the effects of 7-keto on bovine aortic endothelial cells (BAE) and human vascular endothelial cells (HUVEC). DNA fragmentation, chromatin condensation, altered mitochondria and apoptotic bodies were observed in both the bovine and human vascular endothelial cells treated with 7-keto. The ability of a particular oxysterol to induce cell death varies depending on the structure of the oxysterol, the cell line, duration of exposure and the concentration of the oxysterol used.

Table 1.   Summary of Studies Which have Investigated the Apoptogenicity of Oxysterols Using Animal Cell Models

Cell Line	Oxysterols	Result	References
Dog Gall Bladder Epithelial Cell Line	Triol	There was shown to be a greater than 3-fold increase in apoptotic cells, relative to the control on exposure of this cell line to triol.	[22]
Chinese Hamster Ovary Cell Line	OxLDL, 25-OH	Both oxLDL and 25-OH were shown to induce apoptosis in this cell line.	[52,73]
Mouse Thymus Tissue	25-OH, 27-OH	Both 25-OH and 27-OH were shown to induce thymic apoptosis.	[21]
Murine Thymocytes	25-OH	25-OH was shown to induce apoptosis in this cell line.	[25]
P388D1-Murine Macrophage Cell Line	7-Keto, 25-OH	Both oxysterols induced apoptosis, however, 25-OH was a more potent inducer of apoptosis than 7-keto in this cell line.	[24, 57]
RDM4-Murine Macrophage-like Cell Line	25-OH	25-OH was shown to induce apoptosis in this cell line.	[25]
J774-Murine Macrophage Cell Line	Oxysterol mixture comprising of 7 -OH, 7 -OH, -epoxide, triol, 7-Keto, 25-OH, 2-epoxide	There was a time and dose-dependant increase in apoptotic cells on exposure to the oxysterol mixture. 7-keto-induced apoptosis was ameliorated in the presence of an oxysterol mix.	[51, 64]
Bovine Aortic Endothelial Cell Line	7-Keto, 7 -OH, 19-OH, -epoxide, 25-OH	The order of apoptotic potency was found to be: 7 -OH > 7-keto > 19-OH > -epoxide > 25-OH in this cell line.	[19]
Rabbit Aortic Smooth Muscle Cells Line	OxLDL, 7-Keto, 25-OH	There was a dose dependant increase in apoptotic cells in response to incubation with 25-OH, 7-keto or oxLDL.	[56]

Table 2. **Summary of Studies which have Investigated the Apoptogenicity of Oxysterols Using Human Cell Models**

Cell Line	Oxysterols	Result	References
MRC5-Human Fibroblast Cell Line	7 -OH, 7-keto	Oxysterols were cytotoxic to this cell line but there was no evidence of apoptosis.	[80]
CEM-C7-Human Leukemic Cell Line	25-OH	25-OH was shown to be a potent inducer of apoptosis in this cell line.	[20, 107]
U937-Human Monocytic Cell Line	25-OH, 7 -OH, 7 -OH, 7-Keto	7 -OH and 7-keto induced apoptosis in this cell line with 7 -OH the more potent of the two. 7 -OH was not cytotoxic and 25-OH was shown to be cytostatic only.	[26, 48, 49, 58, 60, 80, 97]
THP-1-Human Monocytic Cell Line	7 -OH, 25-OH, 7-Keto	7 -OH was a more potent inducer of apoptosis than 25-OH in this cell line. 7-Keto induced moderate levels of apoptosis.	[55, 104]
HL-60- Human Monocytic Cell Line	25-OH, 7 -OH, 7-Keto	7 -OH was shown to induce apoptosis but 25-OH was found to be cytostatic only.	[23, 97]
Human Arterial Endothelial Cell Line	7 -OH, 7-keto, - epoxide, -epoxide	All oxysterols examined induced apoptosis in this cell line.	[67]
Human Umbilical Vein Endothelial Cell Line	7 -OH, 7-Keto, 25-OH, -epoxide, OxLDL, 7 -OH, triol	7 -OH was not cytotoxic. The order of potency for the remaining oxysterols varied between the studies but it appears that 7-keto and 7 -OH were the more potent inducers of apoptosis.	[88, 91, 103]
Human Umbilical Vein Fibroblast Cell Line	7 -OH, 7-Keto	Oxysterols were cytotoxic to this cell line but there was no evidence of apoptosis.	[103]
Vascular/Aortic Smooth Muscle Cell Line	25-OH, 7 -OH, 27-OH, -epoxide, 7-Keto	All oxysterols examined were shown to induce apoptosis in this cell line, 25-OH and 27-OH were shown to be more potent inducers of apoptosis than 7 -OH or -epoxide.	[36, 72, 103]
HepG2-Human Hepatoma Cell Line	7 -OH	7 -OH was cytotoxic to this cell line but there was no evidence of apoptosis.	[27]

Signal Transduction Pathways of Oxysterol-Induced Apoptosis

The apoptotic process has several common features; an initiation phase during which an intracellular sensor detects the presence of a death-inducing signal and activates a signal transduction pathway; a commitment phase, which is a 'point of no return' after which death signals become irreversible and an execution phase which ultimately leads to the destruction of the cell. There are two major apoptotic pathways, known as the extrinsic, or death receptor pathway and the intrinsic, or mitochondrial pathway. In vascular cells oxysterols have been shown to induce apoptosis through either pathway [29].

The extrinsic pathway requires the binding of a ligand to a death receptor on the cell surface [30]. Many death-triggering signals are related to the tumour necrosis factor receptor (TNFR) superfamily which includes Fas (also known as Apo1 or CD95), TNF and TRAIL (TNF-related apoptosis-inducing ligand) receptors [31]. Ligand binding to death receptors results in oligomerisation of the intracellular death domain and recruitment of an adaptor protein called Fas Associated Death Domain (FADD) [32]. This complex binds to pro-caspase-8 through death effector domain (DED) interactions to form a complex called the death inducing signalling complex (DISC) [33] which is a trigger for downstream activation of effector caspases. In certain cells,

sufficient activation of caspase-8 is initiated at the DISC resulting in direct activation of caspase-3 [34]. In other cells, caspase-8 catalyses the cleavage of BID, a death promoting member of the Bcl-2 family, to a truncated form tBID which binds with Bak, another Bcl-2-related protein, to release cytochrome *c* from the mitochondria thus activating the intrinsic pathway [35]. Few studies, to date, have concentrated on investigating the ability of oxysterols to induce apoptosis *via* the extrinsic pathway. Lee and Chau [36] found that 7 -OH and 25-OH upregulated the expression of death mediators, p53, Fas and Fas ligand in vascular smooth muscle cells. Their study also suggested that reactive oxygen species triggered the apoptotic cascade. Sata and Walsh [37] showed that oxLDL induced apoptosis in vascular endothelial cells through Fas-Fas ligand interaction and that oxLDL acted by sensitising the cells to death signals from the Fas receptor. Ryan *et al.* [38] compared the apoptotic processes induced by 7 -OH and -epoxide in U937 cells. Their results illustrated that -epoxide-induced apoptosis proceeded in the absence of cytochrome *c* release from the mitochondria suggesting that cell death induced by -epoxide may be *via* the extrinsic pathway.

The second major apoptotic pathway, the intrinsic pathway, is mediated by mitochondrial release of proapoptotic molecules. Perturbation of the mitochondria results in the opening of the mitochondrial permeability transition pore (MPTP), a non-specific pore in the inner

mitochondrial membrane thought to open under conditions of elevated calcium concentrations [39]. Opening of the MPTP causes a massive swelling and depolarisation of the mitochondria, a condition referred to as the mitochondrial permeability transition (MPT) [40]. The MPTP, once opened, is thought to play a role in apoptosis through the release of proapoptotic molecules such as cytochrome *c*, Smac/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein of low isoelectric point [pI]) and AIF (apoptosis inducing factor). Release of cytochrome *c*, from the intermembrane space of the mitochondria into the cytosol, provides the signal for the initiation of apoptosome assembly. Once released, cytochrome *c* interacts with apoptotic protease-activating factor-1 (Apaf-1), ATP/dATP and recruits pro-caspase-9 to form the apoptosome [41], which can be regarded as the cytosolic counterpart of the DISC. Active caspase-9 in turn cleaves and activates caspase-3, leading to the morphological and biochemical changes characteristic of apoptosis [42].

Smac/DIABLO may promote caspase activation by binding to inhibitor of apoptosis proteins (IAPs) and directly eliminating IAP inhibition of caspases [43]. AIF is a phylogenetically conserved mitochondrial intermembrane flavoprotein capable of inducing chromatin condensation and DNA fragmentation [44]. The release of AIF from the mitochondria occurs before that of cytochrome *c* and before caspase activation and induces apoptosis by a caspase-independent mechanism [45]. Cytochrome *c* may require the release of Smac/DIABLO and AIF to ensure efficient activation of the apoptotic pathway [46].

The ability of oxysterols to induce apoptosis through the intrinsic pathway has been well studied. Miguet-Alfonsi *et al.* [47] found that 7 -OH and 7-keto induced apoptosis in U937 cells *via* loss of mitochondrial potential, caspase-3 activation, PARP degradation and DNA fragmentation. Both oxysterols were also found to enhance superoxide anion ($O_2^{\bullet-}$) production before and after the loss of mitochondrial potential. Ryan *et al.* [48] reported that, in U937 cells, 7 -OH-induced apoptosis involved a decrease in glutathione levels followed by activation of caspase-9, caspase-3 and degradation of PARP. 7-Keto has been shown to induce apoptosis *via* release of cytochrome *c* from the mitochondria with subsequent caspase-9 and caspase-3 activation in a variety of cell lines [49-51]. Yang and Sinensky [52] reported that, in CHO-K1 cells, 25-OH induced apoptosis *via* the mitochondrial route involving release of cytochrome *c* and the typical caspase cascade.

The Bcl-2 Family

Bcl-2 proteins are crucial regulators of apoptosis. The Bcl-2 family includes anti- and proapoptotic members that control the mitochondrial amplification loop of apoptosis. On the basis of function and sequence similarity they can be divided into three groups: group I (Bcl-2/Bcl-X$_L$/Bcl-w/Mcl-1/A1/Bfl 1) inhibit apoptosis by binding to and sequestering proapoptotic Bcl-2 family members [53]. Group II includes Bax and Bak, promoters of cell death, whose activity is necessary to induce cytochrome *c* release from the mitochondria. Group III is comprised of proapoptotic proteins (Bid/Bad/Bik/Bim) which translocate from the cytosol to the mitochondria, in response to apoptotic stimuli, and then activate Bax or Bak to induce the release of apoptogenic mitochondrial proteins [54].

Bcl-2 is thought to play an active role in oxysterol-induced apoptosis. Lim *et al.* [55] investigated 7 -OH and 25-OH-induced apoptosis in the THP-1 cell line. Both oxysterols decreased the level of antiapoptotic Bcl-2 but had no effect on the level of Bax. Nishio and Watanabe [56] showed that both 7-keto and 25-OH induced apoptosis, in vascular smooth muscle cells, by downregulation of Bcl-2 protein. Harada *et al.* [57] found that murine macrophage-like cells, transfected to overexpress Bcl-2, were partially protected from 7-keto and 25-OH-induced apoptosis. The same study also found that inhibition of caspase-3 suppressed cell death in control cells but had no effect in the cells overexpressing Bcl-2 indicating that Bcl-2 is an upstream regulator of caspase-3. In a similar study, Lizard *et al.* [58] examined the effects of 7 -OH and 7-keto on U937 cells and U4 cells (U937 cells transfected to overexpress Bcl-2). Apoptosis was partially inhibited by Bcl-2 overexpression. U4 cells had a lower proportion of apoptotic cells and a significant reduction in Interleukin-1 secretion compared to the U937 cells.

Caspases

Caspases are a family of cysteine proteases that are the primary drivers of apoptosis. All caspases are present as inactive precursors in cells that must be proteolytically cleaved in order to be activated. Each caspase is synthesized as a zymogen (pro-form) that consists of a pro-domain, a large (~20 kDa) subunit and a small (~10 kDa) subunit [53]. Cleavage and subsequent heterodimerization of the larger and smaller subunits result in caspase activation [42]. An active caspase can cleave and activate other caspases leading to a caspase cascade and ultimately cell death.

To date, a number of mammalian caspases have been identified. Based on the length of their pro-domain caspases can be divided into two distinct groups: group I that contain a relatively long pro-domain (caspases -1, -2, -4, -5, -8, -9, -10, -11, -12, -13) and group II containing a short pro-domain (caspases -3, -6, -7, -14). Caspases -1, -4, -5, -11, -12 and -14 are primarily involved in cytokine processing [34]. Many group I caspases are initiator proteases (caspases -2, -8, -9, -10), activated with the help of adaptor molecules such as Apaf-1 and FADD/MORT1. Group II caspases (not including caspase-14) are also known as effector caspases. They lack the ability to self-activate and appear to require cleavage by activated initiator caspases [59]. Once activated, effector caspases cleave various proteins leading to the biochemical and morphological features characteristic of apoptosis.

The caspase cascade is a critical event in oxysterol-induced apoptosis. Miguet *et al.* [60] found that 7 -OH and 7-keto-induced apoptosis in U937 cells was prevented when cells were co-incubated with a broad-spectrum caspase inhibitor. Nishio and Watanabe [56] showed that 7-keto and 25-OH-induced apoptosis in vascular smooth muscle cells occurred *via* activation of caspase-3. Miguet-Alfonsi *et al.* [47] investigated the apoptotic potency of oxysterols oxidised at C7 (7 -OH, 7 -OH and 7-keto) in U937 cells.

7 -OH and 7-keto induced apoptosis with loss of mitochondrial potential and activation of caspase-3. Agrawal *et al.* [50] found that 7-keto induced apoptosis in fibroblasts *via* caspase-9 activation and subsequent caspase-3 activation. Lim *et al.* [55] examined 7 -OH and 25-OH-induced cell death in the THP-1 cell line. They determined that apoptosis induced by the two oxysterols proceeded *via* activation of caspase-9 and caspase-3 and degradation of the caspase-3 substrate, poly ADP-ribose polymerase (PARP). Ryan *et al.* [48] found similar results in U937 cells treated with 7 -OH and also observed that caspase-8 did not appear to play a major role in this particular apoptotic pathway. Yang and Sinensky [52] demonstrated that in CHO-K1 cells, 25-OH induced apoptosis *via* activation of caspase-3, -8 and -9.

Oxysterols and Mitochondria

The mitochondria are involved in a variety of key apoptotic events including release of caspase-activating molecules (such as cytochrome *c*), changes in electron transport, loss of mitochondrial transmembrane potential, opening of the mitochondrial permeability transition pore and acting as a site of convergence for various pro- and anti-apoptotic Bcl-2 family members [61]. Mitochondrial permeability transition (MPT) refers to the massive swelling and depolarisation of mitochondria that occurs under certain conditions such as excess calcium and oxidative stress [40]. The cause of MPT has been attributed to the opening of the mitochondrial permeability transition pore (MPTP), a non-specific pore in the inner mitochondrial membrane [62]. Opening of the MPTP results in depolarisation of the mitochondria, swelling of the matrix leading to rupture of the outer mitochondrial membrane and the release of various proapoptotic molecules such as cytochrome *c*, AIF and Smac/DIABLO. Miguet-Alfonsi *et al.* [47] found that incubation of U937 cells with either 7 -OH or 7-keto led to loss of mitochondrial transmembrane potential followed by caspase-3 activation and PARP degradation. An earlier study by Miguet *et al.* [60] showed that 7-keto-induced loss of mitochondrial transmembrane potential in U937 cells triggered the release of cytochrome *c* from the mitochondria into the cytosol. Yang and Sinensky [52] treated CHO-K1 cells with 25-OH and found that apoptosis proceeded via mitochondrial release of cytochrome *c* with subsequent caspase activation. Many studies, to date, have shown the ability of 7-keto and/or 7 -OH to induce loss of mitochondrial transmembrane potential, mitochondrial permeability transition and subsequent release of cytochrome *c* [47, 49-51, 64, 65]. However, the ability of other oxysterols to induce mitochondrial permeability transition remains to be clarified.

Oxysterols and Calcium

Alterations in intracellular calcium homeostasis have been implicated in the induction of apoptotic cell death in many experimental systems [63, 66-68]. Certain apoptotic processes have been associated with a sustained increase in cytosolic free Ca^{2+} levels, a depletion of intracellular Ca^{2+} stores or a disruption of mitochondrial function and subsequent oxidative stress that leads to inhibition of the Ca^{2+} transport systems in the plasma membrane, endoplasmic reticulum or mitochondria [69]. A sustained elevation of Ca^{2+} can activate degradative enzymes such as Ca^{2+}-dependent proteases (calpain) and endonucleases responsible for DNA fragmentation [70].

The importance of calcium signalling in oxysterol-induced apoptosis has yet to be fully elucidated. Ares *et al.* [71] showed that an influx of extracellular Ca^{2+} was crucial for the induction of apoptosis in human aortic smooth muscle cells incubated with 25-OH. Cell death induced by 25-OH was inhibited when the L-type Ca^{2+} channel blockers, verapamil or nifedipine, were included in the culture medium. The same study also found that apoptosis induced by 25-OH was preceded by sustained oscillations of cytosolic Ca^{2+}. A further study conducted by Ares *et al.* [72] investigated the cytotoxic effects of 7 -OH in human aortic smooth muscle cells. Apoptosis induced by 7 -OH was associated with an induction of Ca^{2+} oscillations and a depletion of thapsigargin-sensitive Ca^{2+} pools. Rusinol *et al.* [73] showed that apoptosis induced by 25-OH, in CHO-K1 and THP-1 cells, was prevented when the cells were co-treated with nifedipine implicating Ca^{2+} uptake as part of the signalling process. Berthier *et al.* [74] reported that apoptosis induced in THP-1 cells following treatment with 7-keto was associated with an increase in cytosolic-free Ca^{2+} and activation of the calcium-dependent phosphatase, calcineurin. A recent study by Gregorio-King *et al.* [75] probed the cytotoxic effects of 25-OH, 7 -OH and 7-keto in HL60 cells. The calcium channel blocker, nifedipine, prevented apoptosis induced by 25-OH but did not protect against 7 -OH or 7-keto-induced cell death. This study suggests that the role of calcium in oxysterol-induced apoptosis varies between individual oxysterols and different cell types.

Oxysterols and Oxidative stress

The generation of an oxidative stress has been implicated in the earlier stages of apoptosis induced by certain oxysterols. Oxidative stress occurs in cells when the generation of reactive oxygen species (ROS) overwhelms the cell's natural antioxidant defences. Cells are equipped with superoxide dismutases for the elimination of the superoxide radical and catalases and glutathione peroxidases for the elimination of hydrogen peroxide and organic peroxides [76]. A role for oxidative stress in the induction of apoptosis has been suggested from studies where the introduction of low levels of ROS have induced apoptosis and from the observation that certain antioxidants may prevent cell death [77].

7 -OH-induced apoptosis in U937 cells was found to result in a decrease in cellular glutathione (GSH) levels and an increase in the activity of superoxide dismutase (SOD) [26], however the activity of the enzyme catalase remained unchanged. Bansal *et al.* [78] examined the effect of 7 -OH on the oxidative status of macrophages over 24 hours. They reported that 7 -OH decreased intracellular reduced glutathione and increased oxidised glutathione levels thus increasing the redox ratio of the cell and inducing an oxidative stress. Apoptosis induced by both 7 -OH and 7-keto in U937 cells has been shown to be associated with an enhancement of superoxide anion production and a decrease in cellular nitric oxide production [47, 51, 64, 79]. Lizard *et al.* [80] demonstrated that 7-keto-induced apoptosis, similar

to 7β-OH, in U937 cells involved a decrease in GSH levels and production of ROS. Rosenblat and Aviram [81] found that while 7-keto and 7β-OH induced high levels of superoxide anion release in macrophages, β-epoxide did not have the same effect. O'Callaghan *et al.* [82] observed that β-epoxide-induced apoptosis in U937 cells did not affect the GSH concentration or the activity of SOD illustrating that certain oxysterols may induce apoptosis in the absence of an oxidative stress.

Lizard *et al.* [49] investigated the ability of a range of antioxidants (*N*-acetylcysteine, vitamin E, vitamin C, glutathione and melatonin) to protect against 7-keto-induced cell death. While all the antioxidants were potent inhibitors of superoxide anion production, only glutathione, *N*-acetylcysteine and vitamin E prevented apoptosis. Numerous studies have illustrated the ability of α-tocopherol to inhibit oxysterol-induced apoptosis in a variety of cell lines [36, 83, 84]. Uemura *et al.* [85] examined the mechanism underlying α-tocopherol's protective effect on vascular endothelial cells treated with oxysterols. Their study revealed that α-tocopherol exerted its effects not only by scavenging ROS but also by inhibiting caspase-3 activation. These studies suggest that though the generation of an oxidative stress is a factor in apoptosis induced by certain oxysterols, it is not a 'point of no return' and the cell may be rescued from apoptosis subsequent to oxidative damage.

OXLDL

Oxidised low-density lipoprotein (oxLDL) is involved in the initiation and acceleration of atherosclerotic lesions. Numerous studies have demonstrated the ability of oxLDL to induce apoptosis in various cell lines [86]. The cytotoxicity of oxLDL has been attributed to its bioactive lipid components such as oxysterols, lysophospholipids and fatty acid peroxides. Lysophosphatidylcholine is involved in the induction of growth factors, cytokines, adhesion molecules and monocyte chemoattractants [87]. However, a study by Harada-Shiba *et al.* [88] examining the apoptosis-inducing activity of the lipid fractions of oxLDL found that lysophosphatidylcholine did not induce apoptosis in human umbilical vein endothelial cells (HUVECs). Fatty acid hydroperoxides such as 9-hydroxyoctadecadienoic acid (HODE) and 13-HODE and several aldeheydes found in oxLDL increase the production of interleukin-1 beta (IL-1β) by monocytes [87]. 4-Hydroxynonenal (4-HNE) is one of the most abundant aldehydes present in oxLDL and is involved in apoB modifications that alter LDL metabolism and the formation of cell protein-adducts that are detected in atherosclerotic areas [89]. *In vitro* 4-HNE induces derivatization and tyrosine phosphorylation of tyrosine kinase receptors [90]. While there is some evidence that fatty acid hydroperoxides may induce apoptosis, the majority of the research would indicate that the ability of oxLDL to induce apoptosis in vascular cells is largely due to its oxysterol component [29,91].

Studies investigating the signalling pathway of oxLDL-induced apoptosis have found that both the intrinsic and extrinsic pathways of apoptosis may be involved. Chen *et al.* [92] found that oxLDL decreased the expression of the antiapoptotic protein Bcl-2 and induced apoptosis *via* release of cytochrome *c* and Smac, activation of caspase-9 and subsequent activation of caspase-3 in human coronary artery endothelial cells (HCAECs). Meilhac *et al.* [93] reported that in endothelial cells expressing low levels of Bcl-2, oxLDL induced a sustained rise in calcium, which is a common trigger of apoptosis. Cell death induced by oxLDL may occur through either the extrinsic or intrinsic pathway of apoptosis or through both simultaneously. Vicca *et al.* [94] found that in U937 cells exposed to LDL oxidised by hypochlorous acid, apoptosis occurred by both death-receptor-mediated caspase-8 activation and mitochondria-mediated caspase-9 activation. Sata and Walsh [37] reported the ability of oxLDL to induce Fas-mediated apoptosis in endothelial cells. A study by Lee and Chau [36] found that the Fas/Fas ligand-mediated pathway was involved in oxLDL-induced apoptosis in vascular smooth muscle cells. Napoli *et al.* [95] studied the effects of oxLDL in human coronary endothelial cells and smooth muscle cells. Their results demonstrated that apoptosis induced by oxLDL involved both Fas and TNF receptors and occurred *via* the extrinsic and intrinsic pathways of apoptosis.

Exposure to individual oxysterols does not occur *in vivo* as a number of oxysterols are present simultaneously in the oxLDL molecule [96]. The cholesterol oxidation products found in oxLDL include 7β-hydroperoxycholest-en-3β-ol (7β-OOH), 7β-OH, 7-keto, 25-OH, α-epoxide and β-epoxide [8]. It may be more physiologically relevant to expose cells, *in vitro*, to a number of oxysterols concurrently. Certain studies have focused on attempting to decipher possible interactive effects between oxysterols. Aupeix *et al.* [97] and Lyons *et al.* [98] reported that simultaneous treatment of U937 cells with equimolar concentrations of 7β-OH and 25-OH considerably decreased the induction of apoptosis seen in cells treated with 7β-OH alone.

Biasi *et al.* [51] recently compared the proapoptotic effects of a biologically representative mixture of oxysterols with equimolar amounts of 7-keto and found that while 7-keto induced apoptosis *via* the mitochondrial route, the oxysterol mixture did not induce cell death in cultured murine macrophages. The same study also found that when 7-keto and 7β-OH were administered simultaneously the proapoptotic effect of 7-keto was dramatically reduced compared to its toxicity when administered alone. Leonarduzzi *et al.* [64] showed that an oxysterol mixture containing 7-keto counteracted the ability of 7-keto alone to up-regulate the proapoptotic factor p21 and trigger the mitochondrial pathway of apoptosis in murine J774.A1 macrophages. An earlier study by Leonarduzzi *et al.* [99] compared the effects of equimolar concentrations of 7-keto with a mixture of oxysterols representative of that found in oxLDL. 7-keto induced apoptosis in cultured murine macrophages when administered alone. Co-incubation of 7-keto and the oxysterol mixture decreased viability by 10-15% with few cells showing fragmented and/or condensed nuclei, characteristic of apoptotic cell death. The oxysterol mixture significantly up-regulated both the expression and synthesis of transforming growth factor β1 (TGF-β1), known to play a key role in the progression of the fibrotic degeneration of the arterial wall. Leonarduzzi *et al.* [64] stated in a recent publication that "oxysterols in the complex chemical composition found in oxLDLs quench the inherent

toxic effects of single oxidised cholesterol derivatives". However, it is beneficial to determine the toxicity and apoptogenicity of individual oxysterols due to the wide variation in the concentration of single cholesterol oxides in oxLDL.

OXYSTEROLS AND ATHEROSCLEROSIS

Athereosclerosis is a multifactorial disease characterised by the build up of plaque on the inside wall of the arteries. The fatty streak lesions typical of early atherosclerosis are made up mainly of cholesterol-laden macrophages known as foam cells [100]. The majority of the cholesterol that accumulates in macrophage foam cells originates from low-density lipoprotein (LDL) [101]. Native LDL, however, is not taken up at an enhanced rate by macrophages, it is only after its oxidation that the cellular uptake of LDL is increased leading to cholesterol accumulation and foam cell formation [96]. After penetrating the arterial wall, LDL adheres to proteoglycans that entrap it and increase its susceptibility to oxidation, (Fig. **2**). The oxysterol component of the oxLDL molecule downregulates the expression of LDL receptors on vascular cells and encourages the uptake of oxLDL by scavenger receptors, such as CD36, expressed on macrophages, leading to foam cell formation [102].

Oxysterols are present in human macrophages/foam cells and atherosclerotic plaque and have been suggested to play an active role in atherosclerotic plaque development. The induction of apoptosis in cells of the arterial wall is a primary process in the development of atheroma [6]. Lizard *et al.* [103] found that oxysterols were cytotoxic to the three major cell types of the vascular wall; endothelial cells, smooth muscle cells and fibroblasts. There is no direct evidence yet that oxysterols contribute to atherogenesis in humans. However, studies of oxysterols in human and animal atherosclerosis have suggested that raised plasma levels of certain oxysterols may be associated with an increased risk of atherogenesis [104]. Rosenblat and Aviram [81] demonstrated the presence of high levels of oxysterols in macrophages isolated from atherosclerotic apoplipoprotein E deficient mice. A link was suggested between the oxysterols, macrophage NADPH-oxidase activation and cell-mediated oxidation of LDL, a key event during early atherogenesis. Li *et al.* [105] exposed U937 and THP-1 cells to 7-keto and 7 -OH and observed that the cytotoxic effects of these oxysterols were similar to those observed in macrophages within atherosclerotic lesions. Hayden *et al.* [106] examined the proatherogenic effects of 7-keto in THP-1 cells. Their study found that THP-1 cells which had been exposed to 7-keto, for a 7-day period, displayed the morphological features of differentiated macrophages. THP-1 cells induced to differentiate by 7-keto formed foam cells when treated with oxLDL, however the same effect was not observed when cells were treated with 7 -OH or 25-OH.

CONCLUSIONS

The ability of oxysterols to induce apoptosis has generated much interest due to their role in the development of atherosclerosis. To date, much research has focused on
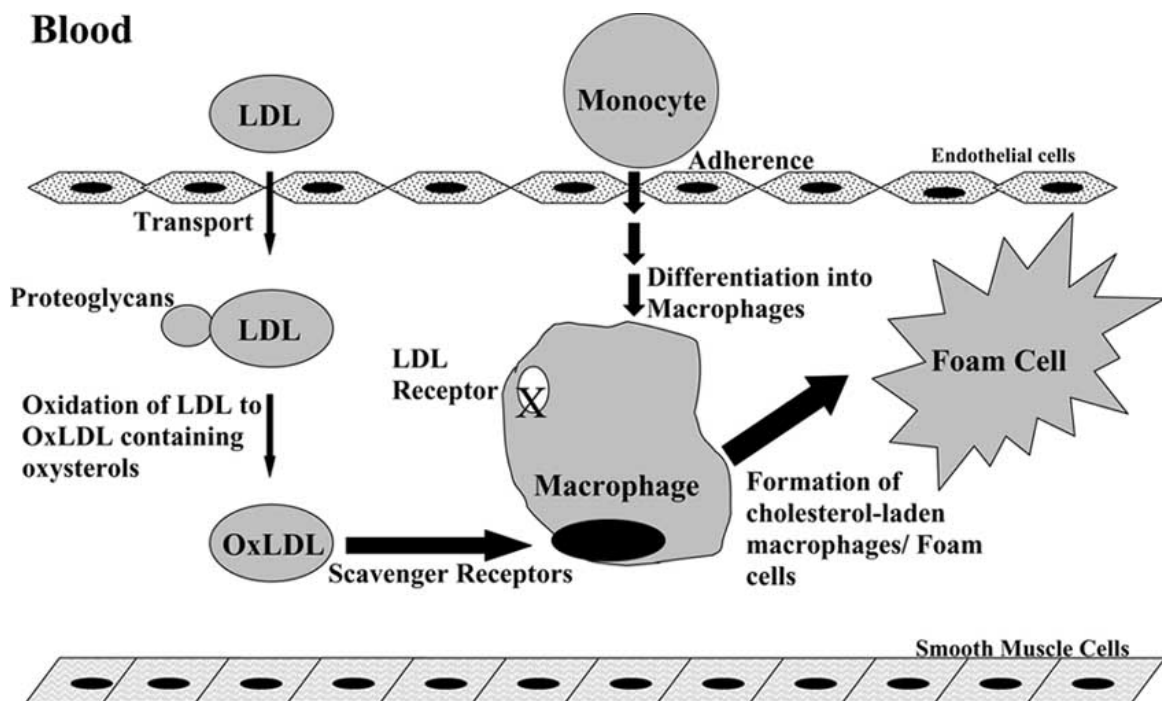

Fig. (2). After penetrating the arterial wall, LDL adheres to preteoglycans which entrap it and increase its susceptibility to oxidation. Oxysterols downregulate the expression of LDL receptors and encourage the uptake of oxLDL by scavenger receptors on macrophages leading to cholesterol accumulation and foam cell formation.

oxysterols oxidised at C7, in particular 7 -OH and 7-keto, both potent inducers of apoptosis. A variety of studies on 7 -OH and 7-keto have illustrated that both oxysterols induce cell death through the intrinsic pathway of apoptosis via generation of an oxidative stress, loss of mitochondrial transmembrane potential, release of cytochrome *c* from the mitochondria, activation of caspase-9 with subsequent activation of caspase-3, PARP degradation and DNA fragmentation [26, 47, 50, 51]. Little is known about the mechanism of cell death induced by other oxysterols such as -epoxide, which is also present in oxLDL at significant levels. Much also remains to be elucidated at the earlier stages of the apoptotic process induced by oxysterols, prior to mitochondrial dysfunction and caspase activation.

Some studies have suggested that generation of the lipid messenger ceramide may be of importance at the initial stages of oxysterol-induced apoptosis. While an increase in ceramide concentration has been shown in cells exposed to oxysterols [88], a study conducted by Miguet *et al.* [60] found that inhibition of ceramide generation did not prevent oxysterol-induced cell death. An increase in intracellular calcium has also been implicated as one of the earlier steps in the apoptotic pathway induced by oxysterols. Preliminary studies have shown that calcium is involved in apoptosis induced by certain oxysterols [71, 73] but the importance of calcium signalling in oxysterol-induced apoptosis has yet to be fully clarified.

The current weight of evidence strongly suggests that oxysterol-induced apoptosis occurs *via* the intrinsic pathway of apoptosis. Few studies, however, have concentrated on examining the ability of oxysterols to induce apoptosis *via* the extrinsic apoptotic pathway. Though many studies have focused on oxysterol-induced apoptosis in a wide variety of cell lines *in vitro*, many questions remain unanswered and much remains to be discovered. A more thorough understanding of the exact mechanisms involved in oxysterol-induced cell death is particularly desirable in the development of therapies for atherosclerosis.

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