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Effects of oxysterols on cell survival and proliferation pathways in human endothelial cells.

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INTRODUCTION

VASCULAR ENDOTHELIUM AND ITS FUNCTIONS

Vascular endothelium

Ever since the endothelium was discovered by microscopical examination, it has always been considered as a lining acting like a barrier. Nevertheless, in the last decades, the recognition of its multiple functions has shown it to be a true regulator of blood flow and tissue homeostasis. Although it is a monolayer that covers the inner surface of the entire vascular system, the total area of this interface in humans has been estimated to be about 350 m^2 (Pries *et al.*, 2000).

The endothelium is the major regulator of vascular homeostasis, it maintains the balance between vasodilatation and vasoconstriction, inhibition and stimulation of smooth muscle cells (SMCs), proliferation and migration, thrombogenesis and fibrinolysis. In response to humoral, neural or mechanical *stimuli*, it synthesizes and releases vasoactive substances.

Endothelium and blood coagulation regulation

All endothelial cells (ECs) secrete a variety of molecules important for the regulation of blood coagulation and platelet functions. The major antiplatelet agents secreted by ECs are prostacyclin (PGI_2) and nitric oxide (NO) (Cines *et al.*, 1998). Both synergistically increase c-AMP content in platelets preventing their aggregation (De Graaf *et al.*, 1992). PGI_2 and NO are constitutively released by ECs but their synthesis is increased in response to molecules involved in the coagulation process (bradykinine and thrombin) or secreted by aggregating platelets (ATP) and thus is necessary to limit the formation of thrombi.

In a quiescent state ECs maintain blood fluidity by promoting the activity of numerous anticoagulant pathways, the most important being ProteinC/ProteinS pathway. This pathway is initiated when thrombin interacts with the EC-receptor thrombomodulin, facilitating the activation of protein C. Then protein C inactivates two cofactors essential for blood coagulation: factor VIII and V. To be effective protein C must form a complex with protein S that is produced by ECs (Stern *et al.*, 1991). The endothelium also participates to fibrinolysis by releasing two factors: tissue-type plasminogen activator (t-PA) and urokinase.

Favourable Effects of the Healthy Endothelium
Promotion of vasodilation
Antioxidant effects
Antiinflammatory effects
Anticoagulant effects
Profibrinolytic effects

Table 1. Favourable effects of the healthy endothelium.

Both the factors allow the degradation of thrombi. T-PA is constitutively released while urokinase is only synthesized by activated ECs (Emeis, 2005). The natural inhibitor of t-PA, is plasminogen activator inhibitor type 1 (PAI-1) and it is also constitutively secreted by ECs. After activation, as can be induced by several *stimuli*, the balance of endothelial properties can be tipped to further platelet aggregation and clot formation through induction of procoagulant and prothrombotic factors and suppression of anticoagulant mechanisms (Gross and Aird, 2000; Preissner, 2000). At least two mediators released by activated ECs promote platelet activation. The first one is the lipid mediator platelet-activating factor (PAF), synthesized by ECs stimulated by thrombin, histamine or cytokines. PAF is a potent platelet activator and can promote platelet adhesion to ECs (Zimmerman *et al*., 1990). The second is the von Willebrand factor (vWF) constitutively secreted into the plasma and the subendothelial matrix by ECs. This factor binds and stabilizes coagulation factor VIII and is a specific factor required for the binding of platelets to exposed extracellular matrix components when the vessel wall is damaged (Savage *et al.*, 1999).

Endothelium and vascular tone regulation

The normal, healthy endothelium regulates vascular tone: the maintenance of vascular tone is accomplished by the release of numerous dilator and constrictor substances. A major vasodilative substance released by the endothelium is NO, originally identified as endothelium-derived relaxing factor (EDRF).

NO is formed in ECs from the conversion of L-arginine to L-citrulline by the enzymatic action of a NADPH-dependent NO synthase (NOs) which requires calcium/calmodulin,

FAD, FMN and tetrahydrobiopterin (BH_4) as the cofactors (Kawashima and Yokoyama, 2004). Three different isoforms of NOs have been identified: neuronal NO synthase (nNOs), inducible NO synthase (iNOs) and endothelial NO synthase (eNOs). As mentioned above, the NO generation by ECs is constitutive but may be enhanced by a wide variety of compounds, including acetylcholine, angiotensin II, bradykinin, histamine, adenine nucleotides, arachidonic acid and almost shear stress. NO diffuses to the vascular SMCs where it stimulates soluble guanylate cyclase, resulting in an increased formation of cyclic GMP and subsequent relaxation (Denninger and Marletta, 1999).

ECs, moreover, release PGI_2, which relaxes the underlying SMCs through activation of adenylate cyclase and subsequent generation of c-AMP.

Endothelium also generates a diffusible relaxing and hyperpolarizing factor (EDHF), its vasodilatator effect is mediated by the activation of potassium channels on the vascular SMCs (Vanhoutte, 1996).

Under some circumstances, endothelium-derived vasoconstrictive factors can be released and contribute to vasoconstriction. One of these factor is endothelin (ET), an endothelium-derived 21 amino acids vasoconstricting peptide, first isolated from the culture supernatant of porcine ECs (Yanagisawa *et al*., 1988). The endothelin family consists of three structurally related peptides, ET-1, ET-2, and ET-3. In the vasculature, proendothelin may be released from the non-luminal surface of the ECs and converted to mature ET extracellularly by membrane-bound endothelin-converting enzymes. ET does not appear to be stored in ECs but is synthesized *de novo* in response to several chemical (thrombin, angiotensin II, cytokines) or physical (shear stress, hypoxia) agents (Rubanyi and Botelho, 1991). Two receptors for ET have been identified in blood vessels: the ET_A receptor is situated on the vascular SMCs and ET_B receptor, which is situated on the ECs. Binding to ET_A receptor stimulates phospholipase C leading to SMCs contraction. The result of ET binding to ET_B receptor on ECs is the release of EDRF/PGI_2, which opposes the vasoconstricting action of ET (Kedzierski and Yanagisawa, 2001).

Endothelium and shear stress

The vascular endothelium is very sensitive to biomechanical stimuli and continuously senses and transduces haemodynamic environments into rapid cellular responses: haemodynamic forces are important modulators of endothelial functions in healthy and pathological conditions. The three major forces experienced by the vascular endothelium are the frictional shear stress imparted by blood flowing, the pulsatile cyclic stretching of the vessel wall and the compressive hydrostatic pressures generated with every cardiac cycle (Davies, 1995). In particular ECs come in contact with significant levels of shear stress that is today recognized as an important biologically active force. Under normal, physiological conditions shear stress serve as an important signal to preserve the antithrombotic, anti-inflammatory, anti-oxidative stress and anti-apoptotic flow-responsive phenotype of the vascular cells through the up regulation of KLF_2, a transcription factor involved in the regulation of multiple endothelial functions and strongly suggested to play an important role in the preservation of the endothelium. (Dekker *et al*., 2005; Parmar *et al*., 2005).

ENDOTHELIAL DYSFUNCTION IN ATHEROSCLEROSIS

As mentioned above, ECs are the main regulators of vascular homeostasis, interacting with circulating cells and SMCs: being the interface between blood and tissue, they are mostly susceptible to changes in blood composition and in blood flow. ECs are the main responder to all these changes and play a central role in the mechanisms underlying the development of vascular disorders, including atherosclerosis. Damages to the endothelium, in fact, modify its physiology leading to the endothelial dysfunction (ED) that is a key early step in the development of atherosclerosis and that is involved in plaque progression and in the occurrence of atherosclerotic complications (Bonetti *et al.*, 2002).

A study by Ludmer and co-workers, using the acetylcholine test, provided the first evidence in humans of impaired endothelium-dependent vasodilation in the presence of atherosclerosis. These investigators observed paradoxical constriction in the arteries of patients with mild coronary artery disease (CAD), as well as in those with advanced CAD, indicating that ED is present in the early stage of atherosclerosis (Ludmer *et al.*, 1986).

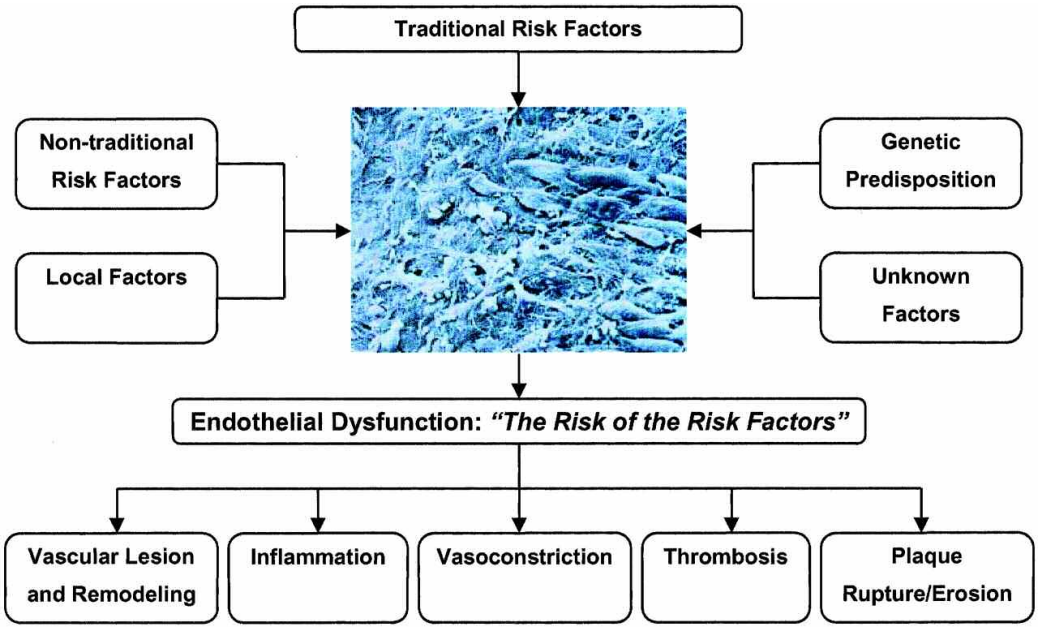


Figure 1. Endothelial dysfunction as the "risk of the risk factors." Traditional and non-traditional risk factors, local factors (shear stress), genetic factors, and yet-unknown factors determine the status of endothelial function (from Bonetti *et al.*, 2003).

Possible causes of ED leading to atherosclerosis include elevated and modified low density lipoproteins (LDL); free radicals caused by cigarette smoking, hypertension, and diabetes mellitus; genetic alterations; elevated plasma homocysteine concentrations; infectious microorganisms like Chlamydia pneumoniae; and combinations of these or other factors. The different forms of injury increase the adhesiveness of the endothelium with respect to leukocytes or platelets, as well as its permeability (Ross, 1999)

Initiation of inflammation

The inflammatory activation of ECs causes increased expression of selectins, vascular and intercellular adhesion molecules-1 (VCAM-1 and ICAM-1).

Activated EC express adhesion molecules on their surface, the glycoproteins P-selectin and E-selectin. These glycoproteins bind P-selectin ligand-1 present on the surface of monocytes in the circulation causing these to adhere and roll to the endothelium.

Subsequently, the monocyte interacts with VCAM-1, that have been shown to be up regulated in cultured ECs in presence of oxidized-LDL (ox-LDL)(Li *et al.*, 2002). Finally the monocyte migrate into the sub endothelial space by a process known as *diapedesis* under the influence of chemoattractant molecules like the chemokine Macrophage Chemoattractant Protein-1 (MCP-1), the expression of whom is induced in ECs by oxidized phospholipidic components of ox-LDL (Subbanagounder, 2002).

Once recruited from the blood, the monocytes differentiate to macrophages into the tissue and here proliferate under the stimulation of ox-LDL (Hamilton *et al*., 1999). Macrophages produce cytokines like Tumour Necrosis Factor-α (TNF-α) and interleukin-x1β (IL-x1β). TNF-α is a small protein causing the release of cytokines involved in the inflammatory response, IL-x1β is a chemoattractant that enhances leucocytes adhesion to the endothelium, promotes SMCs proliferation and activates T-cells (Osterud and Bjorklid, 2003). Furthermore macrophages secrete proteases like Matrix Metalloproteinases (MMPs) that degrade collagenous components of the extracellular matrix. Macrophages express a wide number of scavenger receptors that are capable of taking up ox-LDL, including scavenger receptor-A (SR-A), SR-B1, cluster of differentiation-36 (CD-36), cluster of differentiation-68 (CD-68), and scavenger receptor for phosphatidylserine (PS) and ox-LDL (Li *et al.*, 2002).

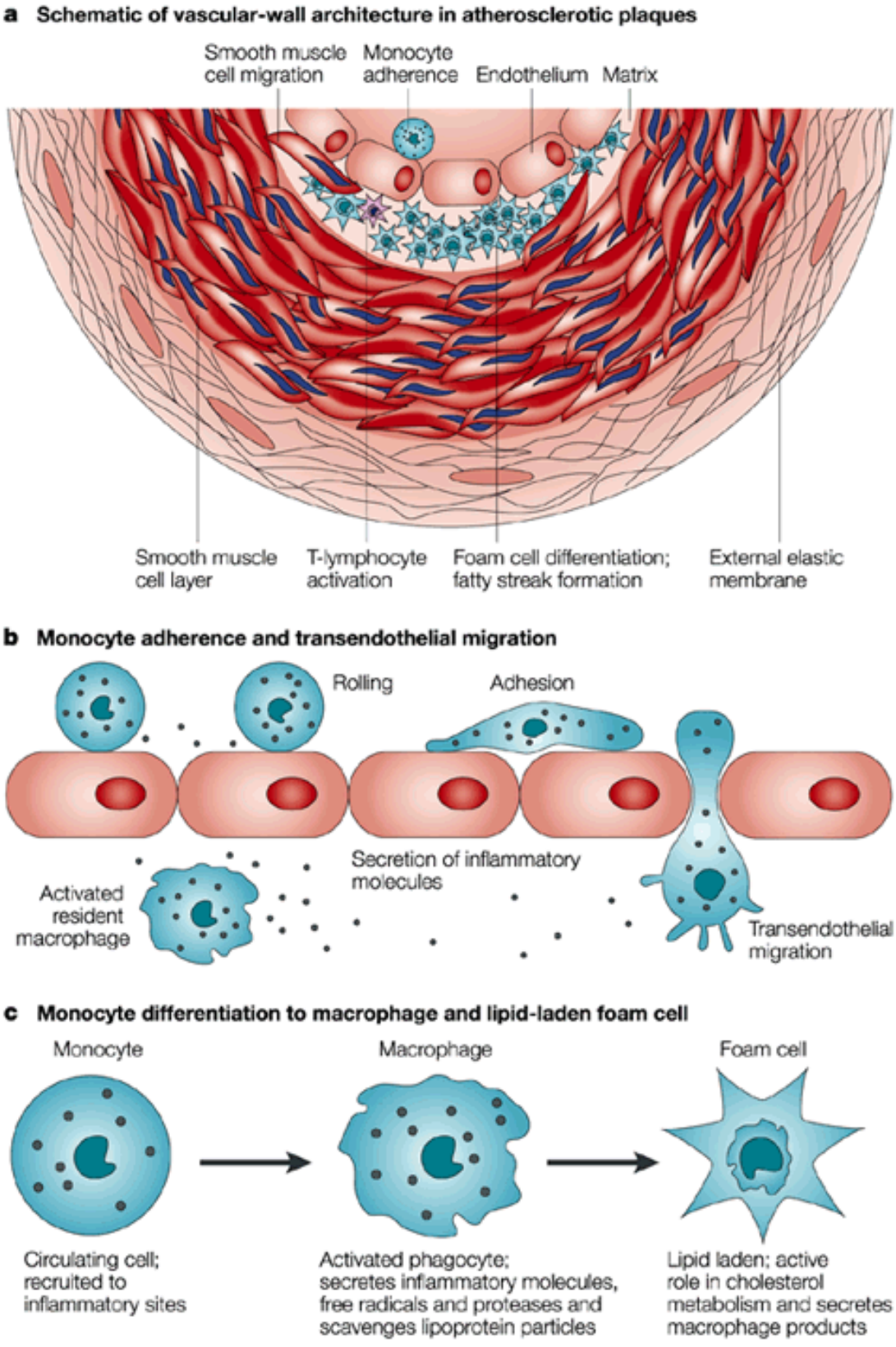


Figure 2. A / Schematic of the early sub-endothelial accumulation of monocytes, macrophages and foam cells, and the formation of fatty streaks. **B /** At the site of a nascent vascular lesion, lipids, lipoproteins or other reactive substances stimulate the endothelium to produce inflammatory

cytokines and chemoattractants. The cytokine-activated endothelium expresses adhesion molecules that lead to the recruitment of peripheral blood monocytes to the inflammatory site. Low-affinity interactions between monocytes and the endothelium, which are mediated by selectins and integrins, lead to capture and rolling of monocytes on the endothelial surface. Activated macrophages within the lesion secrete chemotactic products, including chemokines. In response to these chemokine gradients, cells migrate through the endothelium. **C /** As cells transmigrate across the endothelium, they are activated by inflammatory cytokines that are secreted by the inflamed endothelium and the underlying smooth muscle layer. They differentiate into the metabolically active, secretory and highly phagocytic inflammatory macrophage. As macrophages accumulate, they take up modified lipoproteins and actively accumulate lipid to become foam cells (from Cascieri, 2002).

Uptake of ox-LDL is primarily mediated by CD-36, that recognizes the oxidized phospholipids within the particle, and SR-A that recognizes the proteic component of the particle. Both the receptors are not subjected to negative feedback regulation by the intracellular content of cholesterol and moreover their expression is increased by the exposure of ox-LDL, leading the macrophages to ingest an excess of cholesterol. Since the mammalian cells posses no mechanism for breaking down the sterol back-bone of cholesterol, the excess of cholesterol within the macrophages induce toxic effects leading the formation of foam cells and lastly to cell death (Tabas, 2002). Accumulation of foam cells within the intima leads to the formation of the fatty streak, the initial lesion of atherosclerosis. T lymphocytes also enter the intima facilitated by binding to adhesion molecules including VCAM-1 and in response chemoattractants selective for lymphocytes. These chemokines bind to chemokine receptor CXCR3 expressed by T cells in the atherosclerotic lesion. Once resident in the arterial intima, the T cell may encounter antigens such as Ox-LDL. Upon activation by engagement of the receptor and antigen, the T cell can produce cytokines that can influence the behaviour of other cells present in the atheroma, in particular inducing the proliferation of SMCs (Van Kaer, 2007).

Inflammation in atheroma progression and complications

Fatty streaks evolve into complicated atheroma through multiplication of SMCs, which accumulate in the plaque and lay down an abundant extracellular matrix.

These SMCs are responsible for the deposition of extracellular connective tissue matrix and form a fibrous cap that overlies a core of lipid-laden foam cells, extracellular lipid, and necrotic cellular debris. Growth of the fibrous plaque results in vascular remodelling, progressive luminal narrowing, blood-flow abnormalities, and compromised oxygen supply to the target organs (Kolodgie, 2003).

Denudation of the overlying endothelium or rupture of the protective fibrous cap may result in exposure of the thrombogenic contents of the core of the plaque to the circulating blood. This exposure constitutes an advanced or complicated lesion. The plaque rupture occurs due to weakening of the fibrous cap and may result in thrombus formation, partial or complete occlusion of the blood vessel, and progression of the atherosclerotic lesion due to organization of the thrombus and incorporation within the plaque (Robbie and Libby, 2001).

LDL AND ITS OXIDIDATIVE MODIFICATIONS

The cholesterol that accumulates in the vascular wall derives primarily from plasma lipoproteins. Among these lipoproteins, LDLs appear the most important since clinical and epidemiologic studies have shown that an elevated level of LDL is an important risk factor for coronary heart disease (Witztum and Steinberg, 1991).

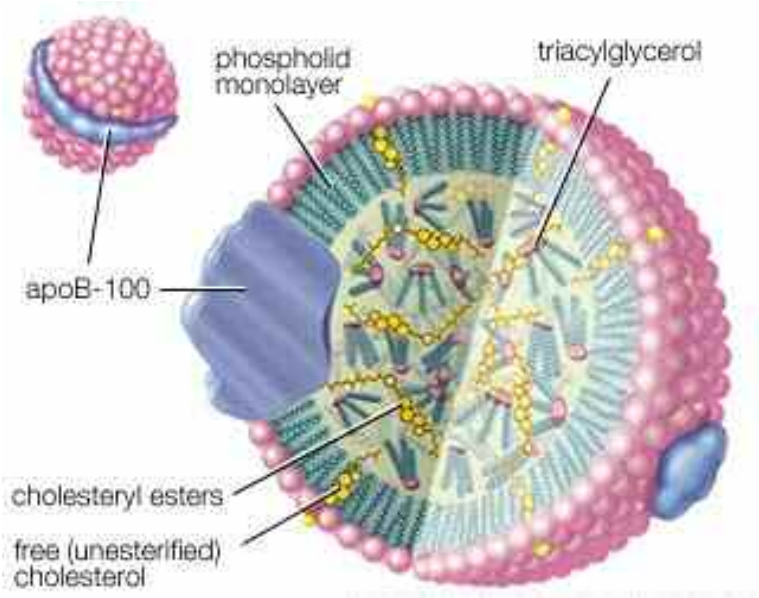


Figure 3. Biochemical composition of low density lipoprotein (LDL). The central core of LDL particles contains 1600 molecules of cholesterol ester and 170 molecules of triglycerides. It is surrounded by a monolayer of 700 phospholipid molecules, consisting primarily of lecithin, small amounts of sphingomyelin and lysolecithin, and 600 molecules of cholesterol. About half of the fatty acids in LDL are polyunsaturated fatty acids (PUFAs), mainly linoleic acid with minor amounts of arachidonic acid and docosahexaenoic acid. These PUFAs are protected against free radical attack and oxidation by antioxidants, primarily α-tocopherol (six molecules per LDL particle), with minor amounts of carotenoids, cryptoxanthin, and ubiquinol-10. The amount of PUFAs and antioxidants varies significantly within individuals, resulting in a great variation in the susceptibility of LDL to oxidation (from Mertens and Holvoet, 2001).

As mentioned before, the fatty streak contains numerous cholesterol-loaded foam cells derived from blood monocytes. The monocytes have very few LDL receptors and, moreover, their LDL receptors are downregulated to prevent excessive uptake of LDL cholesterol.

It was Goldstein and Brown who first proposed that modification of LDL was a prerequisite for macrophage uptake. They showed that chemical modifications of LDL (acetylation, acetoacetylation, modification by malondialdehyde) led to accumulation of cholesteryl esters and to an enhanced macrophage uptake by different receptors called “scavenger receptors.” These differ from classical LDL receptors in that they do not bind native LDL and are not down regulated (Goldstein and Brown, 1979).

These chemical modifications of LDL do not occur in vivo, while LDL oxidation may occur in the vascular wall as demonstrated by immunohistochemical staining of atherosclerotic lesions with specific monoclonal antibodies for ox-LDL (Young and Eneny, 2001).

LDL oxidation must occur in the arterial wall rather than the circulation, moreover, oxidized lipoproteins that may exist or form in plasma are diluted rapidly by either hepatic clearance or accumulation and subsequent degradation in the arterial wall. Consistent with this, the plasma concentrations of ox-LDL, as assessed by immunological techniques, and oxidized lipids, determined by analytical techniques, in healthy humans are extremely low. In sharp contrast to the plasma situation, atherosclerotic lesions contain substantial amounts of oxidized lipids, in particular of oxysterols, oxidative derivatives of cholesterol (Stocker and Keaney, 2004).

Ox-LDL is generated by auto-oxidation in the presence of transition metals, by cell mediated mechanisms and by enzyme-mediated mechanisms (Harada-Shiba *et al*., 1998). The formation of ox-LDL has been reported after incubation of LDL with a variety of cell types including cultured ECs, monocytes, macrophages and SMCs. The oxidation of LDL results in marked changes in LDL composition including an increased lysolecithin content, a decreased content of polyunsaturated fatty acids and their peroxidated products, fragmentation of apolipoprotein B100 and decreased levels of lysine, histidine and proline; an increased negative charge and an increased density. In addition ox-LDL has been claimed to have an increased content of oxysterols (Steinberg *et al.*, 1989).

Biological properties of ox-LDL

As already mentioned, ox-LDLs exhibit several biological properties involved in atherogenesis, like the induction of adhesion protein expression and subsequent entry of mononuclear cells, formation of foam cells and fatty streak, induction of SMCs migration and proliferation, changes in the extracellular matrix, alterations of coagulation pathways and disturbance in the arterial tone regulation (Table 2).

Potential proatherogenic activities of oxidized LDL

Induce monocyte binding to endothelial cells
Mimic effects of platelet-activating factor
Increase tissue factor activity
Increase expression of monocyte colony-stimulating factor and monocyte chemoattractant protein 1
Increase expression of vascular cell-adhesion molecule 1
Induce Fas-mediated apoptosis
Induce expression of interleukins 1 and 8
Inhibit nitric oxide release or function
Increase collagen synthesis in smooth muscle cells
Increase intracellular calcium
Activate nuclear factor-κB
Induce expression of type 1 metalloproteinase

Table 2. Potential proatherogenic activities of oxidized low density lipoprotein (from Steinberg, 2002).

In addition, ox-LDL is cytotoxic to cultured cells including cultured vascular cells. Multiple studies have shown that ox-LDL elicits both necrotic and apoptotic cell death of cultured cells; in fact many studies reporting on ox-LDL-induced apoptosis also indicate that a significant fraction of the dying cell population does not exhibit apoptotic characteristics

(Hsieh *et al.* 2001). Ox-LDL has been shown to induce apoptosis in numerous cell types, including ECs, SMCs, macrophages and lymphoid cells (Mallat and Tedgui, 2000).

On the other hand, recently it has been postulated a dual effect induced by ox-LDL on ECs: it has been reported that incubation of ECs with increasing concentrations of ox-LDL induced proliferation and apoptosis depending on its concentration and the exposure time (Galle *et al*., 2001).

Even if some cellular components of the apoptotic pathways induced by ox-LDL have been identified, the way in which ox-LDL exerts its cytotoxicity is not clear. The toxicity induced by ox-LDL has been linked to their lipidic components like aldheydes, hydroperoxides, lyso-phosphatidylcholine and oxysterols (Lizard *et al*., 1999).

Several studies *in vitro* and *in vivo* suggest a key role of oxysterols in the cytotoxicity induced by ox-LDL on the vascular cells (Imai *et al*., 1980; Peng *et al*., 1985). In particular, Hughes and co-workers identified the components of copper-oxidized LDL responsible for its toxicity to porcine aortic SMCs: 7-ketocholesterol and 7β-hydroxycholesterol appear to be the only responsible for the cytotoxic effect induced to the cells (Hughes *et al*., 1994).

OXYSTEROLS

Oxysterols, also named cholesterol oxidation products, are 27-carbon derivatives of cholesterol resulting from oxidation on either the sterol nucleus or the C_{17} acyl chain (Colles *et al.*, 2001).

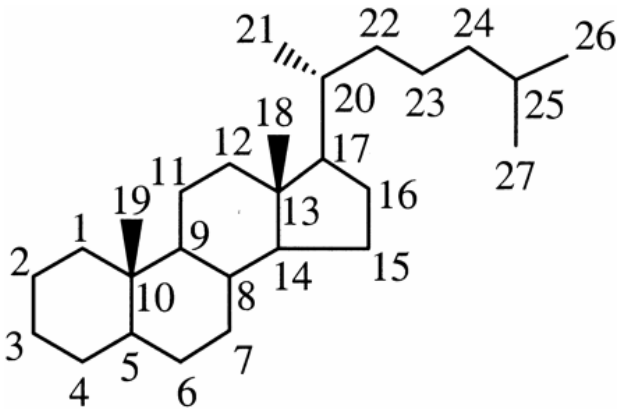


Figure 4. Chemical structure and numbering system for cholestane

Introduction of an oxygen atom in cholesterol drastically reduces its half life and directs the molecule to leave the body. The physical properties of oxysterols facilitate their degradation and excretion, as oxysterols are able to pass lipophilic membranes much more quickly than does cholesterol itself (Lange *et al.*, 1995).

They are present in all mammalian systems, almost invariably accompanied by 10^3-10^6-fold excess of cholesterol, but in spite of their low levels they are generally believed to be important physiological mediators of cholesterol-induced effect (Bjorkhem, 2002).

Oxysterols have been shown to inhibit endogenous cholesterol synthesis lowering the levels of hydroxy-methyl-glutaryl–Co-enzyme-A (HMG-CoA) reductase through a mechanism that is still now a controversial subject (Tamasawa *et al.*, 1997). The suggested mechanisms probably involved are: a) inhibition of enzyme synthesis; b) degradation of enzyme; c) modification of enzyme structure (Guardiola *et al.*, 1996).

Various oxysterols have been detected in appreciable quantities in human tissues and fluids, including human plasma, atherogenic lipoproteins and atherosclerotic plaque.

Oxysterols can be ingested with the diet or formed endogenously. Dietary sources of oxysterols are cholesterol-rich food (diary, eggs, meat products), especially those products

which are heated in air during processing or stored for lengthy periods. For example, home made ghee, used extensively in traditional Indian cooking by heating butter (usually in a copper pot over an open flame), has been estimated to contain 12% of total sterols as oxysterols (Jacobson, 1987).

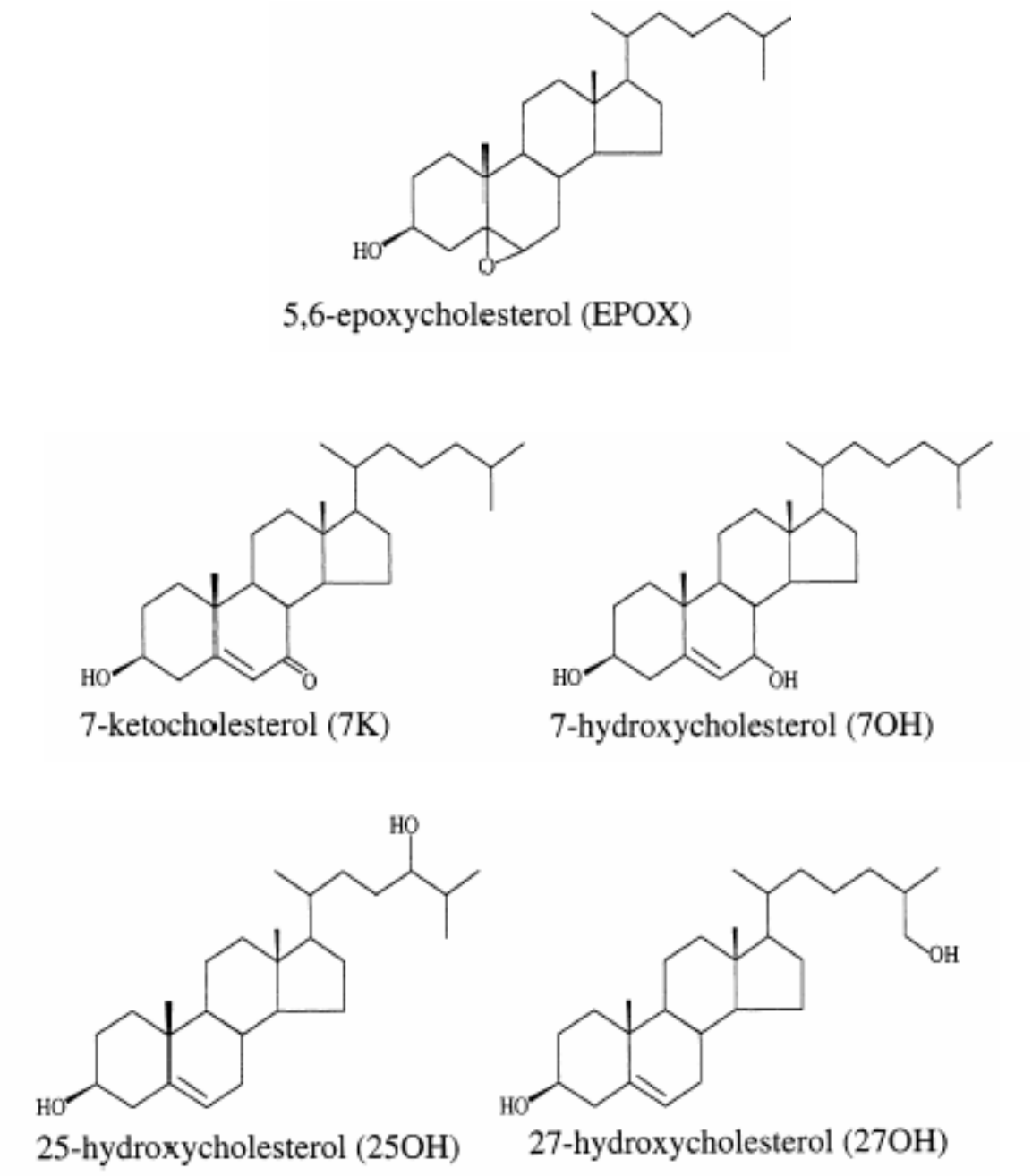


Figure 5. Chemical structures of some common oxysterols.

The most commonly detected oxysterols in foods are the major products of cholesterol autoxidation: 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol, α-epoxycholesterol, β-epoxycholesterol.

In vivo oxysterols may be formed by enzymatic or non-enzymatic oxidation. For example 7α-hydroxycholesterol is formed in the liver by 7α-hydroxylation of cholesterol by the microsomal cholesterol 7α-hydroxylase and, in the same way, 27-hydroxycholesterol is formed by the enzyme cholesterol 27- hydroxylase. In contrast, it is generally considered that 7-ketocholesterol and 7β-hydroxycholesterol are not formed enzymically in mammals, but they are probably products of autoxidation of cholesterol (Brown and Jessup, 1999).

As above mentioned, oxysterols have been detected in human plasma. The types and levels of oxysterols reported in human plasma vary widely. In fact, autoxidation of cholesterol, present at relatively high levels in plasma of normal human subjects, creates the problem of artifactual formation of oxysterols during sample storage, processing and analysis. The problem is highlighted by the dramatic increase of 7-oxysterols between a fresh and aged sample (Smith *et al.,* 1981). To reduce the autoxidation of cholesterol Kudo and co-workers developed a rigorous methodology that allowed the analysis of fresh samples free of haemolysis, including antioxidants and using peroxide-free degassed solvent and including an internal standard in each sample (Kudo *et al*., 1989) .

Dzeletovic and co-workers measured nine oxysterols: the major oxysterols found in the human plasma were 27-hydroxycholesterol, 24-hydroxycholesterol and 7α-hydroxycholesterol (Dzeletovic *et al.*, 1995).

Several studies suggested that an increased plasma concentration of 7β-hydroxycholesterol may be correlated to risk of developing atherosclerosis. In fact, in a Finnish study, a high plasma concentration of 7β-hydroxycholesterol was the strongest predictor of a 3-year increase in carotid wall thickness when compared to 30 variables tested (Salonen, 1997).

Zhou and co-workers analyzed the oxysterol content in the plasma from 105 cardiac catheterized patients with angina and stenosis in their coronary arteries. The result showed that the plasma contained a significantly higher concentration of oxysterols (in particular 7β-hydroxycholesterol, cholesterol 5beta, 6beta-epoxide, cholesterol 5alpha, 6alpha-epoxide and 7-ketocholesterol) than did plasma from 105 age- and sex-matched, non-catheterized and angina-free controls (Zhou *et al.*, 2000). Another study compared established and putative risk factors between 50 years-old Swedish men and as many Lithuanians, who have a four-

fold higher mortality in coronary heart disease. It was found that the plasma concentration of 7β-hydroxycholesterol was higher in Lithuanian men, compared to the Swedes, despite similar risk factors (Zieden *et al.*, 1999).

Oxysterols are present in ox-LDL, also. Breuer reported on the oxysterols present in LDL subjected to oxidation with $CuSO_4$. Substantial formation of 7-ketocholesterol, 7β-hydroxycholesterol and 7α-hydroxycholesterol was reported. Other oxysterols formed in significant amounts were 5β, 6β-epoxycholesterol and (in lesser amounts) 5α, 6α-epoxycholesterol (Breuer *et al.*, 1996). A previous study by Hughes and co-worker isolated the toxic compounds of ox-LDL: the unesterified oxysterols isolated from lipid extracts by solid phase extraction and HPLC were identified as 7-ketocholesterol, 7-hydroxycholesterol. The two cytotoxic oxysterols were the only responsible for the cytotoxicity induced on SMCs and they were present in ox-LDL at sufficient levels to account for its toxicity (Hughes *et al.*, 1994).

Moreover oxysterols are present in the atherosclerotic plaque. Brown reported on HPLC analysis of human atherosclerotic plaques. The major oxysterol detected in advanced lesions was 26-hydroxycholesterol, accompanied, at lower levels, by 7-ketocholesterol, 7β-hydroxycholesterol, and 7α-hydroxycholesterol, while the fatty streak contained higher levels of 7-ketocholesterol and 7β-hydroxycholesterol indicating that at this stage of lesion development oxidative events are particularly frequent or that there is a specific accumulation of these oxysterols in macrophage-foam cells, the predominant cell type in fatty streak (Brown *et al.*, 1994; Brown and Jessup, 1999).

Oxysterols have been reported to induce IL-8β secretion in vascular EC and, consequently the expression of adhesion molecules necessary for the recruitment of monocytes and T lymphocytes found in atherosclerotic plaque (Lemaire *et al.*, 1998). Therefore, potential role of these molecules has been postulated in the initiation of atheromatous lesion.

Moreover oxysterols have been reported to be strongly cytotoxic to a number of tumoral and normal cell species, including vascular cells such as ECs, SMCs and fibroblasts.

Lizard and co-workers reported on the effects of various oxysterols in causing apoptosis in bovine aortic endothelial cells. Various oxygenated derivatives of cholesterol, at rather high concentrations, induced apoptosis as proved by a decreased number of adherent cells, an increased number of non-adherent cells, and an enhanced permeability to propidium iodide. Oxysterols studied (in order of decreasing potency in inducing apoptosis) were 7β-

hydroxycholesterol, 7-ketocholesterol, 19-hydroxycholesterol, 5α, 6α-epoxycholesterol, and 25- hydroxycholesterol (Lizard *et al.*, 1996). Following studies from the same group demonstrated that 7ß-hydroxycholesterol and 7-ketocholesterol can induce both apoptosis and necrosis depending on the cell type of the arterial wall being considered (Lizard *et al.*, 1999).

Clare and co-workers investigated the toxicity of the oxysterols 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol and 26-hydroxycholesterol to human monocyte-macrophages in vitro and showed that the 7-position derivatives were more potent in inducing cell damage (Clare *et al*., 1995).

Because of the effect of these compounds in inducing apoptosis and inhibiting cell proliferation, oxysterols had been proposed as potential cancer chemotherapeutic agents. In fact, in 1984, Chen first described the inhibitory action of three oxygenated sterols (25-hydroxycholesterol, 20α-hydroxycholesterol, and 7-ketocholesterol) on the growth of cultured mammalian cells (Chen, 1984). A large number of oxygenated sterols has been shown to suppress the growth of both normal and transformed cells in culture. It has been reported that 7β-hydroxycholesterol exhibits some degree of selectivity in its effects on tumour cells relative to normal cells (Hieter *et al.*, 1986).

Recently some intracellular targets of oxysterols have been identified. It is the case of the liver X receptors α and β (LXRα and LXRβ), members of the nuclear receptor family of proteins that are critical for the control of lipid homeostasis in vertebrates. Both LXRα and LXRβ are activated by physiological concentrations of sterol metabolites such as 22-hydroxycholesterol, 24-hydroxycholesterol, 27-hydroxycholesterol, and 24, 25-epoxycholesterol (Janowski, 1996). LXRs control the transcription of genes involved in the synthesis and metabolism of cholesterol, including SREBP-1. This gene codes for sterol regulatory element binding proteins, designated as SREBP-1a, SREBP-1c, and SREBP-2, which are transcription factors that are synthesized as membrane-bound precursors and then undergo a specific, two-step cleavage before entering the nucleus. Here they bind to sterol regulatory elements (SRE) in the promoter regions of the genes that encode the LDL receptor and a number of important enzymes involved in sterol biosynthesis (Schroepfer, 2000).

Although oxysterols have been deeply investigated in the last decades and some intracellular target of these molecule have been characterized their mechanism of action remains still unclear. Understanding the complexity of the mechanism of oxysterols, which probably play important roles in atherosclerosis, might allow for the development of new therapies for this disease.

CELL DEATH

NECROSIS

Necrosis is a type of cell death caused by severe (non-physiological) physical or chemical insults and usually affecting groups of cells (rather than individual cells). Morphologically, necrosis is characterized by a disruption of the cellular membrane and a swelling of the cytoplasm and mitochondria, culminating in the complete disintegration of organelles. The process ends with total cell lysis. Biochemical features of necrosis include loss of regulation of ion homeostasis, random digestion of DNA and DNA fragmentation after lysis. Also, the process is uncontrolled and passive and does not require energy. Severely damaged cells do not form membrane-bound vesicles, and thus release their cellular contents. This normally results in inflammatory reactions with oedema and damage to surrounding cells. (Levin, 1998)

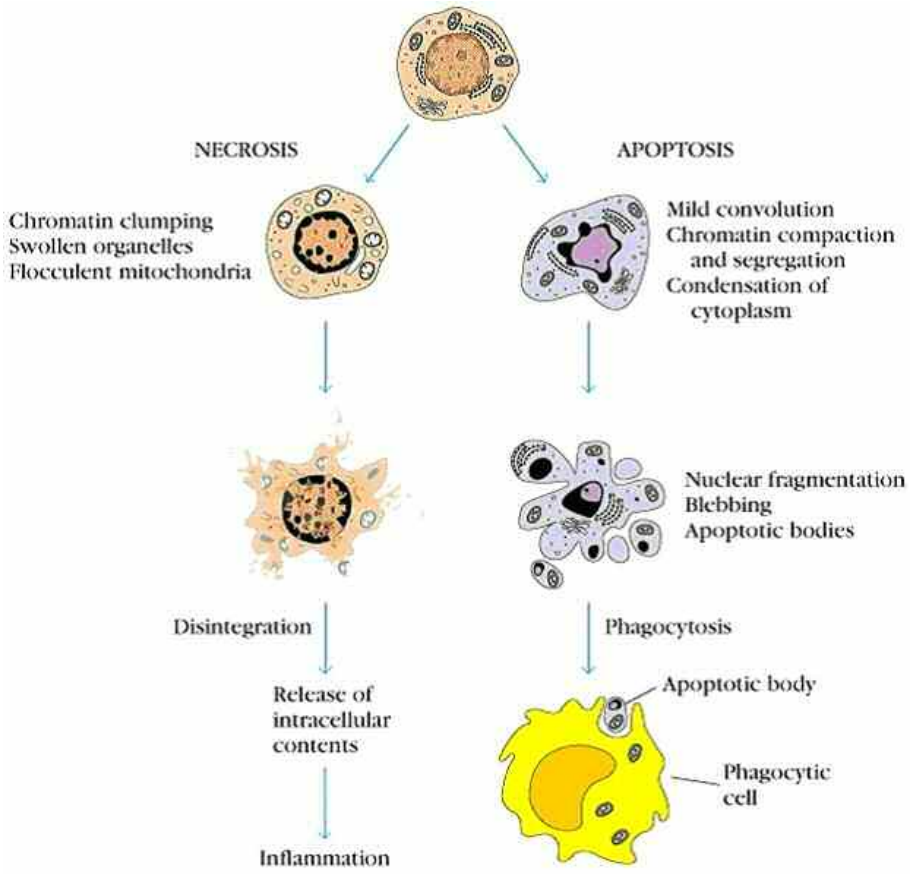


Figure 6. Necrosis versus apoptosis.

APOPTOSIS

Apoptosis is one of the main types of programmed cell death. In contrast to necrosis, apoptosis is carried out in an ordered process that generally confers advantages during an organism's life cycle. In fact apoptotic cell death has been implicated in embryonic development, immune system regulation, morphogenesis and the preservation of tissue homeostasis. Apoptosis is also involved in the pathogenesis of various disease states such as cancer, ischemia-reperfusion damage or infarction, neurodegenerative or neuromuscular diseases, atherosclerosis and heart failure (Hengartner, 2000; Wyllie *et al.*, 1980).

Morphological changes of apoptotic cells

Apoptotic cells display distinctive morphology during the apoptotic process (Figure 7). Typically, the cell begins to shrink following the cleavage of laminas and actin filaments in the cytoskeleton. The breakdown of chromatin in the nucleus often leads to nuclear condensation and in many cases the nuclei of apoptotic cells take on a “horse-shoe” like appearance.

Cells continue to shrink, packaging themselves into a form that allows for their removal by macrophages. These phagocytic cells are responsible for clearing the apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death.

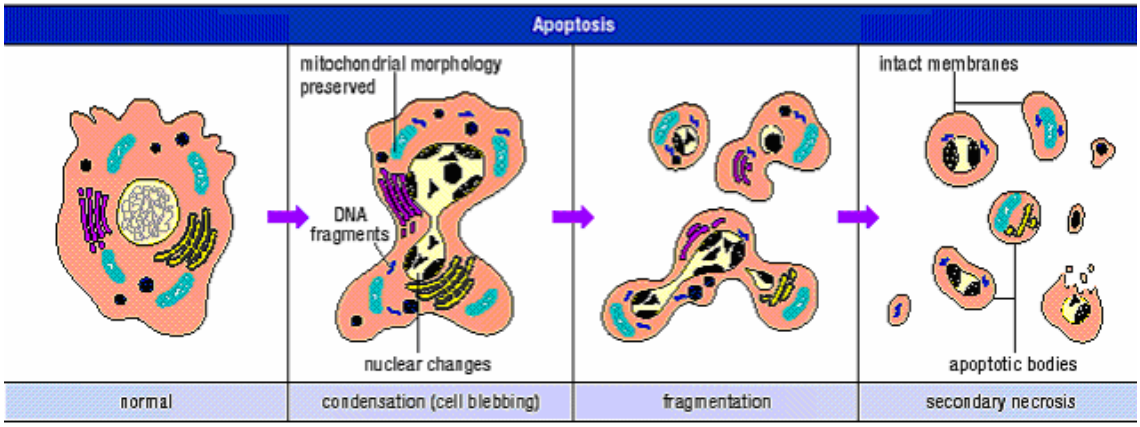


Figure 7. Morphological changes associated with apoptosis.

In order to promote their phagocytosis by macrophages, apoptotic cells often undergo plasma membrane changes that trigger the macrophage response. The end stages of apoptosis are often characterised by the appearance of membrane blebs or blisters process. Small vesicles called apoptotic bodies are also sometimes observed (Doseff, 2004).

Pathways of apoptosis

Intrinsic pathway

Apoptosis can be initiated by intrinsic signals, for example during normal development. This occurs when cells activate an internal program of self-destruction (cell suicide, programmed cell death) in response to an internal clock, withdrawal of survival factors, changes in haemodynamic parameters or loss of contact. Every cell already contains all the components of the suicide machinery and is ready to engage in self-destruction unless it is actively signalled not to do so. The intrinsic pathway is triggered in response to a wide range of death stimuli that are generated from within the cell, such as oncogene activation and DNA damage. The inactivation of this pathway is generally regarded as a hallmark of cancer (Hanahan and Weinberg, 2000).

Upon receiving the stress signal, the proapoptotic proteins in the cytoplasm, BAX and BID, bind to the outer membrane of the mitochondria to signal the release of the internal content. However, the signal of BAX and BID is not enough to trigger a full release. BAK, another proapoptotic protein that resides within the mitochondria, is also needed to fully promote the release of cytochrome c and the intramembrane content from the mitochondria. Following the release, cytochrome c forms a complex in the cytoplasm with adenosine triphosphate (ATP), an energy molecule, and Apaf-1, an enzyme. Following its formation, the complex will activate caspase-9, an initiator protein. In return, the activated caspase-9 works together with the complex of cytochrome c, ATP and Apaf-1 to form an apoptosome, which in turn activates caspase-3, the effector protein that initiates degradation. Besides the release of cytochrome c from the intramembrane space, the intramembrane content released also contains apoptosis inducing factor (AIF) to facilitate DNA fragmentation, and Smac/Diablo proteins to inhibit the inhibitor of apoptosis (IAP). (Hague and Paraskeva, 2004)

Extrinsic pathway

On the other hand, apoptosis can be initiated by binding of Fas ligand, tumour necrosis factor, or other cytokines to death receptors that results in activation of caspase-8. (Haunstetter and Izumo, 1998). In contrast to the more chronic and progressive loss of cells that occurs during normal development, apoptosis triggered by extrinsic signals is generally more acute and massive. Therefore, the capacity for removal of apoptotic cells may be overcome and secondary necrosis of unremoved apoptotic cells is frequent. This may lead to chronic accumulation of cellular debris with the potential for inducing inflammatory and/or auto-immune responses (Casciola-Rosen *et al.*, 1994; Rosen *et al.*, 1995; Tan, 1994). In the extrinsic pathway ligands like Fas ligand (FasL) or TNF-α bind to the death receptors on the target cell. This binding triggers multiple receptors to aggregate together on the surface of the target cell. The aggregation of these receptors recruits an adaptor protein known as Fas-associated death domain protein (FADD) on the cytoplasmic side of the receptors. FADD, in turn, recruits caspase-8, an initiator protein, to form the death-inducing signal complex (DISC). Through the recruitment of caspase-8 to DISC, caspase-8 will be activated and it is now able to directly activate caspase-3, an effector protein, to initiate degradation of the cell. Active caspase-8 can also cleave BID protein to tBID, which acts as a signal on the membrane of mitochondria to facilitate the release of cytochrome c in the intrinsic pathway. (Hague and Paraskeva, 2004).

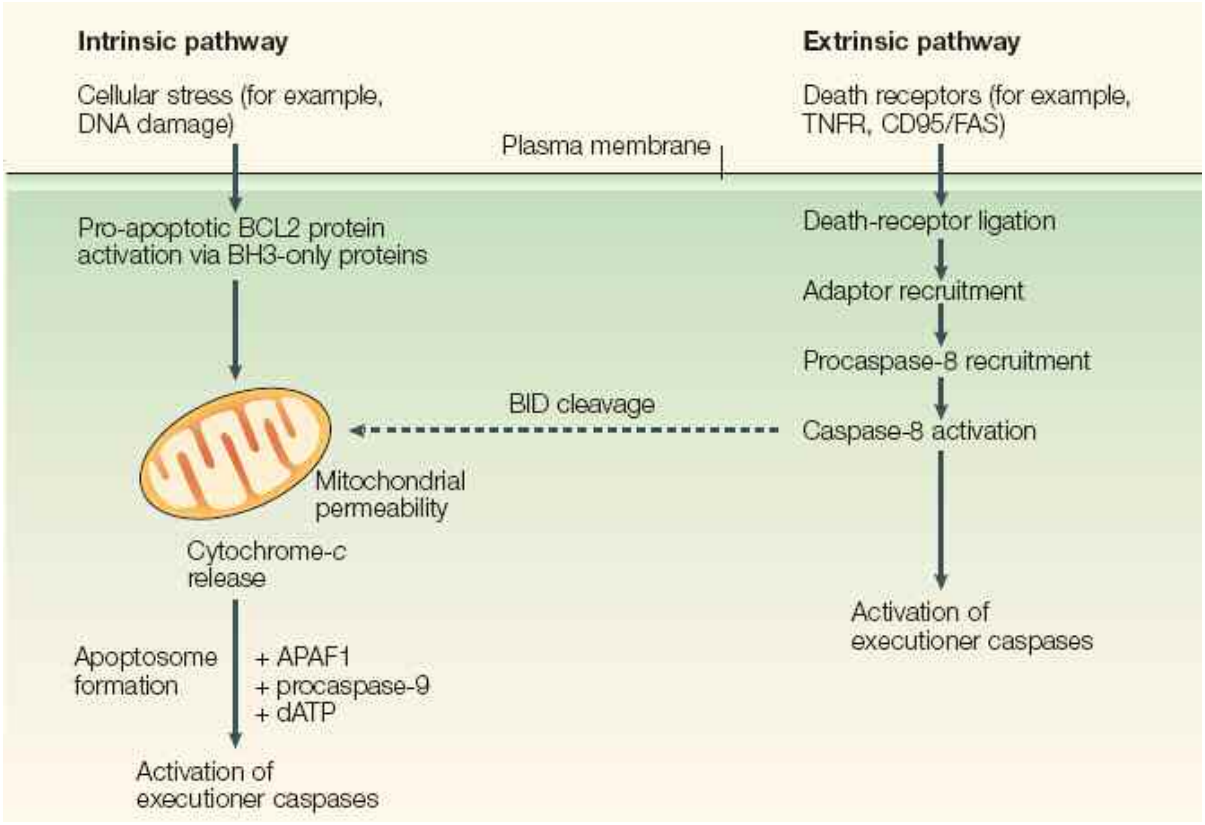


Figure 8. Intrinsic and extrinsic pathways.

Stages of apoptosis

The programmed cell death cascade can be divided into at least four main functionally distinct stages.

The *initiation or signalling phase* during which cells receive the death-inducing signal. This may be accomplished by attachment of death-promoting molecules (TNF-α, FasL) to death receptors (TNFR1, Fas) on the cell surface with subsequent recruitment of death domain proteins (FADD, TRADD, RIP) required for caspase-8 activation (which initiates the lethal proteolytic cascade) (Ashkenazi and Dixit, 1998). Otherwise this phase can be initiated by intrinsic signals that directly or indirectly activate the mitochondrial pathway by inducing the release of cytochrome c and the formation of the apoptosome complex with Apaf-1 and pro-caspase-9.

The *control and effector phase* during which activation of caspases occurs with loss of mitochondrial membrane potential (Green and Kroemer, 1998). Caspases are a family of cysteine proteases with aspartate specificity that have been implicated in the transduction and execution of the apoptotic programme. Caspases are present as inactive pro-enzymes, most of which are activated by proteolytic cleavage. Over a dozen of caspases have been identified in humans and more of them appear to be implicated in apoptosis. They are responsible for the deliberate disassembly of a cell into apoptotic bodies. Caspase-8, caspase-9 and caspase-3 are situated at pivotal junctions in apoptotic pathways (Thornberry and Lazebnik, 1998).

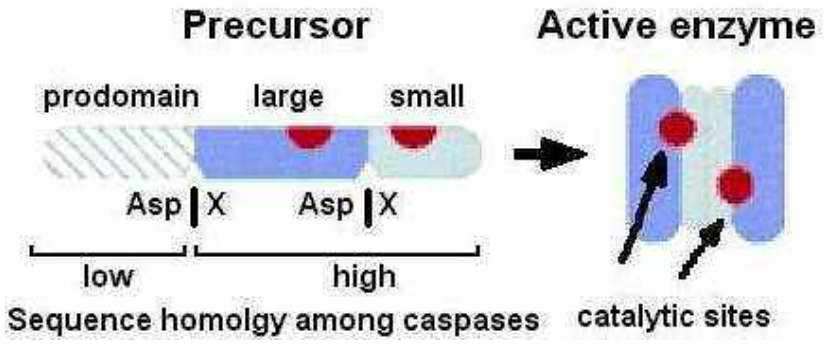


Figure 9. Caspases are synthesized as precursors that undergo proteolytic maturation. The NH_2-terminal domain, which is highly variable in length (23 to 216 amino acids) and sequence, is involved in regulation of these enzymes.

The execution phase is controlled by the BCL-2 family members acting upstream from the caspases, through inhibition of cytochrome c or AIF release from the mitochondrion or through binding and sequestration of Apaf-1 away from caspase-3 (Kroemer, 1997).
The BCL-2 family of proteins contains both inhibitors (BCL-2, BCL-XL) and inducers (BCL-XS, BAX, BID, BAD, BAK) of apoptosis acting as homodimers and heterodimers. The balance between anti-apoptotic and pro-apoptotic BCL-2 family members is critical to determine if a cell undergoes apoptosis. After an appropriate signal, BAX or BAK undergo a conformational change and move to the mitochondrial membrane where they cause release of cytochrome c into the cytosol (Goping *et al*., 1998; Griffiths *et al*., 1999).

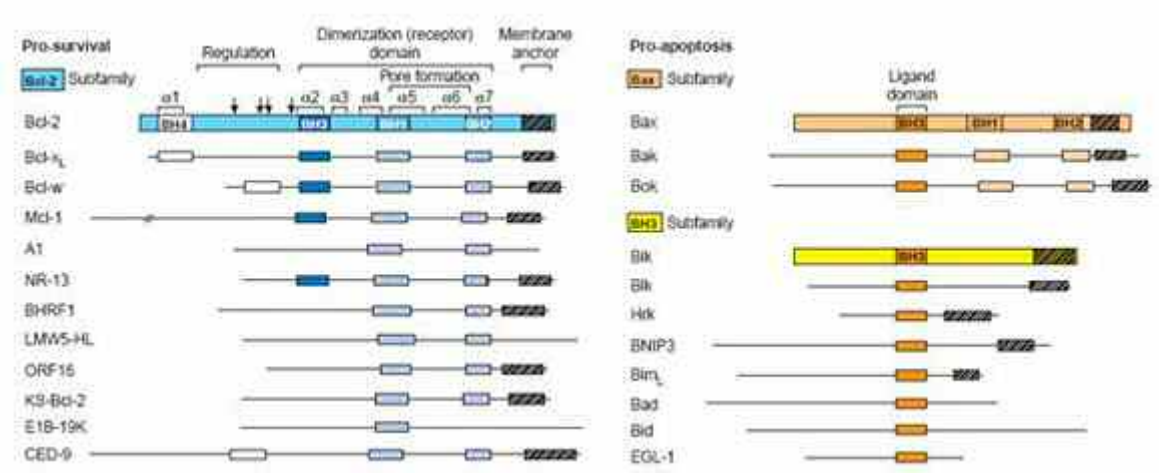


Figure 10. BCL-2 family proteins. The family of Bcl-2 proteins is subdivided into two classes: anti-apoptotic members such as BCL-2 and BCL-x_L which protect cells from apoptosis, and pro-apoptotic members such as BAX and BAK and the large group of BH3, death proteins which trigger or sensitize for apoptosis. The two classes are in turn comprised of 3 groups, according to their content of BCL-2 homology (BH) domains (Antonsson, 2001; Huang and Strasser, 2000).

The *structural alterations and DNA degradation phase*, where caspase activation leads to the cleaving of lamin, the intermediate filament of the nuclear envelope, of poly (ADP) ribose polymerase (PARP) and of the inhibitor of caspase-activated deoxyribonuclease (ICAD/DFF45) (Nicholson *et al.*, 1995; Enari *et al.*, 1998; Liu *et al*., 1997; Sakahira *et al.*, 1998). Fragmentation and degradation of genomic DNA results in an irreversible loss of viability.

The phase of *recognition of apoptotic cells and removal of apoptotic bodies*. After the completion of the apoptotic processes, dead cells are removed from the tissue as a result of specific recognition and phagocytosis by adjacent professional and non-professional cells through a variety of mechanisms that implicate PS, thrombospondin/CD36 binding site, the vitronectin receptor or CD14 (Devitt *et al.*, 1998; Fadok *et al.*, 1992; 1998; Savill *et al*., 1993).

In addition cells undergoing apoptosis generate reactive oxygen species (ROS) that may induce membrane peroxidation (Ashkenazi and Dixit, 1998). Indeed, apoptotic cells present oxidatively modified moieties on their surface that are structurally analogous to the surface of

the ox-LDLs (Chang *et al*., 1999). These moieties, serve as ligands for the recognition and phagocytosis by macrophages through their scavenger receptors, including SR-A, CD36, CD68 and SR-B1 (Bird *et al*., 1999; Chang *et al*., 1999).

Complement activation seems to be required for efficient uptake of apoptotic cells within the systemic circulation (Mevorach *et al*., 1998). The transverse redistribution of plasma membrane PS is followed by the shedding of membrane particles which is a common feature of apoptotic cell death (Aupeix *et al*., 1997).

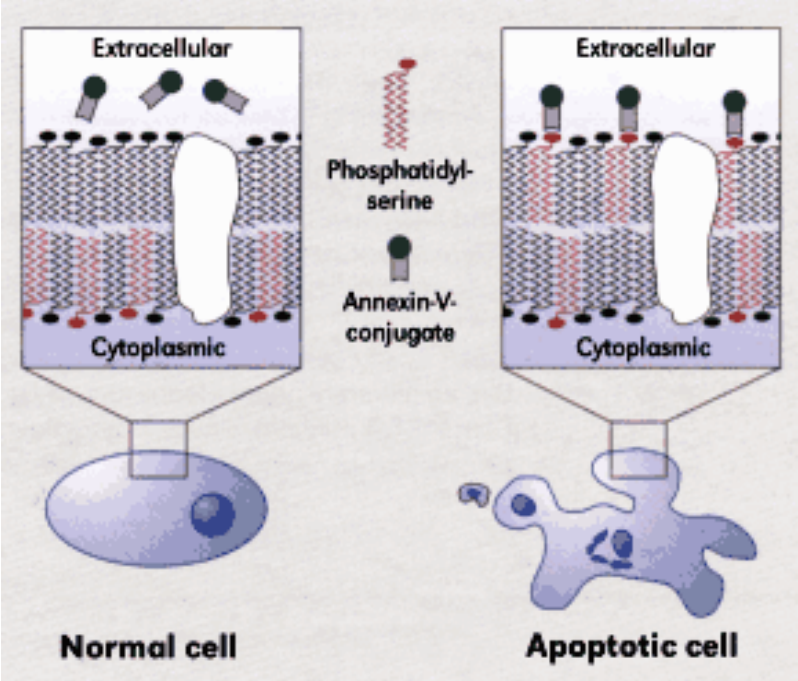


Figure 11. Exposure of phosphatidylserine (PS) on the external leaflet of the cell membrane. Loss of membrane phospholipidic asymmetry and consequent exposure of PS in the exoplasmic leaflet of the plasma membrane is one of the early hallmarks of cells undergoing apoptosis, and is a critical event in their recognition by some macrophage populations (Fadok *et al*., 1992).

CELL SURVIVAL AND PROLIFERATION

Growth Factors

Cell communication is crucial for cell survival and development. In the last fifties many polypeptides able to stimulate cell proliferation have been discovered, they are called growth factors (GF). GF regulate cell growth, differentiation and apoptosis through the binding to receptors on the cell surface. Among these proteins, vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) are important GF for ECs in vitro. These peptides may be critically important in vasculogenesis, atherogenesis, and vascular remodelling in response to injury (Bauters *et al.*, 1999).

VEGF is a 46-kD dimeric peptide with target cell specificity for ECs in vitro. The peptide was first isolated from bovine pituitary follicular cells and stimulates ECs to proliferate and to express collagenase in vitro. bFGF is a 16-kD monomeric peptide which targets a wider variety of cell types in vitro, inducing the proliferation of SMCs and fibroblasts in addition to ECs (Lazarous *et al.*, 1996). EGF is a 6-kD protein with target cell specificity for epithelial cells.

Many GF bind and activate transmembrane glycoproteins of the receptor tyrosine kinase (RTK) family. All RTKs contain an extracellular ligand binding domain, a single transmembrane domain, and an intracellular part that contains a tyrosine kinase domain and several regulatory tyrosines, which are modified through auto- or trans-phosphorylation. Many of these receptors use common intracellular signalling molecules and pathways to mediate the signals induced by the GF. Binding of growth factors to their receptors results in receptor dimerization and subsequently kinase activation. Kinase activation induces phosphorylation of tyrosine residues located at the cytoplasmic tail of the receptor, as well as phosphorylation of effector proteins, which are physically recruited to the active receptor (Nicola, 1994).

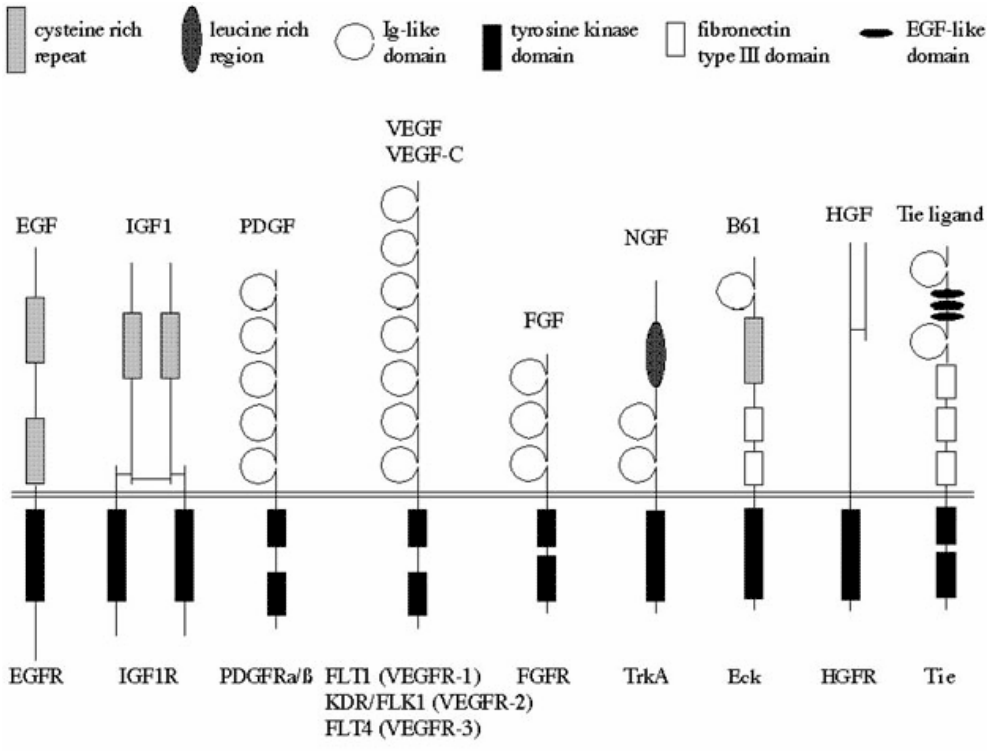


Figure 12. Receptor Tyrosine Kinases.

This step simultaneously initiates multiple signal transduction pathways. The four best characterized signalling pathways induced by RTKs are the mitogen-activated protein kinase cascades (MAPK), the lipid kinase phosphatidylinositol 3 kinase (PI3K), the phospholipase Cγ (PLCγ) pathway and the Signal Transducers and Activator of Transcription (STAT) (Katz *et al*., 2007).

PI3K/Akt signalling pathway

PI3K is a heterodimeric enzyme that comprises two subunits, the p85 regulatory subunit and the p110 catalytic subunit. PI3K activation may be achieved by binding of its p85 regulatory subunit to an activated receptor. Alternatively, RTK signalling may activate the small G protein Ras, which in turn recruits PI3K to the plasma membrane. Once recruited to the plasma membrane, PI3K phosphorylates phosphatidylinositol-4, 5-bisphosphate (PIP_2) on the 3-OH group forming the second messenger phosphatidylinositol-3, 4, 5-trisphosphate (PIP_3) at the inner leaflet of the plasma membrane. PIP_3 activates Akt/PKB protein kinase, a

serine/threonine kinase (Cantley, 2002). This reaction can be inhibited by the major PtdIns (3, 4, 5) P3-phosphatase (PTEN).

Recruitment of Akt to the plasma membrane enables activation and subsequent phosphorylation of various substrate proteins, including major effectors of apoptosis, as well as several transcription factors (Katz *et al*., 2007). Phosphorylation of BAD by Akt prevents the translocation of the proapoptotic protein to the mitochondrial membrane. Akt may also directly block caspase activation by phosphorylating caspase-9. In addition to these direct effects on components of the cell death machinery, Akt phosphorylates transcription factors that regulate cell survival and glycogen synthase kinase 3 (GSK-3), a kinase that regulates apoptosis, cell metabolism and protein synthesis (Song, 2005).

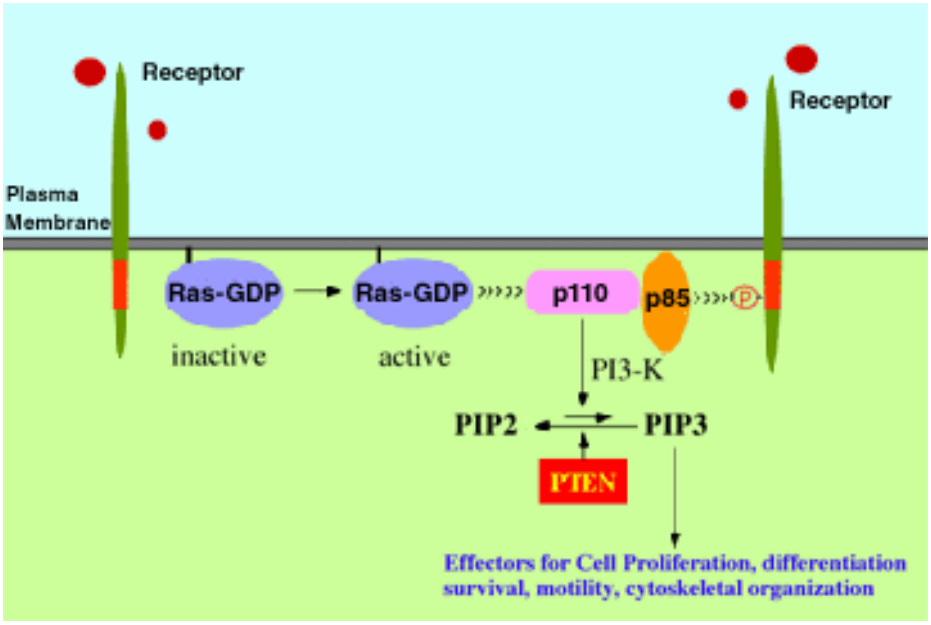


Figure 13. Phosphatidylinositol 3 kinase activation.

MAPK signalling pathway

MAPKs are serine-threonine kinases that mediate important regulatory signals in the cell. The generic MAPK signalling pathway is shared by at least three distinct cascades: the extracellular signal-related kinases (ERK), Jun amino-terminal kinases (JNK) and p38-MAPK. The members of the different MAPK groups are involved in several cellular responses, like gene transcription, induction of cell death or maintenance of cell survival, malignant transformation, and regulation of cell-cycle progression. Each pathway follows the same conserved three kinase activation module consisting of MAPK, MAPK kinase (MAPKK, MKK or MEK), and MAPK kinase kinase (MAPKKK, MEKK) (Roux and Blenis, 2004).

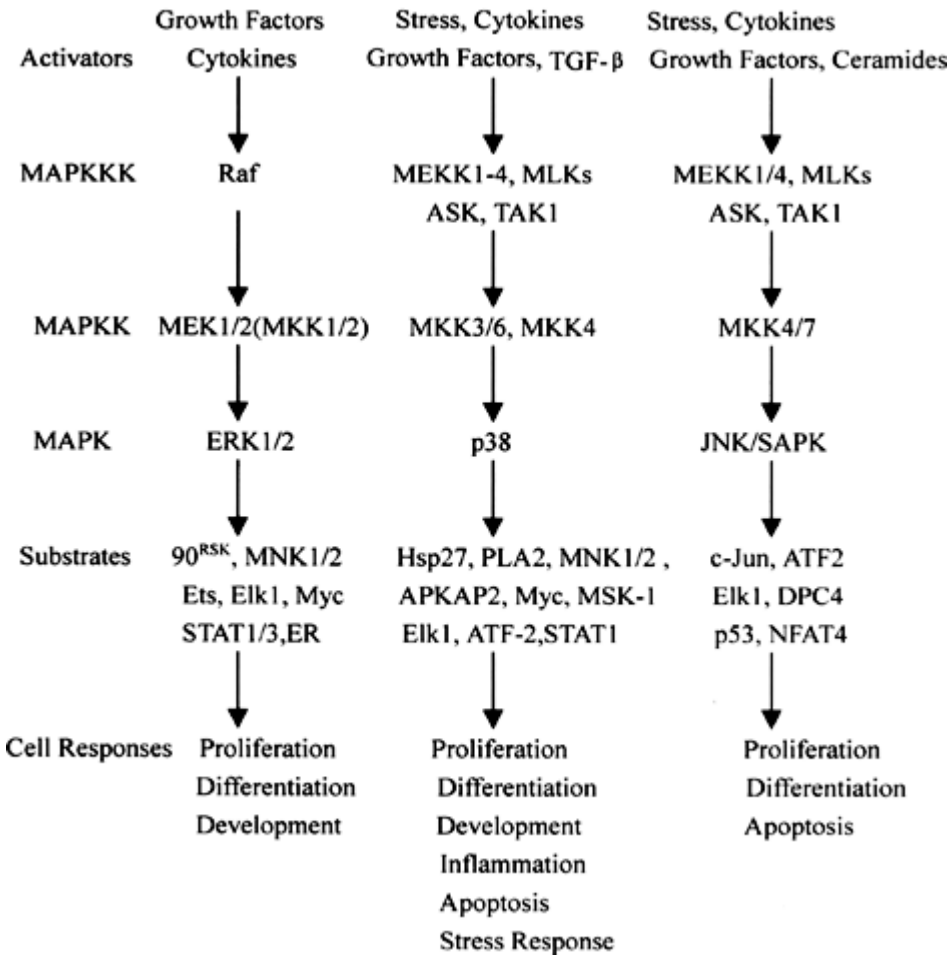


Figure 14. Major mitogen-activated protein kinase cascades in mammalian cells.

ERK signaling pathway

ERK signalling pathway is the best characterized MAPK signaling pathway. The ERK cascade functions in cellular proliferation, differentiation, and survival, and its inappropriate activation is a common occurrence in human cancers. The best known ERK pathway is ERK1/2. The binding of GF to the RTK leads to the interaction of the adaptor protein Gbr2 with the guanine nucleotide exchange factor, Sos. Consequently, Sos is recruited to the plasma membrane, where it promotes the activation of a small G protein, namely Ras. Active Ras can then activate the MAPKKK protein, Raf. Upon activation, Raf induces the phosphorylation of serine residue in the activation loop of MEK (MAPKK). Thereafter, activated MEK1/2 phosphorylates the MAPK proteins, ERK1/2 (Katz *et al.*, 2007).

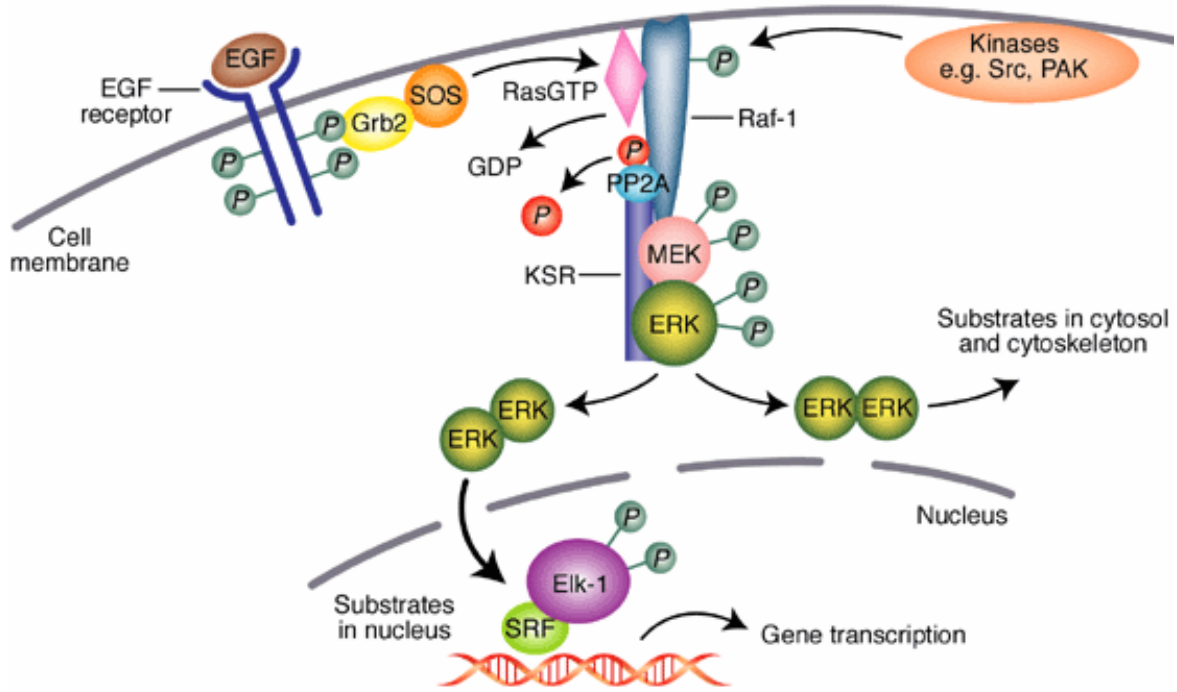


Figure 15. Organisation and function of extracellular signal-regulated kinase (ERK) pathway.

ERK1/2 phosphorylate a variety of nuclear, cytosolic and cytoskeletal targets. For example ERK promotes cell survival by inhibition of caspase-9 (Allan *et al.*, 2003) or by inactivation

of proapoptotic proteins BAD (Scheid and Duronio, 1998). In the nucleus, ERK1/2 dependent phosphorylation activates the transcription factor Elk-1 that binds to the serum response element (SRE) to induce gene transcription in response to serum and growth factors. Other transcription factors, like c-Fos and CREB, are activated by ERK1/2. (Kohno and Pouyssegu, 2006).

JNK signalling cascade

The c-Jun amino-terminal kinase (JNK), also known as the stress activated protein kinase (SAPK), are activated by a variety of environmental stresses, inflammatory cytokines, growth factors and G-protein coupled receptor agonists..

Stress signals are delivered to this cascade by small GTPases of the Rho family (Rac, Rho, cdc42). As with the other MAPKs, the membrane proximal kinase is a MAPKKK, typically MEKK1–4, or a member of the mixed lineage kinases (MLK) that phosphorylates and activates MKK4 (SEK) or MKK7, the SAPK/JNK kinases. SAPK/JNK translocates to the nucleus where it can regulate the activity of multiple transcription factors including c-Jun, JunA, JunB, ATF2 and Elk (Weston and Davis, 2002).

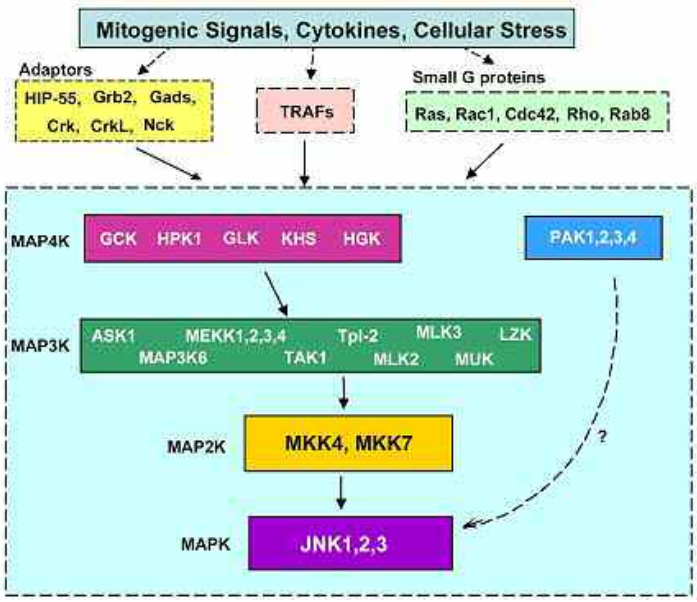


Figure 16. The c-Jun amino-terminal kinase (JNK) signalling cascade.

Although JNK activation is predominantly associated with promotion of cell death, under certain conditions it enables cell survival and even tumor progression (Katz *et al*., 2007).

p38 MAPK signalling pathway

p38 MAPKs are members of the MAPK family that are activated by a variety of environmental stresses and inflammatory cytokines. As with other MAPK cascades, the membrane-proximal component is a MAPKKK, typically a MEKK or a mixed lineage kinase (MLK). The MAPKKK phosphorylates and activates MKK3/6, the p38 MAPK kinases. MKK3/6 can also be activated directly by ASK1, which is stimulated by apoptotic stimuli. Activated p38 has been shown to phosphorylate several cellular targets, including cytosolic phospholipase A2, the microtubule-associated protein Tau, and the transcription factors ATF-1 and -2, MEF2, Sap-1, Elk-1, NF-kB, Ets-1, and p53 (Roux and Blenis, 2004).

PLCγ signalling pathway

Binding of a GF to the membrane receptor results in activation of PLCγ via a G-protein-dependent phenomenon. The activated PLC hydrolyzes PIP_2 to produce diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 causes the release of endogenous Ca^{2+}. Free cytosolic Ca^{2+}, together with DAG, can then activate certain members of the protein kinase C (PKC) family. The PKC family is a multigene family of closely related serine/threonine kinases. There are at least 10 isoforms, which can be subdivided into three subfamilies according to their structure and cofactor regulation: the conventional or classical (α, β1, β2, γ) are regulated by both Ca^{2+} and diacylglycerol (DAG); the novel (δ, ε, θ, η) are regulated by DAG but not by Ca^{2+}, and the atypical (ζ, ι/λ) PKCs require neither Ca^{2+} or DAG regulation (Guo *et al*., 2004).

Activation of PLC-γ in several cell types, including EC, is suggested to stimulate cell proliferation, differentiation and tubulogenesis (Singh *et al.*, 2005; Takahashi *et al.*, 2001).

STAT signalling pathway

RTKs may directly phosphorylate and activate in the cytoplasm STAT-family transcription factors, thereby promoting their translocation to the nucleus. Originally identified as substrates activated by cytokines, several STATs have been shown to undergo phosphorylation by multiple RTKs.Upon phosphorylation-induced dimerization and translocation to the nucleus, STAT proteins elevate transcription of genes involved in cell proliferation (Katz *et al*., 2007).

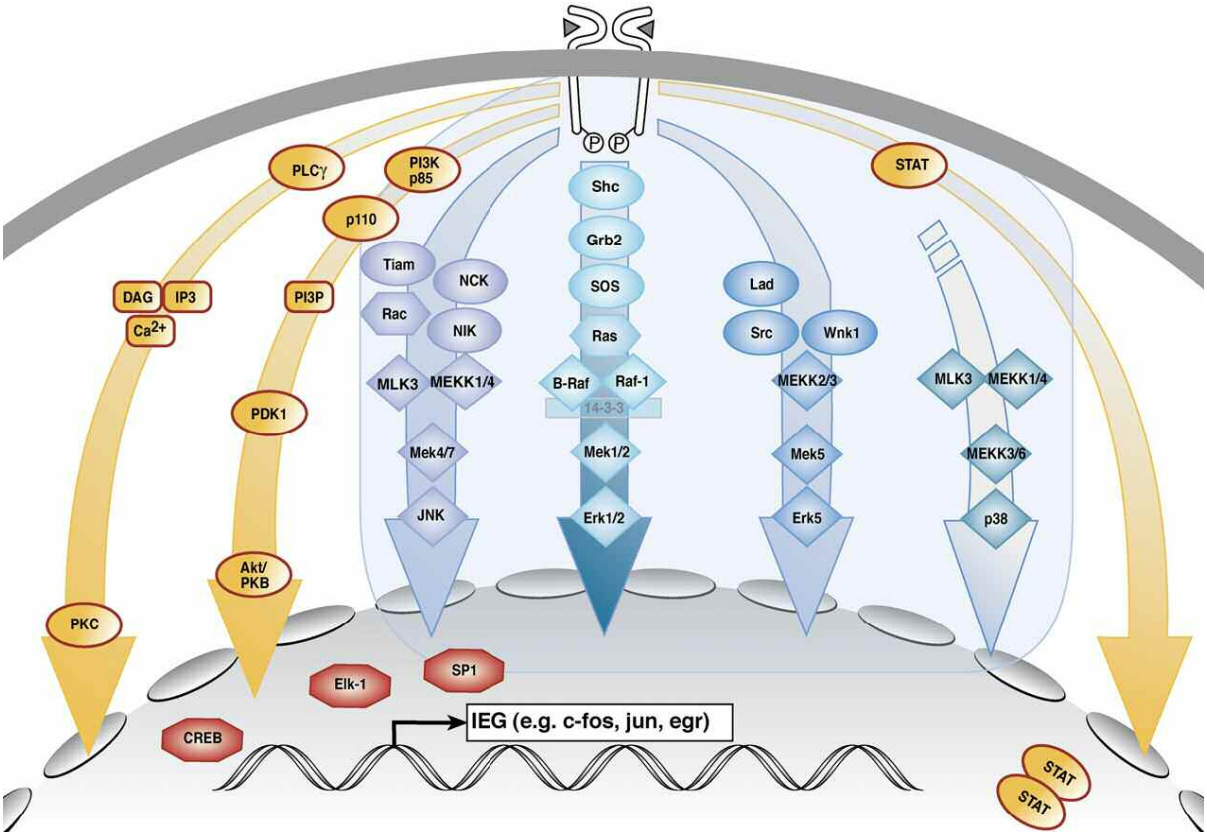


Figure 17. Pathways activated by growth factors. (from Katz *et al.*, 2007)

AIM

Ox-LDL is a proatherogenic lipoprotein, accumulating in the vascular wall and contributing to the pathogenesis of vascular dysfunction in the early development of atherosclerosis. Enhanced serum levels of ox-LDL are predictive for endothelial dysfunction and coronary heart disease. Ox-LDLs are involved in the pathogenesis of atherosclerosis because of their cytotoxic and pro-apoptotic effect on vascular cells (Salvayre *et al*. 2002). However a dual effect of ox-LDL on ECs has been demonstrated: they induce a proliferative effect at lower concentrations (concentration range between 1 and 50 μg/mL) and a proapoptotic effect at higher concentrations (>50 μg/mL) (Galle *et al*. 2001; Seibold *et al*., 2004). Numerous products of LDL oxidation are potent cytotoxic compounds, including many oxysterols, modified phospholipids, lysophosphatidylcholine and various aldehydes. Because of their relative toxicities and their abundance in *in vivo* lesions, oxysterols have been most extensively studied (Scott, 2003). In particular 7-ketocholesterol and 7β-hydroxycholesterol, the main oxysterols present in ox-LDL, have been demonstrated to be highly cytotoxic at concentrations higher than 40 μg/mL. In fact they induce apoptosis or necrosis to several cell lines, including vascular cells (Lizard *et al*., 1996; Lizard *et al*., 1999). The aim of this work was to investigate the effect of lower concentrations (< 20μg/mL) of 7-ketocholesterol and 7β-hydroxycholesterol on the viability of human umbilical vein endothelial cells (HUVEC) and to study the pathways involved in the actions of these compounds.

MATERIALS AND METHODS

CHEMICALS

Cell culture media, fetal bovine serum (FBS), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], PD98059, UO196, SP600125, staurosporine, 7β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol, cholesterol, basic FGF and Propidium Iodide (PI) were purchased from Sigma.

Type 2 collagenase was purchased from Worthington.

Mouse monoclonal IgG_{2a} antibody to phosphorylated ERKs (phos-ERK-1 and phos-ERK-2) and rabbit polyclonal IgG to overall ERKs (ERK-1 and ERK-2) were purchased from Santa Cruz. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG and [^{3}H]-thymidine were purchased from Amersham.

EnzChek Caspase-3 Assay Kit #2, and Annexin V-Alexa Fluor 488 were purchased from Molecular Probes.

Staurosporine was dissolved in methanol (500 μM) and stored at -20°C.

PD98059 was dissolved in dimethylsulfoxide (25 mM) and stored at -20°C.

UO196 was dissolved in dimethylsulfoxide (25 mM) and stored at -20°C.

SP600125 was dissolved in ethanol (10 mM) and stored at -20°C.

Oxysterols and cholesterol were dissolved in ethanol (5mg/mL) and stored at -20°C.

Type 2 collagenase was freshly dissolved in PBS (1 mg/mL).

MTT was dissolved in PBS (12 mM) and stored at 4-8°C.

CELL CULTURE METHODS

ISOLATION OF HUVEC

HUVEC were isolated in our laboratory as described by Jaffe et al. (1973). Human umbilical cords were obtained at normal delivery or caesarean section.

Procedure:

Immediately after delivery, the cords were placed into sterile MEM solution containing penicillin (400 U/mL) and streptomycin (400 µg/mL), and stored at 4°C. The isolation of HUVEC from the cord took place within 24 hours from the delivery.

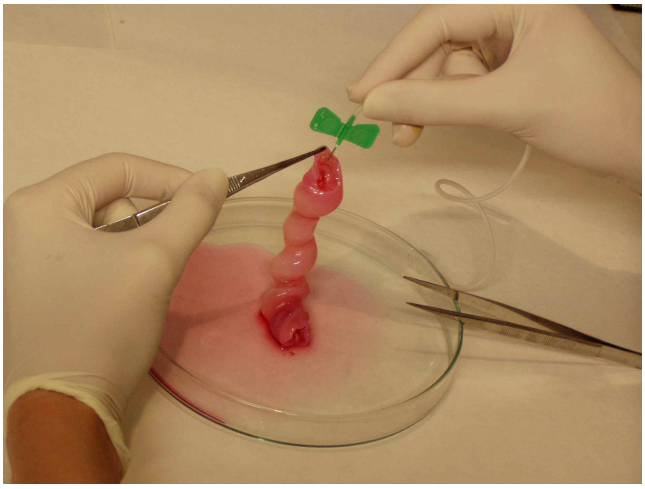


Figure 18. Extraction of human umbilical vein endothelial cells

Under the hood, three syringes of 20 mL, two surgical clamping clips, one cannula (Scalp Vein Set 19G), sterile scissors and sterile gloves were prepared. Before the isolation of HUVEC, the umbilical cords were checked, damaged cords were discharged, the suitable cords were used for the isolation.

The cannula was introduced in one extremity of the vein and tightly maintained with a sterile surgical clamp. The vein was washed with 20 mL of MEM to remove any blood clots. Subsequently the other extremity of the cord was clamped and a solution of collagenase type 2 was injected. The cord was incubated for 18 minutes at 37 °C in an atmosphere of 5% CO_2.
Afterwards the content of the cord was flushed out with 20 mL of MEM with 20% FBS and collected into a sterile tube .The resulting cell suspension was centrifuged for 5 min at 500xg.
The cells were resuspended in 5 mL of M199 containing 20% (v/v) FBS with 2% (v/v) antibiotics and plated into one 25 cm flask. This flask was then incubated at 37 °C and 5% CO_2.
The following day HUVEC were washed twice with 5 mL of MEM and the medium was replaced with a further 5 mL of M199 containing 10% (v/v) FBS, 5 ng/mL bFGF, 25 U/mL heparin, 4 mM L-glutamine, 100 U/mL penicillin-G and 100 μg/mL streptomycin.

CELL CULTURE

HUVEC were grown on 0.5% (v/v) gelatin-coated culture flasks in M199 containing 10% FBS, 5 ng/mL bFGF, 25 U/mL heparin, 4mM L-glutamine, 100 U/mL penicillin-G and 100 μg/mL streptomycin. The medium was changed every two days to remove non-adherent cells. HUVEC were used for the experiments from passages two to six.

CELL TREATMENT

Subconfluent HUVEC were trypsinized and plated in 96-, 12- or 6-well plates (for cell viability, flow cytometry and caspase-3 activity assay, respectively) or 5-cm petri dish (for western blotting) in complete M199 plus bFGF.
Cells were allowed to settle overnight.
HUVEC were then washed with phosphate-buffered saline (PBS) without calcium and magnesium and incubated with or without oxysterols in M199 complete medium in the absence of bFGF for the indicated time and concentrations.
Control samples were treated in the presence of 0.4% ethanol, the maximal concentration of solvent used to dissolve oxysterols.

Pretreatment with the inhibitors UO196 and PD98059 and SP600125 were performed in fresh cell culture medium without bFGF 30 min before the addition of oxysterols. Control samples were added of 0.1% DMSO, the maximal concentration of solvent used to dissolve the inhibitors. Subsequently oxysterols were added directly in the cell culture medium containing the inhibitors.

Staurosporine was added to the cell culture medium containing the oxysterols with or without the inhibitors during the last three hours of treatment. Control samples were added of 0.01% methanol, the maximal concentration of solvent used to dissolve staurosporine.

Solutions:

PBS: 137 mM NaCl; 2.7 mM KCl; 8.1 mM Na_2HPO_4 7 H_2O; 1.76 mM KH_2PO_4

METHODS FOR THE DETECTION OF CELL VIABILITY AND PROLIFERATION

MTT REDUCTION ASSAY

The determination of cell viability was performed with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay. This assay was first described by Mosmann in 1983. The yellow tetrazolium salt (MTT) is reduced in the mitochondria of living cells to form purple formazan crystals. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable cells. Formazan crystals are solubilized by the addition of DMSO. The colour can then be quantified by multiwell scanning spectrophotometer.

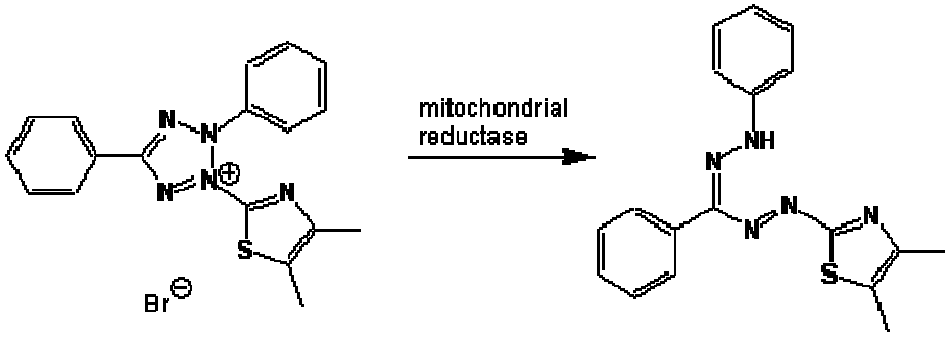


Figure 19. Reduction of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) to formazan.

Procedure:

HUVEC (10,000 cells/well) were plated in 200µl media per well in a 96 well plate. Six wells were left empty for blank controls. The cells were incubated at 37°C and 5% CO_2 and settled overnight. The day after, the cells were incubated with or without oxysterols at the indicated concentrations and time.

Four hours before the end of the treatment 10 µl of stock solution of MTT was added to each well.

At the end of the treatment, medium was carefully removed and the formazan crystals formed were dissolved in 100 µl of DMSO.

MTT reduction was quantified by measuring the light absorbance with a multilabel plate counter (VICTOR^2-Wallac) at 570 and 630 nm. Background absorbance from control wells (media without cells) was subtracted.

Solutions:

MTT : 12 mM (5 mg/mL) in PBS

[^{3}H]-THYMIDINE INCORPORATION ASSAY

[^{3}H]-thymidine incorporation assay was used to quantify DNA synthesis and hence, cell proliferation.

Procedure:

HUVEC where plated in 96-wells plate (5000-10000 cells/well) in 200 µL cell culture medium plus bFGF, incubated at 37°C and 5% CO_2 and settled overnight.
The day after the cells were incubated with or without oxysterols at the indicated concentrations for 48 hours. 1 µCi/well [^{3}H]-thymidine was added twenty-four hours before the end of the treatment.
At the end of the treatment cells were washed with PBS; then a lysis solution was added to each well and the cells were incubated for 30 min at room temperature.
A scintillation solution (Ultima Gold, Packard) was added to detect the incorporation of [^{3}H]-thymidine. The radioactivity for each sample was measured using a β-Counter (TRI-CARB 2100 TR, Packard).

Solutions:

[^{3}H]-thymidine: 0.2 µCi/µL in PBS
Lysis solution: 1%SDS; 4 mM EDTA

METHODS FOR THE DETECTION OF APOPTOSIS

MEASUREMENT OF CASPASE-3 ACTIVITY

The detection of apoptosis through the activation of the enzyme Caspase-3 was determined with the EnzChek Caspase-3 assay kit#2. A fluorogenic synthetic peptide Z-DEVD-Rhodamine 110 was used as a substrate. This substrate is a nonfluorescent bisamide that is first converted by caspase-3 to the monoamide and then to the bright, green-fluorescent rhodamine 110 (excitation/emission maxima ~496/520 nm).

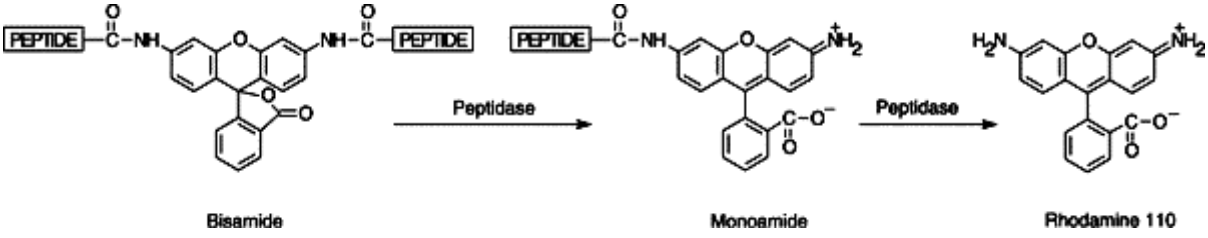


Figure 20. Z-DEVD-Rhodamine 110 is first converted by caspase-3 to the monoamide and then to rhodamine 110.

Procedure:

HUVEC (300,000 cells/well) were plated in 6-well culture plate, incubated at 37°C and 5% CO_2 and settled overnight. The day after the cells were incubated with or without oxysterols at the indicated concentrations and time. After the treatment, cell culture media was removed and the cells were washed twice with PBS. HUVEC were lysed in 200 µL lysis buffer on ice and were centrifuged at 12000g for 20 min at 4 °C. The supernatant, after the measurement of the protein content through the Lowry procedure, was used for the analysis.

50 µL of cell lysate were added to 50 µL of reaction buffer with caspase substrate (50 µM) in a 96-well microplate (1508-0010 Black 96-well microplate, PerkinElmer).

The blank controls (50 µL reaction buffer with caspase substrate and 50 µL lysis buffer) showed the background absorbance and were subtracted from the experimental readings.

The microplate was incubated at room temperature in the dark for 30 minutes. Fluorescence was measured at 485 nm for excitation and 535 nm for emission with a multilabel plate

counter (VICTOR^2-Wallac). Fluorescence signal was normalized for protein content (Lowry assay).

Solutions:

Lysis buffer: 100 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.01% Triton X-100.
Reaction buffer : 20 mM PIPES, 4 mM EDTA, 0,2% CHAPS, 100 mM DTT; pH 7,4

FLOW CYTOMETRIC DETECTION OF APOPTOSIS

Cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose PS which is translocated to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. This PS exposure represents a hallmark (early and widespread) in detecting dying cells. Annexin V is a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca^{2+}. By conjugating a fluorescent probe to Annexin V it is possible to identify and quantify apoptotic cells on a single-cell basis by flow cytometry.

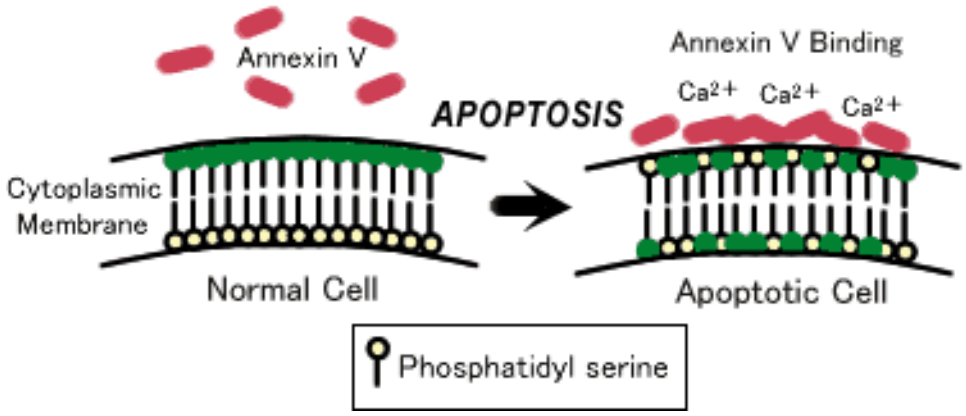


Figure 21. Detection of early stage of apoptosis through binding of Annexin-V to phosphatidyl serine.

Changes in PS asymmetry, which is analyzed by measuring Annexin V binding to the cell membrane, were detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost.

Staining cells simultaneously with Alexa Fluor 488-Annexin V (green fluorescence) and the non-vital dye propidium iodide (red fluorescence) allows (bivariate analysis) the discrimination of intact cells (Annexin-/PI-), early apoptotic (Annexin+/PI-) and late apoptotic or necrotic cells (Annexin+/PI+).

Procedure:

HUVEC (180,000 cells/well) were plated in 12-well culture plate, incubated at 37°C and 5% CO_2 and settled overnight.

The day after the cells were incubated with or without oxysterols at the indicated concentration and time.

After the treatment, cell culture media was removed and the cells were washed with PBS, trypsinized, centrifuged and the resulting pellet was resuspended in 100 µL of a binding buffer. 2 µL annexin V-Alexa Fluor 488 and 1.25 µL propidium iodide (PI) were added to the cell suspension.

The samples were incubated for 15 min at room temperature in the dark.

The samples were diluted to 500 µl with binding buffer and analysed by flow cytometry (EPICS XL, Beckman Coulter) equipped with EXPO^{TM} 32 ADC software. Fluorescence was measured at 488 nm for excitation and 525 (Annexin-V) and 620 (PI) nm for emission.

For each sample, 10,000 events selecting cell size and granularity by forward and side scatters.

Solutions:

Binding buffer: 40 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4.

WESTERN BLOTTING ANALYSIS

CELL LYSATES OF HUVEC

Procedure:

HUVEC (800,000 cells/dish) were plated in 5 cm petri, incubated at 37°C and 5% CO_2 in cell culture medium plus bFGF and settled overnight.
The day after the cells were incubated with or without oxysterols for the indicated time.
After the treatment, cell culture media was removed, the cells were washed with PBS and lysed in 150 µl of lysis buffer at 4°C.
Cell lysates were centrifuged at 12000g for 15 min at 4°C. The supernatant was quantified for the protein content (Besadoun-Weinstein procedure).
20 µg of proteins for each sample were added to 1/5 of the volume of Laemmli buffer. The samples were boiled for 5 min at 95°C and then they were subjected to 10% SDS-PAGE (10% resolving gel and 4% stacking gel).

Solutions:

Lysis buffer: 10 mM Tris-Hcl pH 7.4; 0,5% Sodium Dodecyl Sulphate (SDS); 10% glycerol; 1% Triton-X 100; 0.75% DOC; 75 mM NaCl; 10 mM EDTA; 0.5 mM PMSF; 2 mM $NaPO_4$; 10 µg/mL leupeptin; 25 µg/mL aprotinin; 1.25 mM NaF; 1 mM $Na_4P_2O_7$.

Laemmli buffer: 70 mM Tris HCl pH 6.8; 22% glycerol; 2% SDS; 4% β-mercaptoethanol.

WESTERN BLOTTING

Procedure:

Samples were loaded into the appropriate gel and run in running buffer, for 1h at 32mA (2002 power supply LKB Bromma). A molecular weight marker (BenchMark Prestained Protein Ladder, Invitrogen) was used as benchmark.
Proteins were transferred to PDVF membrane (Hybond-P, Amersham), previously soaked in methanol. The blot "sandwich" was placed into the Mini-Transblot electrophoresis system (Bio-Rad) filled with transfer buffer.
A constant current of 200 mA was applied for 4 h at 4°C.
Subsequently, non specific binding sites were blocked by incubating the membrane for 1 hour at room temperature in a blocking solution (5% milk in TBS-Tween-20).
The membrane was exposed to the primary antibody at the dilution 1:10,000 in TBS-Tween-20 containing 2.5% non fat-dried milk or 5% BSA at 4°C overnight.
Then the membrane was washed 3 times for 15 min with TBS-Tween 20 at room temperature under gentle agitation, the membrane was incubated with horseradish peroxidase (HRP)-linked secondary antibody at the concentration 1:100,000 in TBS-Tween 20 for 1 hour at room temperature.Then the membrane was washed 3 times for 15 min with TBS-Tween 20.
The signal was detected by chemiluminescence using the ECL-Advance Western blotting detection kit (Amersham).
After 5 min of incubation at room temperature the membrane was exposed to Hyperfilm (Amersham Biosciences) in an X-ray film cassette.
The film was developed in developing solution (Kodak). When the bands were at the desired density, the films was transferred into the fixing solution (Kodak), then washed in water and air-dried. Molecular weight standards were used to identify appropriate antibody binding.

Solutions:

Running buffer: 25 mM Tris; 192 mM glycin; 1% SDS.
Transfer buffer: 25 mM Tris; 192 mM glycin.
TBS-Tween 20: 10 mM Tris, 150 mM NaCl, 0,1% Tween-20.

PROTEIN ASSAYS

Protein content of cell lysates was determined by Lowry procedure (1951) or Bensadoun and Weinstein procedure (1976).

LOWRY PROCEDURE

Increasing concentrations of Bovine Serum Albumine (BSA) were used to develop a standard curve. Each sample was measured in duplicate.

50 µL cell lysate were added to 200 µL of an aqueous solution of deoxycholate (DOC), then bidistillated H_2O was added up to 1 mL.

100:1:1 mixture of solutions A, B1, B2 was prepared.

5 mL of the mixture were added to each sample and immediately mixed. The samples were let sit for exactly 10 min at 25 °C.

0,5 mL Folin-Ciocalteu's phenol reagent 1N was added to each sample, the resulting solution was fast mixed.

The tubes were let stand for at least 30 min but (no more than 1 hour).

Absorbance was read at 750 nm.

The absorbance was plotted as function of µg BSA (i.e. µl BSA). The blank OD was subtracted from each unknown and read the amount, in µg, on the standard curve.

<u>Solutions:</u>

Folin-Ciocalteu's phenol reagent (Sigma): 1N in bidistillated H_2O.

DOC: 10% in bidistillated H_2O

Solution A: 2% Na_2CO_3 in 0.1N NaOH

Solution B1: 1% $CuSO_4.5H_2O$ in bidistillated H_2O

Solution B2: 2% NaK tartrate in bidistillated H_2O

BESADOUN-WEINSTEIN PROCEDURE

Increasing concentrations of Bovine Serum Albumine (BSA) were used to develop a standard curve. Each sample was measured in duplicate.

50 µL cell lysate were added to 25 µl of DOC, then bidistillated H_2O was added up to 3 mL .

The tubes were let stand for at least 10 min at 25°C.

1 mL trichloroacetic acid (TCA) 24% was added to each sample to precipitate proteins.

The samples were centrifuged for 20 min at 12000g.

To each sample 300 µl of an aqueous solution of SDS and 200 µl of bidistillated H_2O were added. The solution was mixed.

100:1:1 mixture of solutions A, B1, B2 was prepared.

3 mL of the mixture were added to each sample and immediately mixed. The samples were let sit for exactly 10 min at 25 °C.

0.5 mL Folin-Ciocalteu's phenol reagent 1N was added to each sample, the resulting solution was fast mixed.

The tubes were let stand for at least 30 min but (no more than 1 hour).

Absorbance was read at 750 nm.

The absorbance was plotted as function of µg BSA (i.e. µl BSA). The blank OD was subtracted from each unknown and read the amount, in µg, on the standard curve.

<u>Solutions:</u>

Folin-Ciocalteu's phenol reagent (Sigma): 1N in bidistillated H_2O.

DOC: 10% in bidistillated H_2O

Solution A: 2% Na_2CO_3 in 0.1N NaOH

Solution B1: 1% $CuSO_4.5H_2O$ in bidistillated H_2O

Solution B2: 2% NaK tartrate in bidistillated H_2O

TCA: 24% in bidistillated H_2O.

STATISTICAL ANALYSIS

Results are presented as mean ± standard error of the mean (SE).

Statistical significance of the differences between experimental groups were calculated by one-way ANOVA followed by the Dunnett's or Bonferroni's Multiple Comparison Test; $P<0.05$ was considered to be statistically significant. Graphs and statistical analysis were performed using GraphPad Prism version 3.03 for Windows, GraphPad Software.

RESULTS

EFFECT OF 7-KETOCHOLESTEROL AND 7β-HYDROXYCHOLESTEROL ON CELL VIABILITY

The effect of 7-ketocholesterol and 7β-hydroxycholesterol on HUVEC viability was assessed by the MTT reduction assay. For this purpose HUVEC were exposed for 24, 48, 72 or 96 hours to increasing concentrations of 7-ketocholesterol and 7β-hydroxycholesterol (1-20 µg/mL).

As shown in figure 22 both oxysterols induced a dual effect on cell viability associated with MTT reduction in HUVEC. 7-ketocholesterol induced an increase in cell viability at concentrations lower than 20 µg/mL compared to the control, this effect appears to be strong after 24 hours (fig. 22A) and it is maintained even after 96 hours (fig. 22D). The exposure of the cells to the highest concentration of 7-ketocholesterol leaded to a strong decrease in cell viability in particular after 72 or 96 hours of treatment (fig. 22C, 22D).

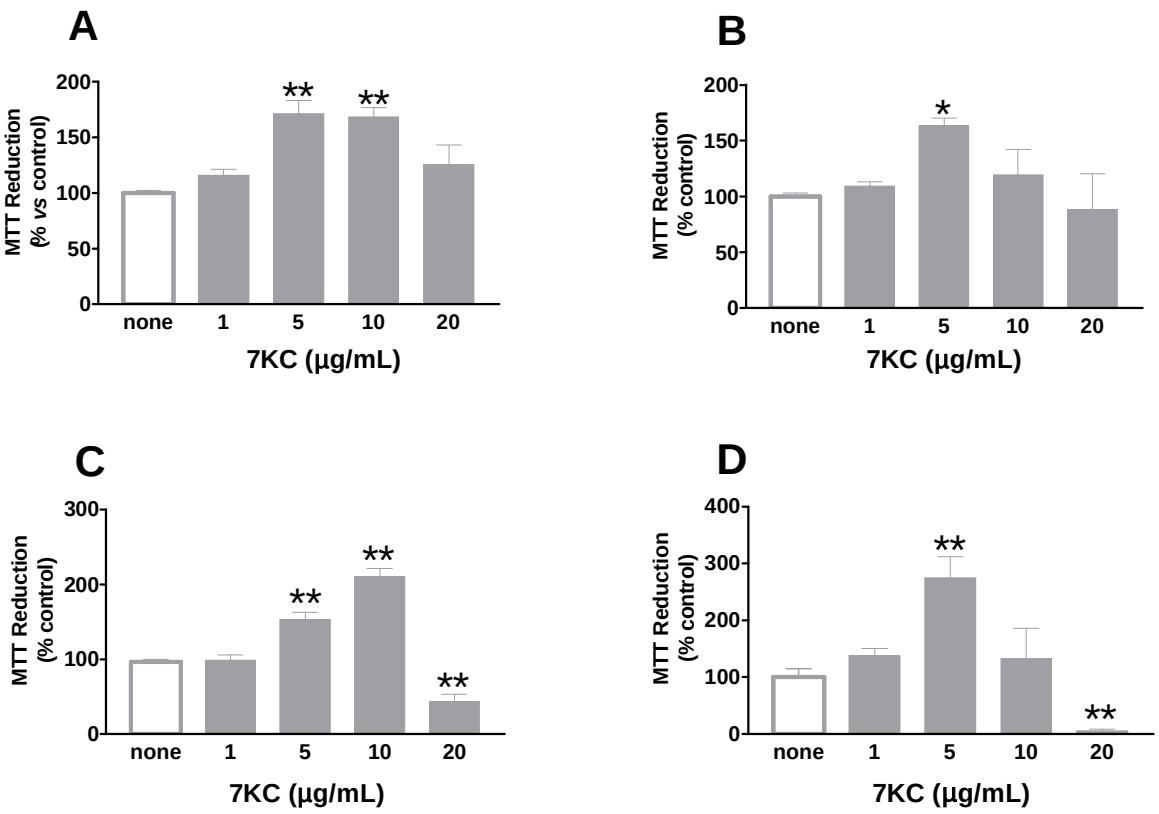


Figure 22. Effect of 7-ketocholesterol (7KC) on HUVEC viability. Cells were incubated for 24 h (A), 48 h (B), 72 h (C) or 96 h (D) in cell culture medium without bFGF containing increasing concentrations of 7KC. Cell viability was determined by MTT reduction assay. Data are expressed as means ± S.E. of four independent experiments performed in sixtuplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test.*P<0.05; **P<0.01 (*vs* control).

Similar results were obtained when HUVEC were exposed to increasing concentrations of 7β-hydroxycholesterol. In fact, as shown in figure 23, 7β-hydroxycholesterol induced an increase in cell viability at concentrations lower than 20 µg/mL compared to the control. This increase was evident after 24 hours (fig. 23A) and it was maintained even after 96 hours (fig. 23D). The treatment of HUVEC with 20 µg/mL of 7β-hydroxycholesterol induced a strong decrease in cell viability visible after 24 hours of treatment.

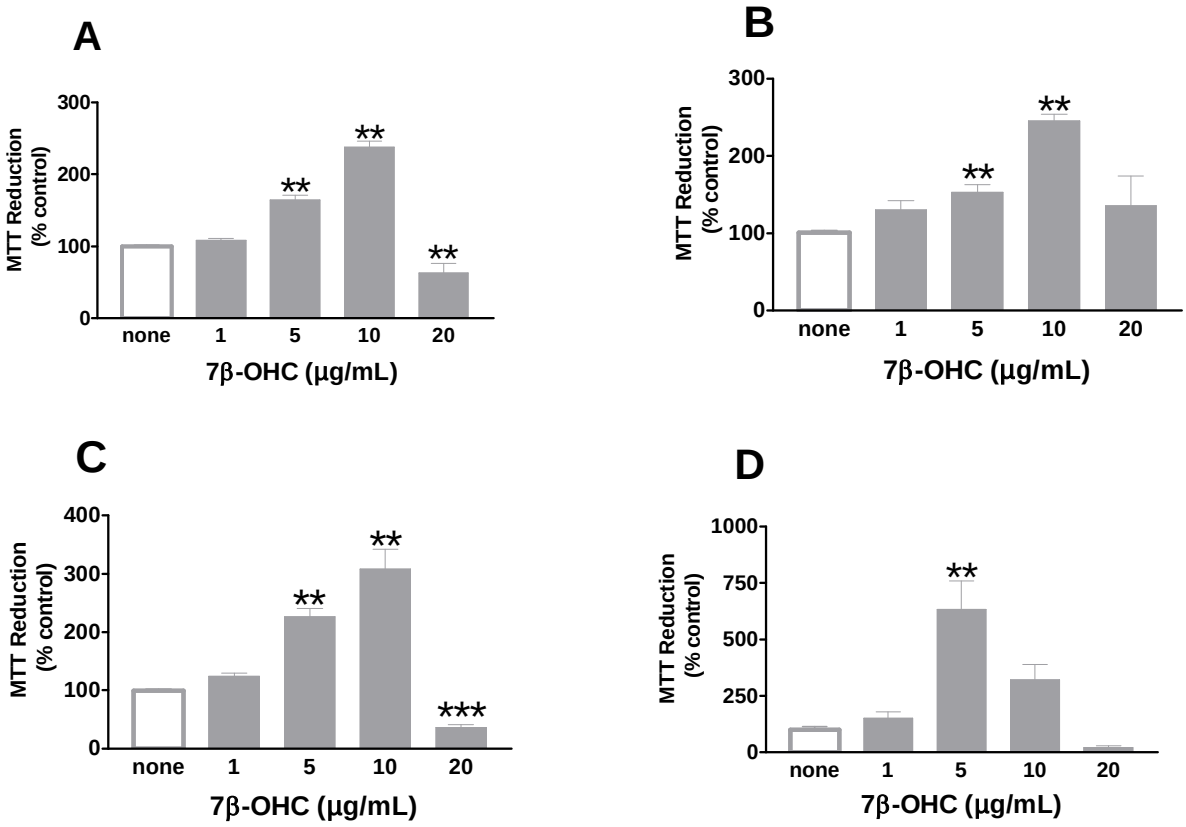


Figure 23. Effect of 7β-hydroxycholesterol (7βOHC) on HUVEC viability. Cells were incubated for 24 h (A), 48 h (B), 72 h (C) or 96 h (D) in cell culture medium without bFGF containing increasing concentrations of 7β-OHC. Cell viability was determined by MTT reduction assay. Data are expressed as means ± S.E. of four independent experiments performed in sixtuplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test.**P<0.01, ***P<0.001 (*vs* control).

TOXIC EFFECT OF 7 KETOCHOLESTEROL AND 7β-HYDROXYCHOLESTEROL

The cytotoxic effect of 7-ketocholesterol and 7β-hydroxycholesterol in HUVEC was first investigated by Lizard and co-workers, who showed that both compounds induce apoptosis or necrosis in several ECs types, including HUVEC (Lizard *et al*., 1996; Lizard *et al*., 1999).
We confirmed these data in our laboratory investigating the effect of 7-ketocholesterol and 7β-hydroxycholesterol at the concentration of 20 µg/mL in HUVEC.
Two hallmarks of early apoptosis were measured: caspase-3 activation and PS translocation. In fact, the enzyme caspase-3 is an important effector caspase involved in the development of apoptosis, while PS is exposed on the surface of the apoptotic cells.
HUVEC were treated with 20 µg/mL 7-ketocholesterol for 6 (fig. 24A) or 10 hours (fig. 24B) and the activation of caspase-3 was measured. As shown in figure 24B, 7 ketocholesterol did not change caspase-3 activity after 6 hours of treatment (24A) whereas it induced a three fold increase in caspase-3 activity after 10 hours of treatment.

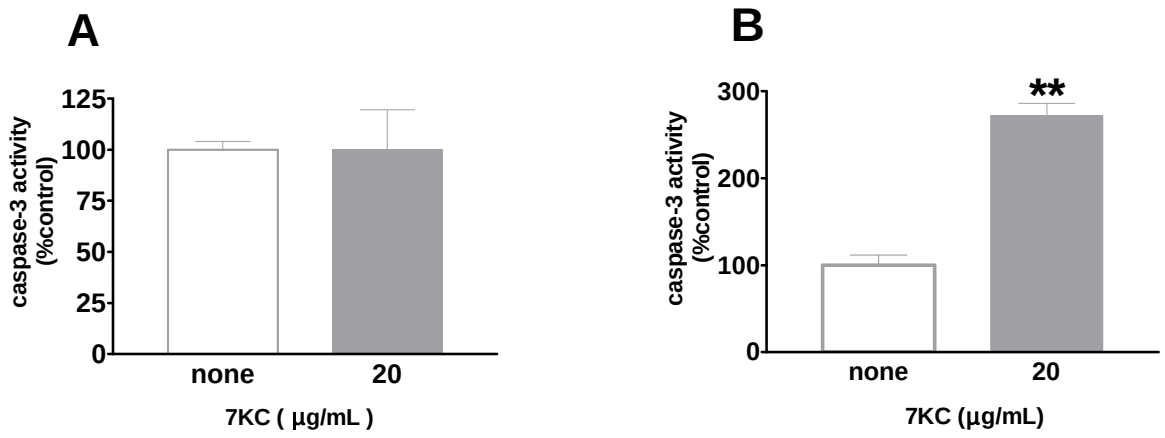


Figure 24. Effect of 7-ketocholesterol on caspase-3 activity in HUVEC. Cells were incubated for 6 h (A) or 10 h (B) in cell culture medium in absence of bFGF with or without 20 µg/mL 7KC. The caspase-3 activity was measured on cell lysates. Data are expressed as means ± S.E. of four independent experiments performed in duplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test. **P<0.01 (*vs* control).

To confirm the apoptotic effect induced by of 7 ketocholesterol at the concentration of 20 µg/mL, PS translocation was measured. HUVEC were treated for 8 hours with 20 µg/mL 7 ketocholesterol, and then analysis of the binding of annexin V and PI was performed. As shown in figure 25, 7-ketocholesterol induced a strong increase in the number of apoptotic cells (annexin-V positive) and late apoptotic (annexin-V and PI positive) cells.

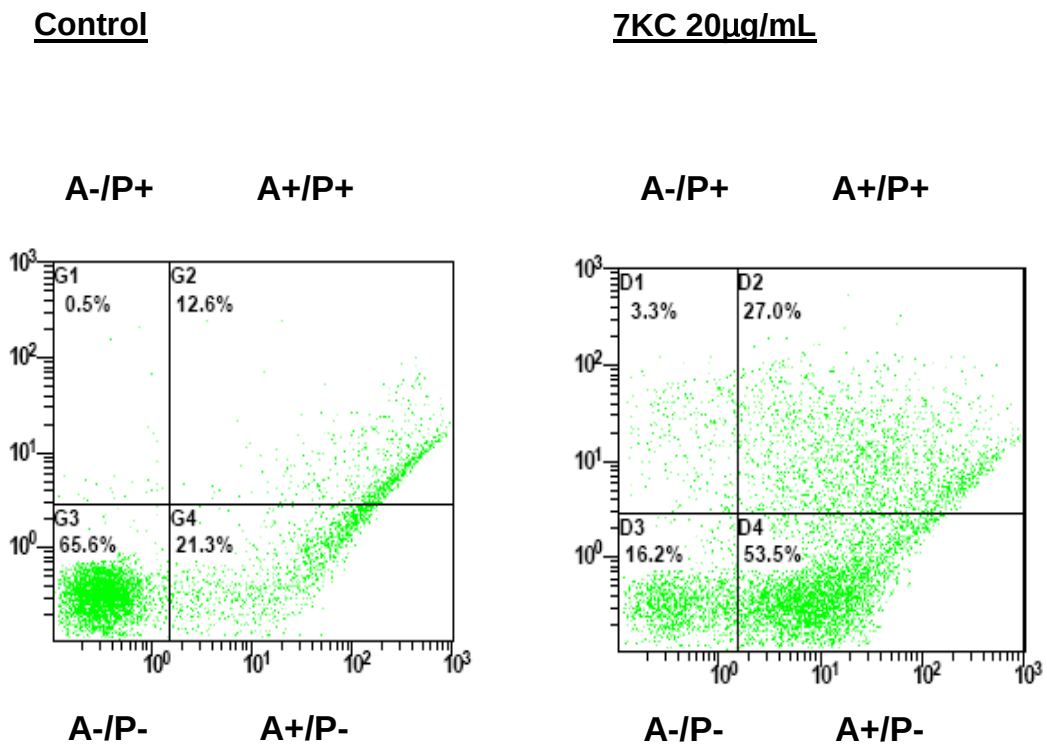


Figure 25. Representative cytograms of flow cytometric analysis of annexin-V and propidium iodide (PI) binding in HUVEC treated with 7 ketocholesterol (7KC). HUVEC were incubated in cell culture medium without bFGF in the absence (control) or in the presence of 20 µg/mL 7KC. After 8h treatment the cells were stained with PI and Alexa Fluor 488-conjugated annexin-V and then flow cytometric analysis of annexin V binding was performed. The cytograms represent annexin-binding (ordinate) versus PI-binding (abscissa). Viable cells (A-/P-), apoptotic cells (A+/P-), late apoptotic cells (A+/P+), and necrotic cells (A-/P+) are shown in the respective quadrants.

Different from 7 ketocholesterol, treatment of HUVEC with cytotoxic concentrations (20 µg/mL) of 7β-hydroxycholesterol did not induce caspase-3 activation (fig. 26).

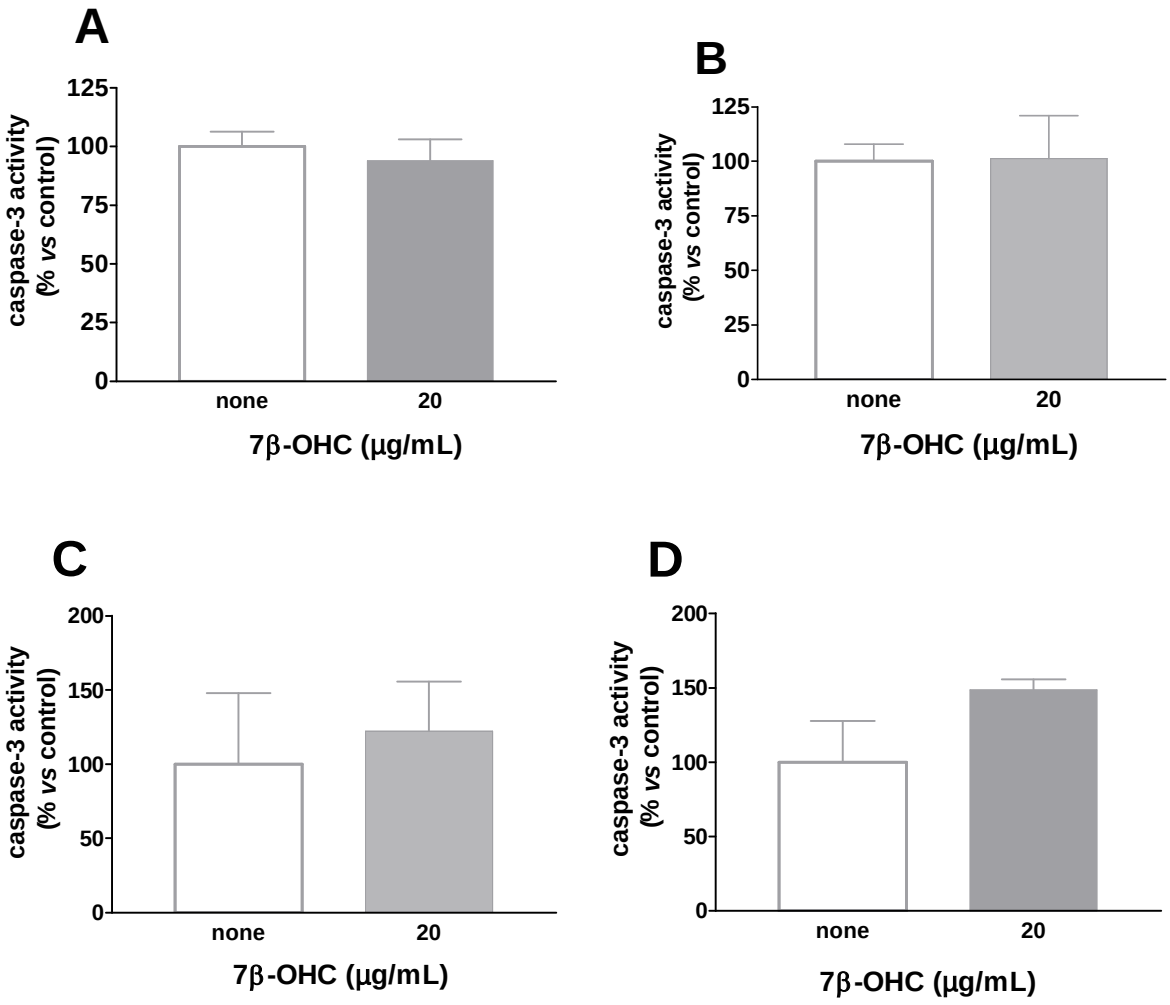


Figure 26. **Effect of 7β-hydroxycholesterol (7β-OHC) on caspase-3 activity in HUVEC.** Cells were incubated for 6 h (A), 10 h (B), 13 h (C) or 15 h (D) in cell culture medium in absence of bFGF with or without 20 µg/mL 7β-OHC. Data are expressed as means ± S.E. of four independent experiments performed in duplicate. Statistical analysis was performed by one-way ANOVA test.

To confirm that the cytotoxic effect induced by 7β-hydroxycholesterol was not due to apoptosis, PS translocation was determined. As shown in the figure 27, treatment with 20 µg/mL 7β-hydroxycholesterol for 8 hours induced a strong increase in the number of cells incorporating PI, a feature of cells undergoing necrosis.

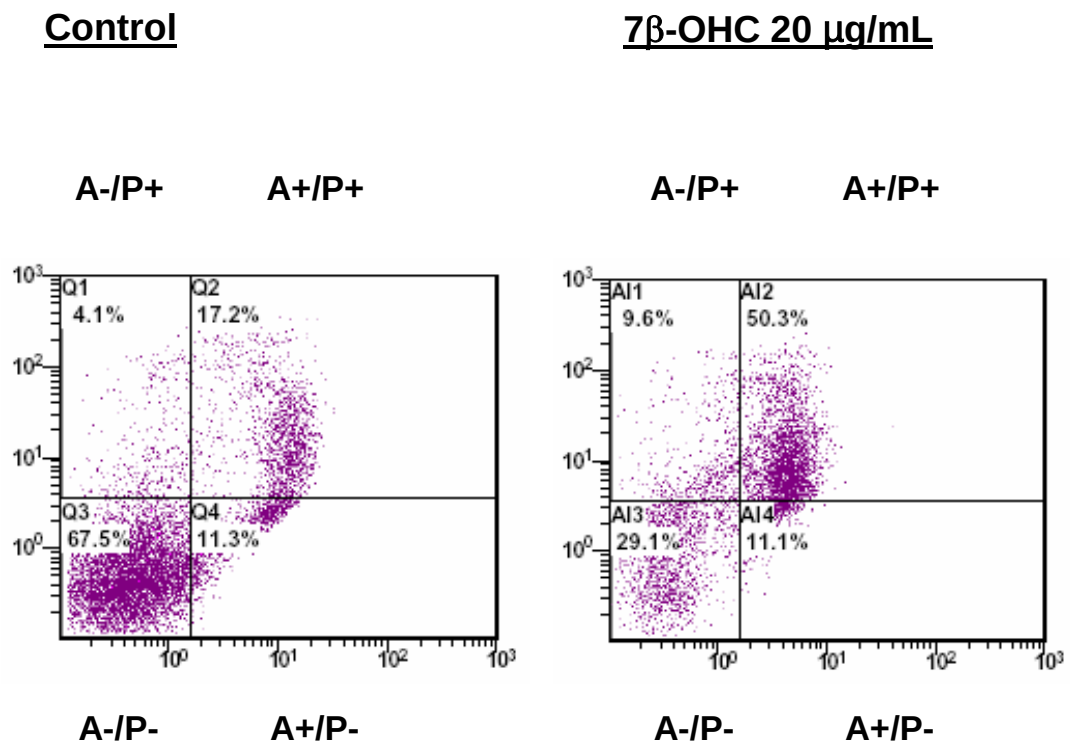


Figure 27. Representative cytograms of flow cytometric analysis of annexin-V and propidium iodide (PI) binding in HUVEC treated with 7β-hydroxycholesterol (7β-OHC). HUVEC were incubated in cell culture medium without bFGF in the absence (control) or in the presence of 20 µg/mL 7β-OHC. After 8h treatment the cells were stained with PI and Alexa Fluor 488-conjugated annexin-V and then flow cytometric analysis was performed. The cytograms represent annexin-binding (ordinate) versus PI-binding (abscissa). Vital cells (A-/P-), apoptotic cells (A+/P-), late apoptotic cells (A+/P+), and necrotic cells (A-/P+) are shown in respective quadrants.

EFFECT OF 7β-HYDROXYCHOLESTEROL ON CELL PROLIFERATION

The increase in HUVEC viability induced by 7-oxysterols at concentrations below 20 µg/mL could be due to an increase in cell proliferation as well as to an antiapoptotic effect. In fact treatment of cells was performed in bFGF deprivation: this condition has been recently shown to be proapoptotic in HUVEC (Vinci *et al*., 2004; Trevisi *et al*., 2004). Thus, 7β-hydroxycholesterol could induce an increase in cell vialbility also protecting cells from apoptosis induced by bFGF deprivation. The increase in cell proliferation and the antiapoptotic effect were studied using 7β-hydroxycholesterol as a model because of its stronger and faster effect.

Cell proliferation was measured by the determination of [^{3}H]-thymidine uptake. As shown in figure 28, 7β-hydroxycholesterol caused a two fold increase in cell proliferation compared to the control, the increase was not as strong as the one caused by bFGF.

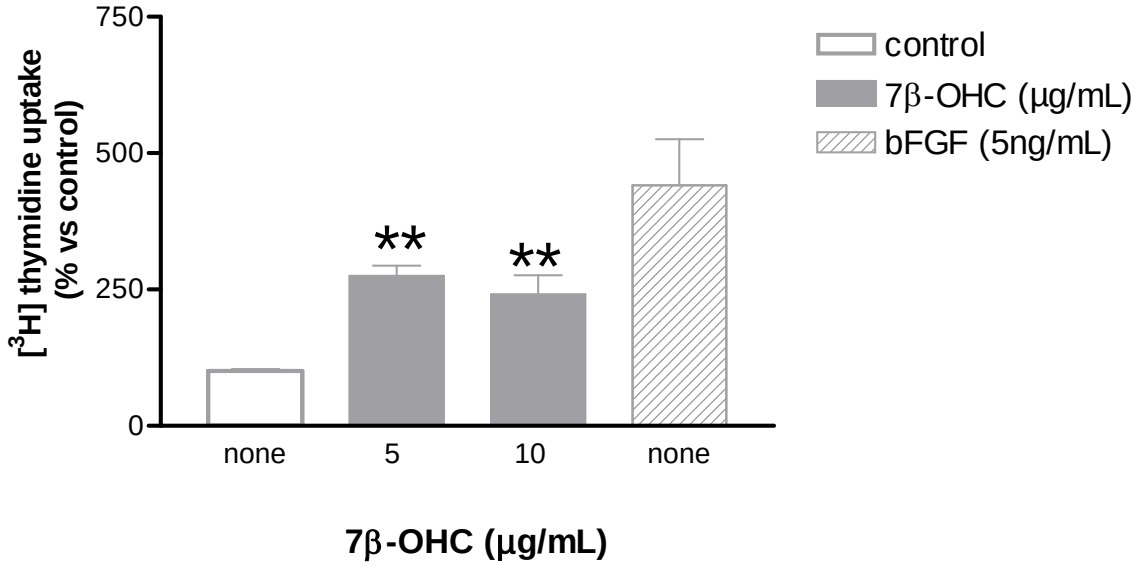


Figure 28. Effect of 7β-hydroxycholesterol (7β-OHC) on cell proliferation in HUVEC. Cells were incubated for 48 h in cell culture medium with or without bFGF in the presence of increasing concentrations of 7β-OHC. Cell proliferation was determined by [^{3}H] thymidine uptake. Data are expressed as mean ± S.E. of three independent experiments performed in quadruplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test. **P<0.01 vs control..

The effect of 7β-hydroxycholesterol was also investigated in the presence of different GF (bFGF, EGF and VEFG). Cell proliferation was detected measuring the uptake of [^{3}H] thymidine.

HUVEC were treated with 7β-hydroxycholesterol in the presence of increasing concentrations of bFGF (fig. 29) or EGF (fig. 30). In both cases 7β-hydroxycholesterol enhanced the effect on cell proliferation stimulated by the GF. The additive effect induced by 7β-hydroxycholesterol increased until reaching a value representing the maximal proliferation allowed in these experimental conditions. A similar effect was observed in the presence of increasing concentrations of VEGF (fig. 31).

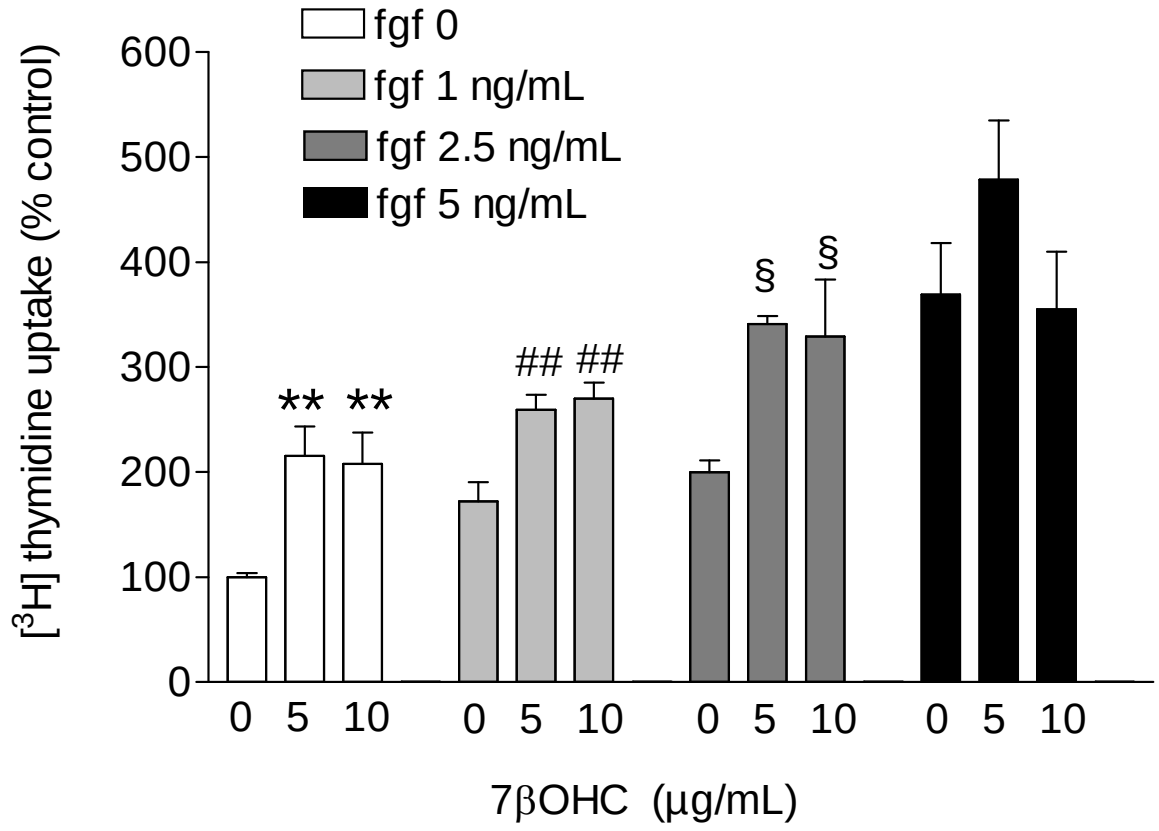


Figure 29. Effect of 7β-hydroxycholesterol (7β-OHC) on HUVEC proliferation in presence of bFGF. Cells were incubated for 48 h in cell culture medium containing different concentrations of bFGF with or without increasing concentrations of 7βOHC. Cell proliferation was determined by [^{3}H] thymidine uptake. Data are expressed as mean ± S.E. of three independent experiments performed in quadruplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test. ***P*<0.01, ## *P*<0.01, § *P*<0.05 (*vs* corresponding controls without 7βOHC).

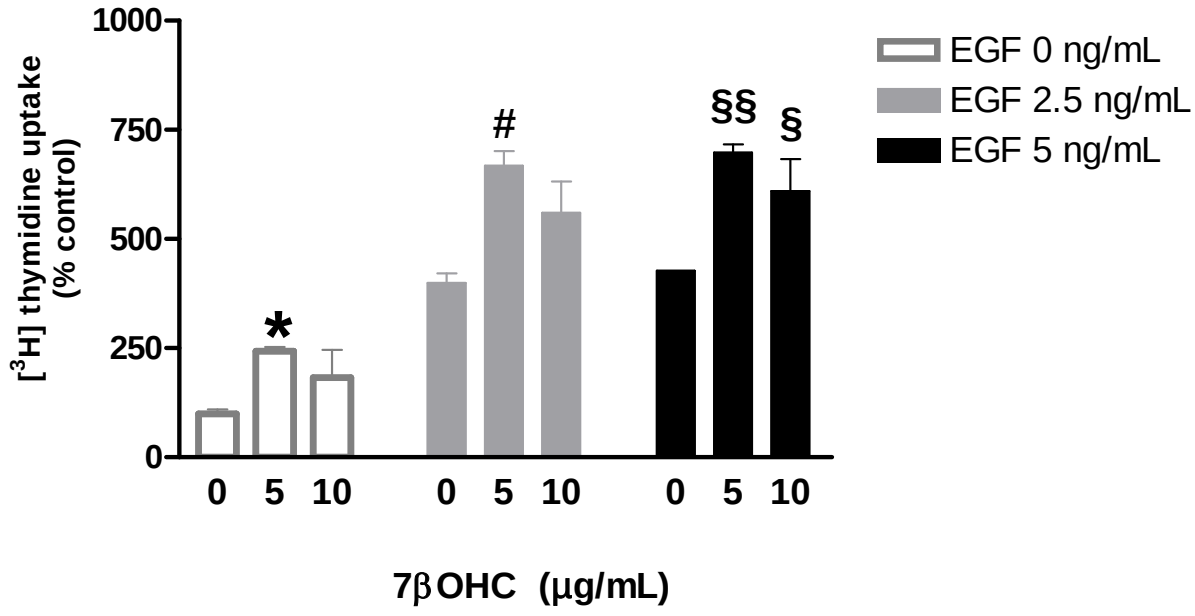


Figure 30. Effect of 7β-hydroxycholesterol (7β-OHC) on HUVEC proliferation in presence of EGF. Cells were incubated for 48 h in cell culture medium containing different concentrations of EGF with or without increasing concentrations of 7β-OHC. Cell proliferation was determined by [^{3}H] thymidine uptake. Data are expressed as mean ± S.E. of three independent experiments performed in quadruplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test **P*<0.05, # *P*<0.05, § *P*<0.05, §§ *P*<0.01 (*vs* corresponding controls without 7βOHC).

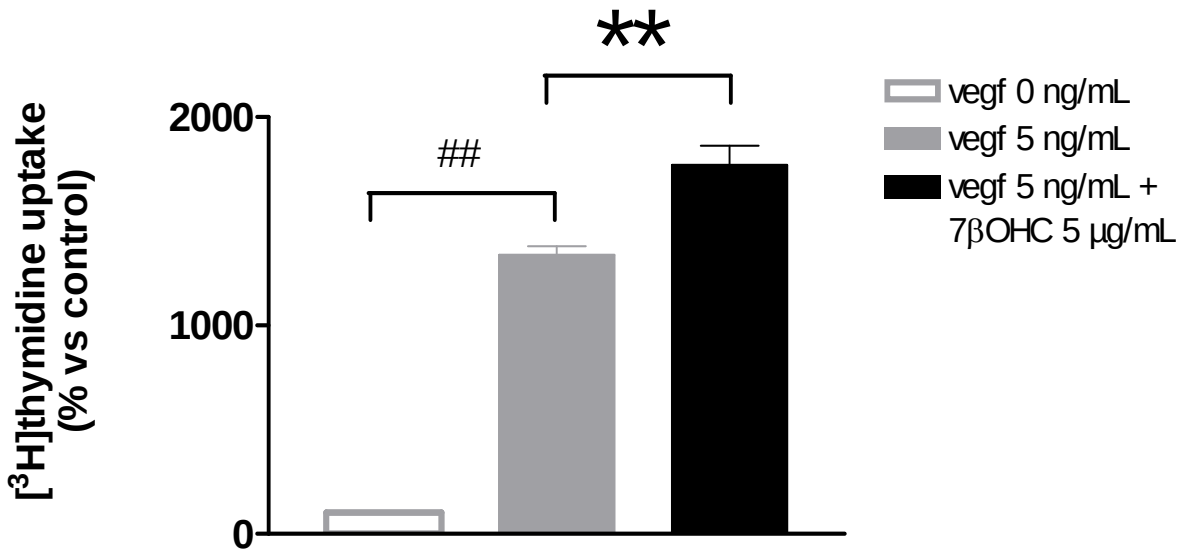


Figure 31. Effect of 7β-hydroxycholesterol (7β-OHC) on HUVEC proliferation in presence of VEGF. Cells were incubated for 48 h in cell culture medium with or without 5 ng/mL VEGF in presence or in absence of 5 µg/mL 7β-OHC. Cell proliferation was determined by [^{3}H] thymidine uptake. Data are expressed as mean ± S.E of two independent experiments performed in quadruplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test. ##P<0.01, **P<0.01.

ANTIAPOPTOTIC EFFECT OF 7ß-HYDROXYCHOLESTEROL

EFFECT OF 7β-HYDROXYCHOLESTEROL ON APOPTOSIS INDUCED BY bFGF DEPRIVATION

As mentioned above, the increase in cell viability induced by 7-oxysterols could be due to a protective effect against apoptosis induced by bFGF deprivation. Thus the possible antiapoptotic effect of 7β-hydroxycholesterol was investigated. Activation of the enzyme caspase-3 and translocation of PS were measured in HUVEC treated with 7β-hydroxycholesterol at concentrations below 20 µg/mL. As shown in figure 32, 7β-hydroxycholesterol induced a decrease in caspase-3 activity observed after 6 hours of treatment.

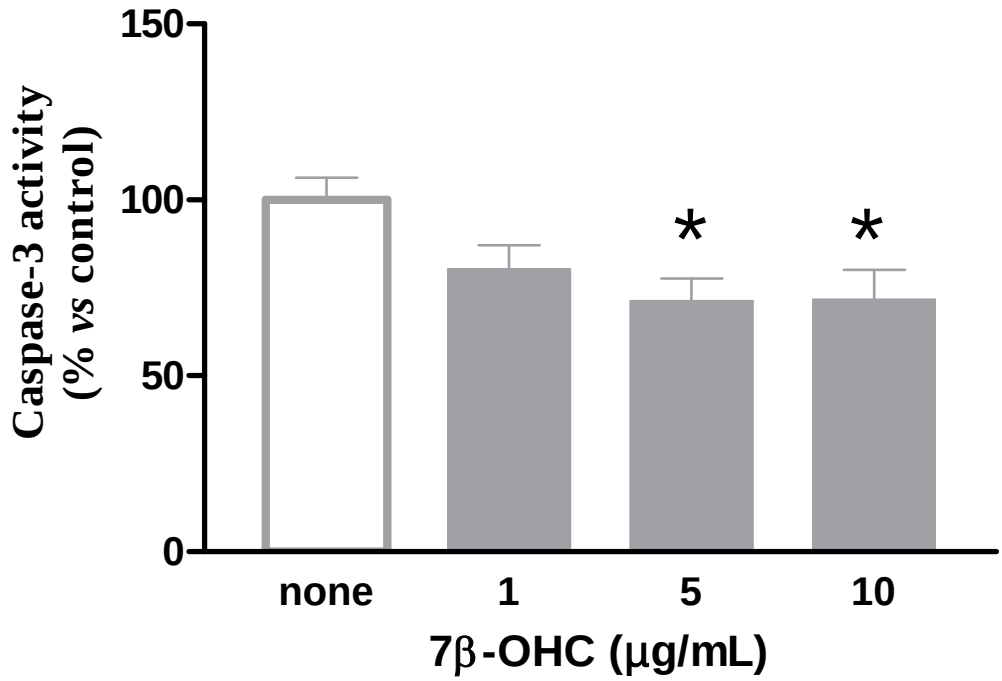
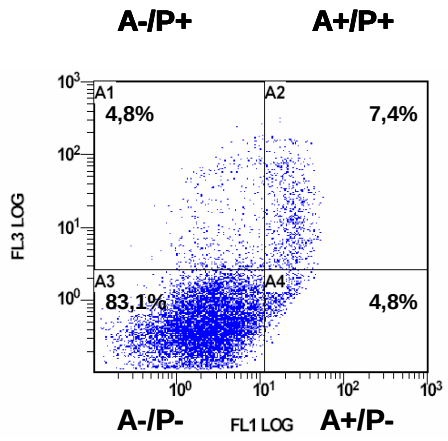


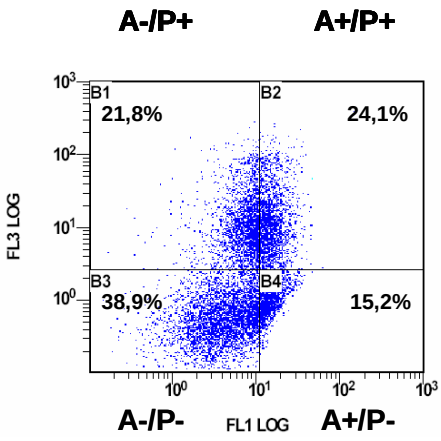
Figure 32. Effect of 7β-hydroxycholesterol (7βOHC) on caspase 3 activity in HUVEC. Cells were treated for 6 h in cell culture medium without bFGF in the presence of increasing concentrations of 7β-OHC. At the end of the treatment caspase-3 activity was measured. Data are expressed as mean ± S.E of four independent experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test. *P<0.05 (*vs* control).

The protective effect of 7β-hydroxycholesterol against apoptosis induced by bFGF deprivation was confirmed by analysis of PS translocation. HUVEC were treated for 24 hours in cell culture medium without bFGF in the presence of increasing concentrations of 7β-hydroxycholesterol, then flow cytometric analisys of annexin V was performed. As shown in figures 33 and 34 7β-hydroxycholesterol decreased the percentage of annexin-V positive cells.

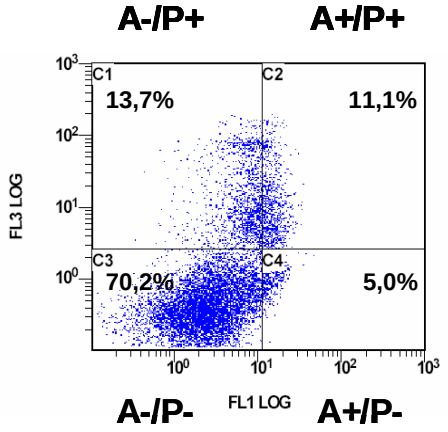
A) bFGF (5 ng/mL)



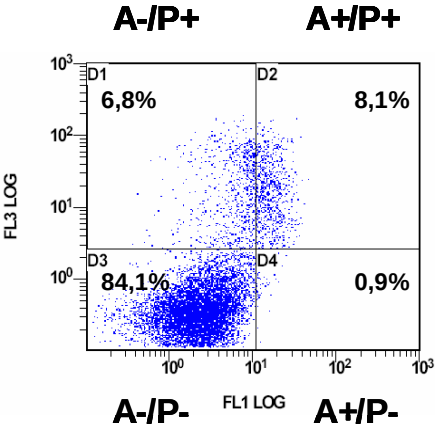
B) Control



C) 7β-OHC (1 µg/mL)



D) 7β-OHC (5 µg/mL)



E) 7β-OHC (10 µg/mL)

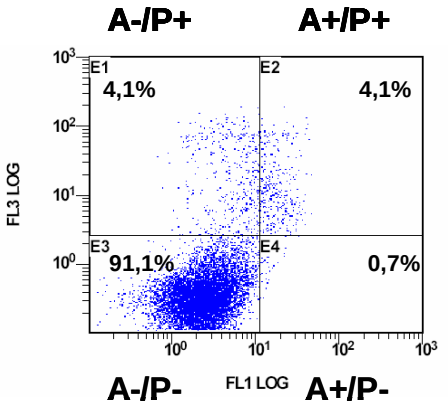


Figure 33. Representative cytograms of flow cytometric analysis of annexin-V and propidium iodide (PI) binding in HUVEC treated with 7β-hydroxycholesterol (7β-OHC). HUVEC were incubated in cell culture medium with (A) or without bFGF and exposed to 0 (B), 1 (C), 5 (D) or 10 μg/mL (E) of 7β-OHC. After 24 h treatment the cells were stained with PI and Alexa Fluor 488-conjugated annexin-V and flow cytometric analysis was performed. The cytograms represent annexin-binding (ordinate) versus PI-binding (abscissa). Viable cells (A-/P-), apoptotic cells (A+/P-), late apoptotic cells (A+/P+), and necrotic cells (A-/P+) are shown in the respective quadrants.

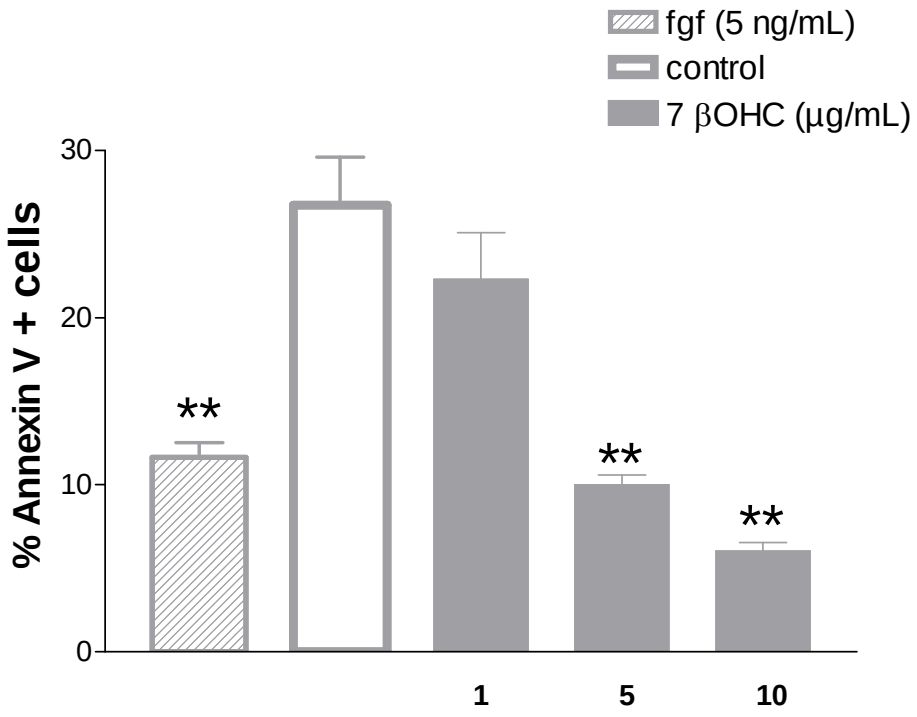


Figure 34. Effect of 7β-hydroxycholesterol (7β-OHC) on phosphatidylserine translocation in HUVEC. HUVEC were incubated for 24 h in cell culture medium with or without bFGF with increasing concentration of 7β–OHC, at the end of the treatment flow cytometric analysis of annexin V binding was performed; data are expressed as mean ± S.E of four independent experiments performed in duplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test. **P<0.01 (*vs* control).

EFFECT OF 7ß-HYDROXYCHOLESTEROL ON STAUROSPORINE-INDUCED APOPTOSIS

To further investigate the antiapoptotic effect of 7β-hydroxycholesterol other experiments were performed in the presence of an apoptotic stimuli, staurosporine. Staurosporine is a potent inducer of apoptosis in several cell lines, including HUVEC (Mosnier *et al*., 2003; Vinci *et al*., 2004).

HUVEC were pretreated for 15 hours with 10 μg/mL 7β-hydroxycholesterol, then staurosporine was added for 3 hours. Caspase-3 activity and PS translocation were determined. As shown in fig. 35, staurosporine (50 nM) induced a three fold activation of the enzyme caspase-3, that was inhibited by the pretreatment with 7β-hydroxycholesterol.

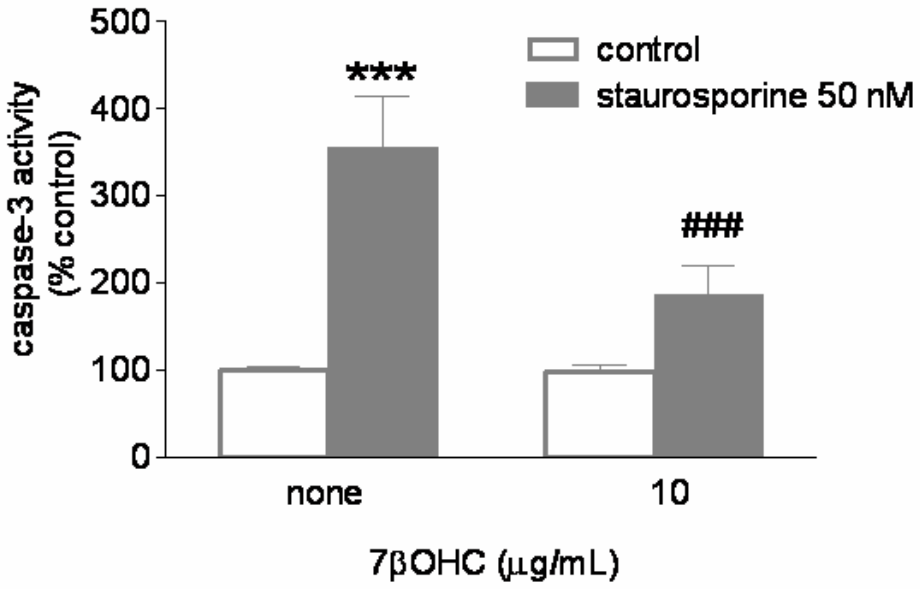


Figure 35. Effect of 7β-hydroxycholesterol (7β-OHC) on caspase-3 activation induced by staurosporine in HUVEC. Cells were pretreated for 15 h with or without 10 μg/mL of 7β-OHC in culture medium without bFGF. Then staurosporine 50 nM was added for 3 h and caspase-3 activity of cell lysates was measured. Data are expressed as mean ± S.E. of three experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA test, Bonferroni post test.****P*<0.001 (*vs* control), ###*P*<0.001 (*vs* cells exposed to staurosporine).

The protective effect of 7β-hydroxycholesterol on apoptosis induced by staurosporine, was confirmed by the analysis of annexin-V binding (fig. 36 and 37).

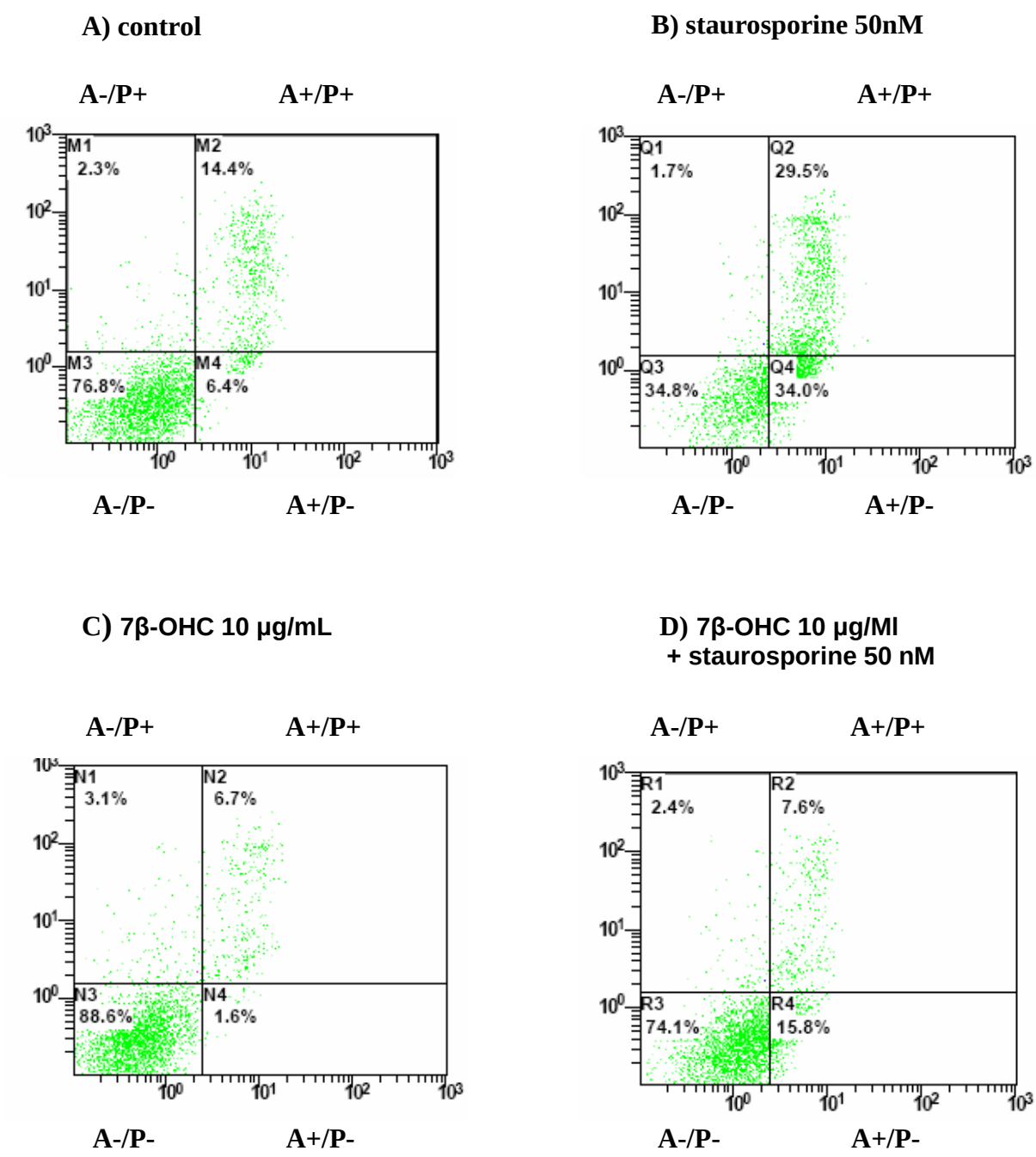


Figure 36. Representative cytograms of flow cytometric analysis of annexin-V and propidium iodide (PI) binding in HUVEC treated with 7β-hydroxycholesterol (7β-OHC). HUVEC were preincubated for 15 h in cell culture medium in absence of bFGF with (C-D) or without 10 µg/mL 7β-OHC (A-B), then staurosporine 50 nM was added (B-D) for 3 h. After the treatment HUVEC were stained with PI and Alexa Fluor 488-conjugated annexin-V and flow cytometric analysis was performed. The cytograms represent annexin-binding (ordinate) versus PI-binding (abscissa). Viable cells (A-/P-), apoptotic cells (A+/P-), late apoptotic cells (A+/P+), and necrotic cells (A-/P+) are shown in the respective quadrants.

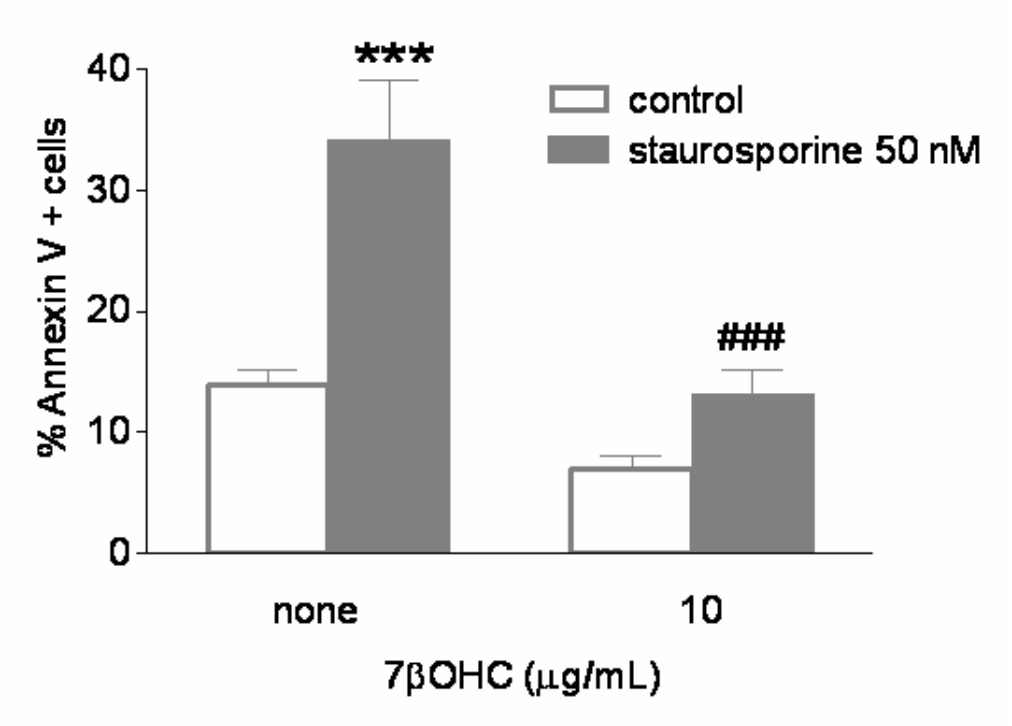


Figure 37. Effect of 7β-hydroxycholesterol (7β-OHC) on phosphatidylserine translocation induced by staurosporine in HUVEC. Cells were pretreated for 15 h with or without 10 μg/mL of 7β-OHC in culture medium without bFGF. Then staurosporine 50 nM was added for 3h. At the end of the treatment flow cytometric analysis of annexin V binding was performed; data are expressed as mean ± S.E of three independent experiments performed in duplicate. Statistical analysis was performed by one-way ANOVA test, Bonferroni post test. ***P<0.001 (*vs* control).###*P*<0.001 (*vs* cells exposed staurosporine).

SPECIFICITY OF THE EFFECT FOR 7-OXYSTEROLS

In order to define the molecular features of the antapoptotic action of 7β-hydroxycholesterol, 7β-hydroxycholesterol and cholesterol (at concentrations below 20 μg/mL) were compared. HUVEC were treated for 24 hours in cell culture medium in absence of bFGF with increasing concentrations of 7β-hydroxycholesterol or cholesterol. As shown in figures 38 and 39, cholesterol did not protect cells from apoptosis induced by bFGF deprivation.

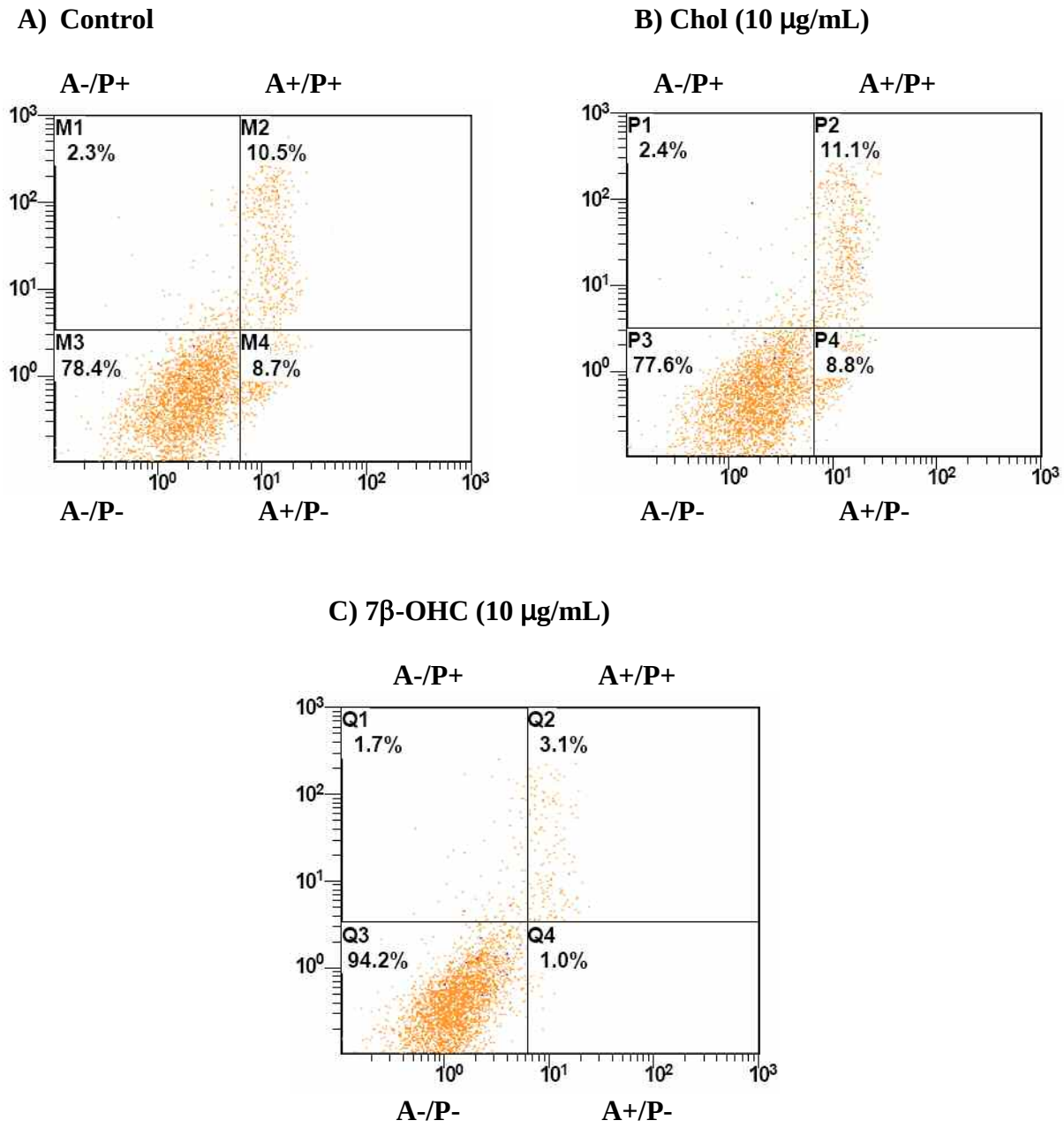


Figure 38. Representative cytograms of flow cytometric analysis of Annexin-V and propidium iodide (PI) binding in HUVEC treated with 7β-hydroxycholesterol (7β-OHC) or cholesterol (chol). HUVEC were incubated for 24 hours in cell culture medium in absence of bFGF with 10 µg/mL chol (B) or with 10 µg/mL 7β-OHC (C). Control cells were treated in culture medium without bFGF (A). After the treatment HUVEC were stained with PI and Alexa Fluor 488-conjugated annexin-V, then flow cytometric analysis was performed. The cytograms represent annexin-binding (ordinate) versus PI-binding (abscissa). Viable cells (A-/P-), apoptotic cells (A+/P-), late apoptotic cells (A+/P+), and necrotic cells (A-/P+) are shown in the respective quadrants.

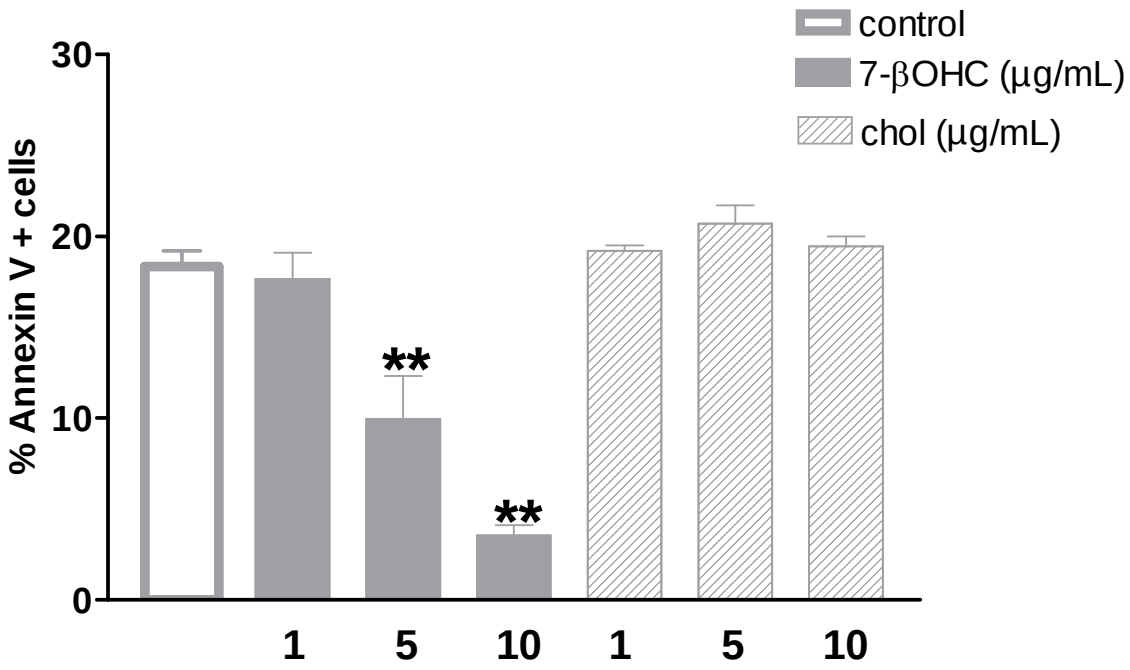


Figure 39. Effect of 7β-hydroxycholesterol (7β-OHC) and cholesterol (chol) on apoptosis induced by bFGF deprivation in HUVEC. HUVEC were incubated for 24 h in cell culture medium without bFGF with increasing concentrations of 7β-OHC or chol. At the end of the treatment flow cytometric analysis of annexin V binding was performed. Data are expressed as mean ± S.E of two independent experiments performed in duplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test. **P<0.01 (*vs* control).

The action of 7-ketocholesterol and 7β-hydroxycholesterol was also compared to another oxysterol commonly present in the atherosclerotic plaque, 25-hydroxycholesterol.

HUVEC were treated for 24 hours without bFGF in presence of 10 µg/mL 7-ketocholesterol, 7β-hydroxycholesterol or 25-hydroxycholesterol, then cell viability was measured through MTT reduction assay. As expected, 7-ketocholesterol and 7β-hydroxycholesterol induced an increase in HUVEC viability whereas the treatment with 25-hydroxycholesterol did not affect cell viability.

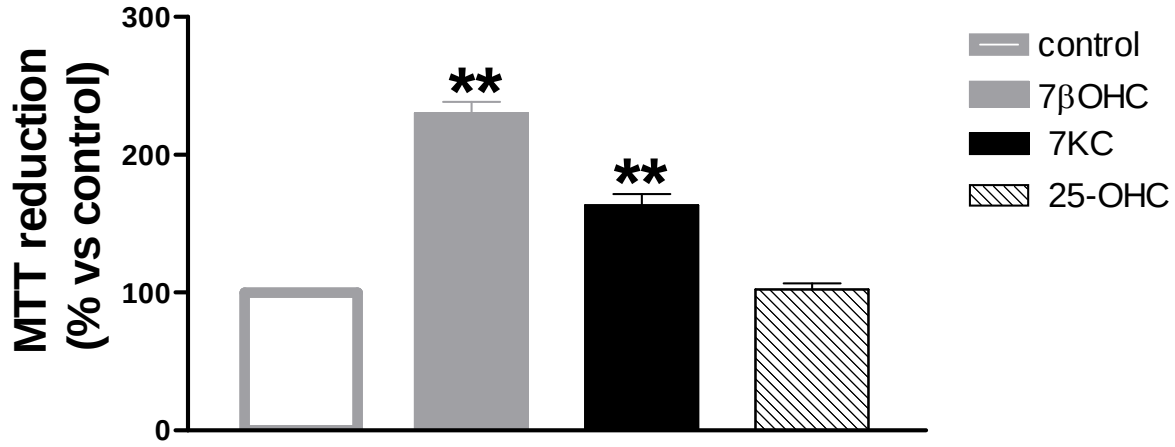


Figure 40. Effect of 7-ketocholesterol, 7β-hydroxycholesterol and 25-hydroxycholesterol on MTT reduction in HUVEC. HUVEC were treated in cell culture medium without bFGF with 10 µg/mL 7-ketocholesterol, 7β-hydroxycholesterol or 25-hydroxycholesterol for 24 h. Cell viability were determined through MTT reduction assay. Data are expressed as mean ± S.E of two independent experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test.**P<0.01 (*vs* control).

ROLE OF JNK IN THE EFFECTS INDUCED BY 7β-HYDROXYCHOLESTEROL

MAPKs play an important role in cell proliferation and survival: the balance between activated ERK and stress-activated JNK-p38 pathways may be important in determining whether a cell survives or undergoes apoptosis. It is not clear if oxysterols induce the activation of the JNK pathway but a recent study showed that 7-ketocholesterol activates the JNK signalling pathway in inducing apoptosis (Pedruzzi *et al*., 2004). Other studies demonstrated that ERK1 and ERK2, but not JNK, are involved in the effects induced by 7β-hydroxycholesterol and 22-hydroxycholesterol (Ares *et al*., 2000; Yoon *et al*., 2004). Thus, to study if the dual effect induced by 7-oxysterols was due to the activation of the JNK pathway, a specific JNK inhibitor, SP600125, was used.

HUVEC were treated for 24 hours with 7β-hydroxycholesterol in the presence of increasing concentrations of SP600125, then MTT reduction was measured. As shown in figure 41, SP600125 did not inhibit the increase in cell viability induced by 7β-hydroxycholesterol.

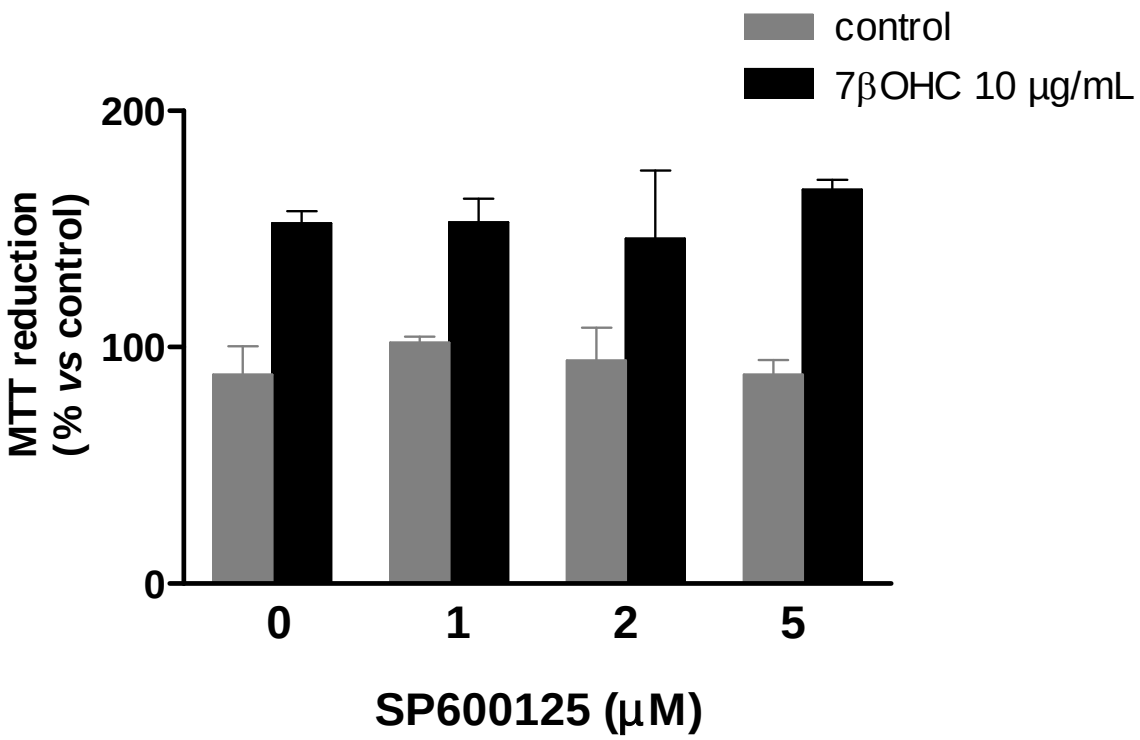


Figure 41. Effect of 7β-hydroxycholesterol (7βOHC) on HUVEC viability. Cells were incubated for 24 h in cell culture medium without bFGF with or without 7β-OHC containing increasing concentrations of SP600125. Cell viability was determined by MTT reduction assay. Data are expressed as means ± S.E. of two independent experiments performed in quadruplicate.

ROLE OF ERK IN THE EFFECTS INDUCED BY 7β-HYDROXYCHOLESTEROL

As mentioned above, ERKs are implicated in proliferation as well as in the survival pathways, for this reason the role of ERKs in the effects induced by 7β-hydroxycholesterol was investigated.

After a bFGF deprivation for 4 hours, HUVEC were incubated with 10 μg/mL 7β-hydroxycholesterol for different periods of time and analysis of ERK phosphorylation was performed. As shown in figure 42, 7β-hydroxycholesterol did not cause an increase in ERK phosphorylation. ERK activation was not observed also after longer treatments (5 and 24 hours).

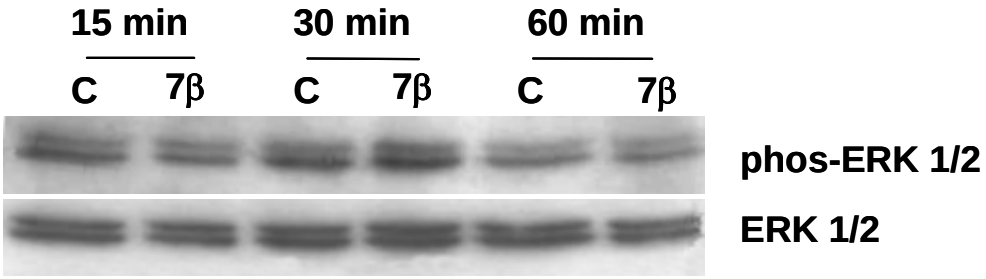


Figure 42.Representative experiment of the effect of 7β-hydroxycholesterol on ERKs phosphorylation in HUVEC. HUVEC were deprived of bFGF for 4 h, then they were incubated for the indicated times in the absence or in the presence of 10 μg/mL. Cell lysates (20 μg) were processed for western blotting and the membranes were exposed to the primary antibody specific for human phos-ERK1 and phos-ERK2 or against overall ERK1 and ERK2.

In spite of the lack of ERK phosphorylation induced by 7β-hydroxycholesterol , pretreatment of HUVEC with a selective MEK inhibitor (PD98059 or UO126) was able to antagonize the protective effect of the oxysterol against apoptosis induced by staurosporine treatment (fig.43).

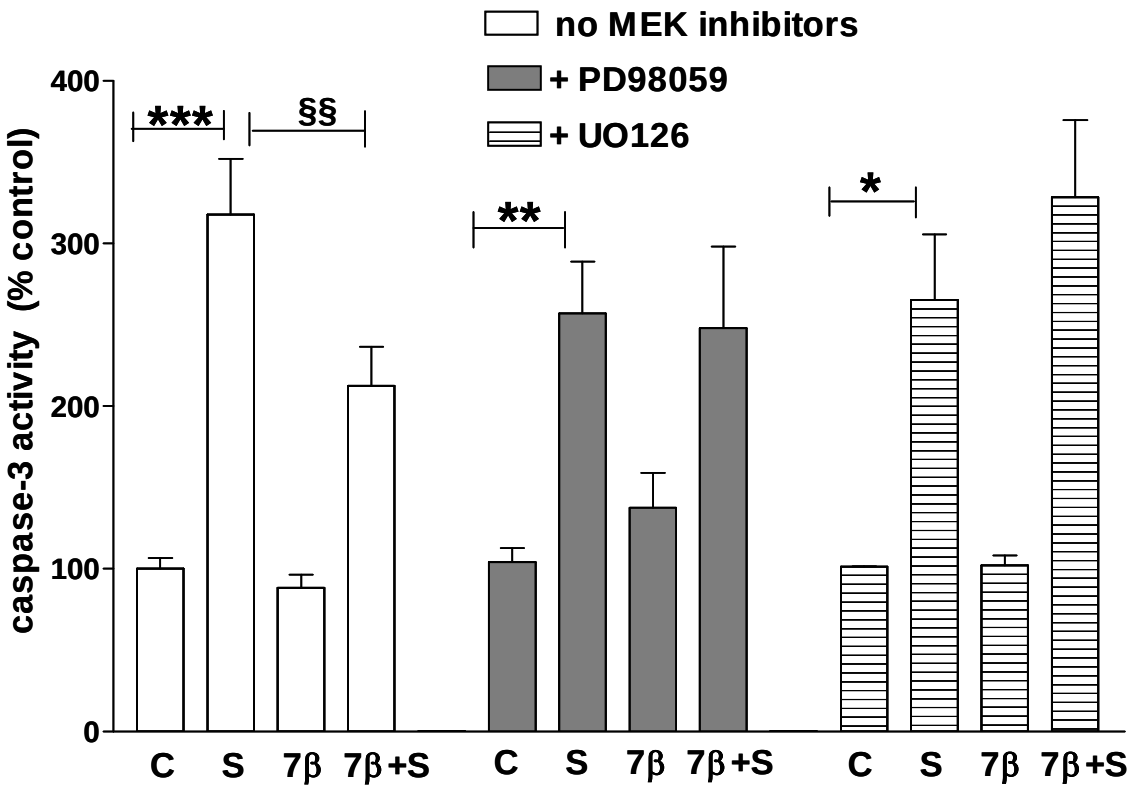


Figure 43. Effect of MEK inhibitors on the antiapoptotic effect of 7β-hydroxycholesterol (7β-OHC). HUVEC were pretreated for 30 min with or without 25 μM PD98059 or 25 μM UO126, then the cells were incubated for 2h with or without 10 μg/mL of 7β-OHC in culture medium without bFGF. Afterwards staurosporine 50 nM was added for 3h and caspase-3 activity of cell lysates was measured. Data are expressed as mean ± S.E. of three experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA test, Bonferroni's post test.***P<0.001, **P<0.01, *P<0.05, §§P<0.01.

The involvement of MEK activation in the effect of 7β-hydroxycholesterol was also observed when apoptosis was induced by bFGF deprivation. In fact, treatment of HUVEC with the MEK inhibitor PD98059 blocked the increase in cell viability induced by 7β-hydroxycholesterol (fig. 44).

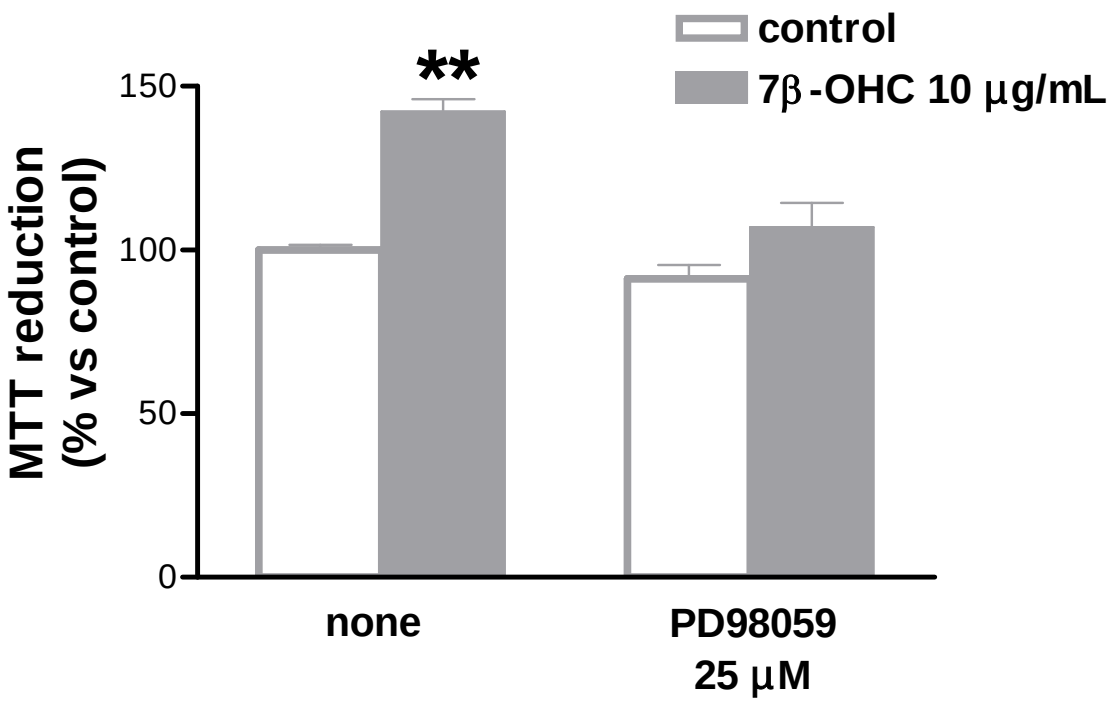


Figure 44. Effect of PD98059 on the increase in MTT reduction induced by 7β-hydroxycholesterol in HUVEC. HUVEC were treated in cell culture medium without bFGF with or without 25 μM PD98059 for 30 min. Then the cells were incubated with or without 10 μg/mL 7β-hydroxycholesterol for 24 h. Cell viability were determined through MTT reduction assay. Data are expressed as mean ± S.E of four independent experiments performed in sixtuplicate. Statistical analysis was performed by one-way ANOVA test, Bonferroni's post test. **P<0.01 (*vs* control).

The role of MEK on the proliferative effect of 7β-hydroxycholesterol was also studied. As shown in figure 45, UO196 was able to block completely both 7β-hydroxycholesterol and bFGF induced proliferation. The MEK inhibitor also blocked basal [^{3}H] thymidine uptake without affecting cell viability, as assessed by microscope observation.

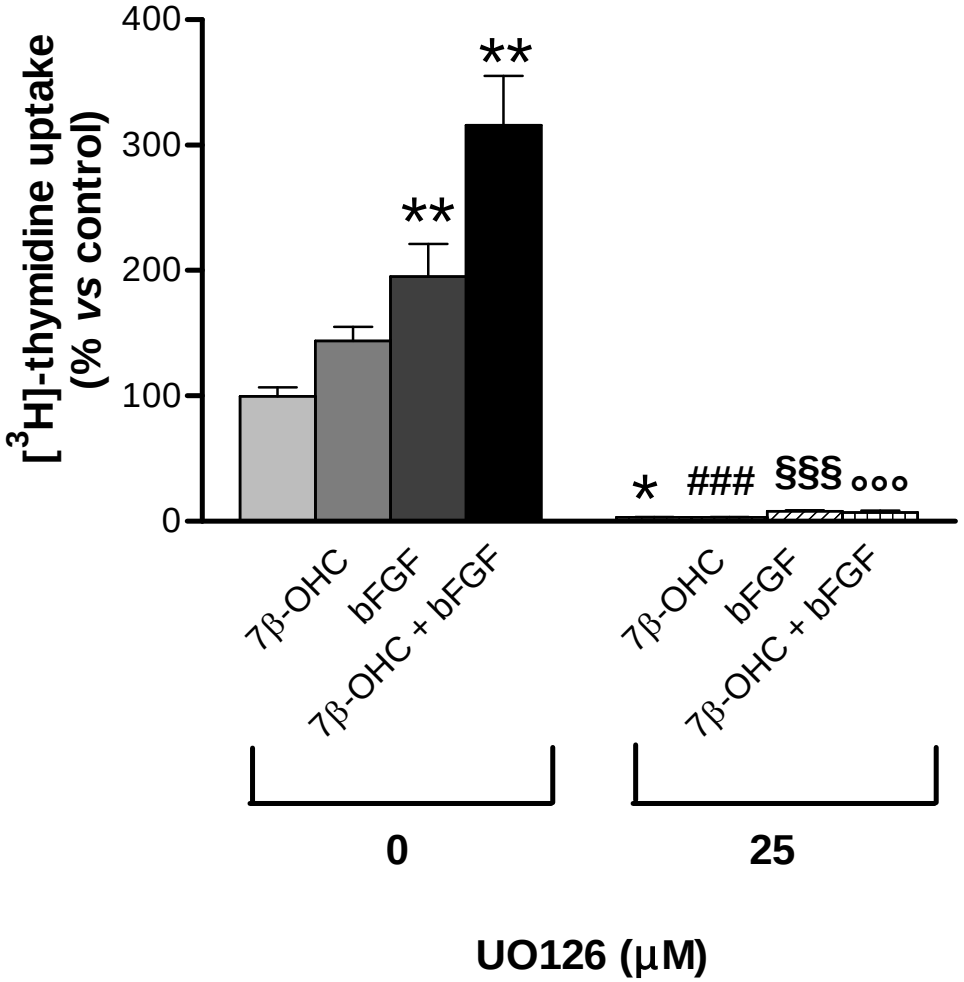


Figure 45. Effect of UO126 on [^{3}H]-thymidine incorporation in HUVEC. HUVEC were treated in cell culture medium with UO126 (25 μM) for 30 min. Cells were incubated for 30 h in cell culture medium in the presence or not of bFGF (5 ng/mL) with or without 7βOHC (10 μg/mL). Cell proliferation was determined by [^{3}H] thymidine uptake. Data are expressed as mean ± S.E. of two independent experiment performed in quadruplicate. Statistical analysis was performed by one-way ANOVA test, Dunnet's post test.*P<0.05 **P<0.01 (*vs* control), ### P<0.001, §§§ P<0.001, °°°P<0.001 (*vs* control)

DISCUSSION

The main result of this thesis is the observation that 7-ketocholesterol and 7β-hydroxycholesterol induce a dual effect on cell viability in HUVEC: treatment of the cells with 7-ketocholesterol or 7β-hydroxycholesterol at the concentration of 20 μg/mL causes cell death, while treatment with oxysterols at concentrations between 1 and 10 μg/mL induces an increase in cell viability. To our knowledge, a similar effect was never observed for oxysterols, components of ox-LDL, which are considered to be responsible for the genesis and development of atherosclerosis (Berthier *et al*. 2005).

Interestingly, a dual effect of ox-LDLs on ECs has been recently demonstrated. In fact a proapoptotic effect is observed at concentrations above 50 μg/mL and a proliferative effect is observed at lower concentrations (Galle *et al*., 2001; Seibold *et al*., 2004).

Concerning the cytotoxic effect of the oxysterols studied, our data suggest that these compounds activate different pathways leading to various modes of cell death. In fact 7-ketocholesterol provokes cell apoptosis by caspase-3 activation, while it is not clear if 7β-hydroxycholesterol induces cell death through necrosis or through an apoptosis independent on caspase-3. In fact, even if apoptosis centres on the activation of caspases, mounting evidence indicates that a cell that has been treated with an apoptotic inducer can also initiate a suicide programme that does not rely on caspase activation. This kind of cell death is called caspase-independent cell death (CIDC) and one of the hallmarks is the presence of annexin-V negative (occasionally positive) cells (Chipuk and Green, 2005).

Thus, even if it remains unclear how 7β-hydroxycholesterol causes cell damage, our data confirm that both 7-ketocholesterol and 7β-hydroxycholesterol at concentrations ≥ 20 μg/mL induce cell death, as already demonstrated by Lizard and co-workers. Studies from this group, in fact, showed that both oxysterols enhance cell detachment, reduce cellular viability and induce both apoptosis and necrosis on several cell types of the vascular wall (Lizard *et al.*, 1996; Lizard *et al.*, 1999).

Regarding the increase in cell viability observed at concentrations below 20 μg/mL, it is important to underline that cells are treated in cell culture medium without bFGF, a condition that is known to induce cell apoptosis in HUVEC (Vinci *et al.*, 2004; Trevisi *et al.*, 2004). Therefore, the increase in cell viability observed between 24 and 96 hours of treatment with 7-ketocholesterol and 7β-hydroxycholesterol at concentrations ranging between 1-10 μg/mL could be due to an augmented cell proliferation or to an antiapoptotic effect. To investigate

these possibilities, 7β-hydroxycholesterol was taken as a model for its ability to induce a stronger increase in cell viability.

7β-hydroxycholesterol stimulates HUVEC proliferation with a lower potency respect to all the GFs tested (bFGF, EGF and VEGF). Furthermore, when added together, 7β-hydroxycholesterol and GFs have an additive effect.

Besides the proliferative effect, 7β-hydroxycholesterol protects HUVEC from apoptosis induced by bFGF deprivation as demonstrated by the reduction in the number of apoptotic cells and the decrease in caspase-3 activation. A protection against bFGF deprivation is not observed when the cells are treated with similar concentrations of cholesterol.

This result, supported by the data obtained measuring cell viability in the presence of 7 ketocholesterol and 25-hydroxycholesterol, suggests that the effect possessed by 7-oxysterols is correlated to the oxygenated moiety in C-7 position. This oxidation could allow the binding of oxysterols to a target receptor, or simply affect the physical-chemical properties of the plasma membrane producing a deep modification in the cell signalling.

Pretreatment with 7β-hydroxycholesterol protects HUVEC also from apoptosis induced by staurosporine, a broad spectrum inhibitor of protein kinases (Feng 2002). When staurosporine and 7β-hydroxycholesterol are added simultaneously, cell death occurs inevitably suggesting that a pretreatment with the oxysterol is necessary to the prevention of the apoptotic process.

Other studies have demonstrated the protective effect of low concentrations of oxysterols against cytotoxic stimuli. In fact, a recent study has shown that pretreatment of PC12 cells (a cell line derived from a pheochromocytoma of the rat adrenal medulla) with sublethal concentrations of 7β-OHC protectes cells against the oxidative stress induced by 6-hydroxydopamine (6-OHDA) (Chen *et al*., 2006). Another study demonstrates that low concentrations of 20-α-hydroxycholesterol and 25-hydroxycholesterol (0.25-0.5 μg/mL), markedly increase the resistance of L-cell cultures (mouse fibroblasts) to the lethal effects of a cytolytic toxin, streptolysin, in function of hydroxycholesterol concentration, incubation time, and density of the cell cultures (Duncan and Buckingam, 1978).

Taking into account the similarity of the effects caused by lower concentrations of 7β-hydroxycholesterol with those induced by GFs, some of the pathways involved in the action of these factors were investigated. MAPKs play an important role in cell proliferation and survival: the balance between activated ERK and stress-activated JNK-p38 pathways is important to determine whether a cell survives or undergoes apoptosis. A recent study

showed that 7-ketocholesterol activates the JNK signalling pathway in inducing apoptosis (Pedruzzi *et al*., 2004). Other studies demonstrated that ERK1 and ERK2, but not JNK, are involved in the effects induced by 7β-hydroxycholesterol and 22-hydroxycholesterol (Ares *et al*., 2000; Yoon *et al*., 2004). Our results show that JNK are not activated under 7β-hydroxycholesterol treatment of HUVEC.

Another pathway, the Ras/Raf/MEK/Erk pathway plays a critical role in cell survival. Indeed, Ras-activated Raf operates by phosphorylating and activating MEK 1/2. MEKs have very narrow substrate specificity, restricted to ERKs. Phosphorylation of these kinases by MEKs results in phosphorylation of further downstream targets (Katz *et al*., 2007). To investigate the exact role of ERKs in the antiapoptotic effect of 7β-hydroxycholesterol, we used two specific MEK inhibitors: U0126 and PD98059. Our data show that although 7β-hydroxycholesterol does not activate ERKs, MEK activity is essential for the protective and proliferative effects of 7β-hydroxycholesterol.

In the last years some intracellular target of oxysterols have been identified. In fact oxysterols have been shown to bind and activate the nuclear LXR receptors (Michael, 2005). LXRs directly regulate multiple genes involved in cholesterol metabolism and transport, and genes like ATP-binding cassette transporter A1 (ABC-A1), which is involved in hepatic cholesterol efflux. Moreover LXRs activate SREBP-1c transcription leading to the activation of lipogenesis (Bobard *et al.*, 2005). Recently, evidence is accumulating that SREBPs are not only involved in cholesterol-regulated events but are also gene regulatory targets of intracellular signalling pathways like Akt/PI3K and MAPK cascades. In fact SREBP-1 has been demonstrated to be upregulated by insulin growth factor through activation of Akt (Smith *et al.*, 2007). Moreover it has been shown that overexpression of members of the MAPK cascade (MEK1, MEKK-1) leads to SREBP-related stimulation of LDL receptor promoter activity. *In vitro* studies have demonstrated that SREBP-1a is a direct substrate of ERK1 and ERK2 being phosphorylated at serine 117, a site involved in SREBP-1a-mediated induction of the LDL receptor gene by insulin and PDGF (Roth *et al.*, 2000).

In conclusion, although the mechanism of the antiapoptotic and proliferative actions of oxysterols requires further investigations, this work show new evidence that 7β-hydroxycholesterol, and probably other 7-oxysterols, are not only products of oxidation of cholesterol involved in its metabolism, but they might also play an important role in the intracellular signalling regulating cell survival and proliferation.

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ABBREVIATIONS

7KC	7-ketocholesterol
7β-OHC	7β-hydroxycholesterol
ABCA1	ATP-binding cassette transporter A1
AIF	apoptosis inducing factor
Akt	protein chinase B
ApoE	apolipoprotein E
ATP	adenosine triphosphate
AV	annexin V
BCL-2	B-cell CLL/lymphoma 2
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CAD	coronary artery disease
CD	cluster of differentiation
COX	cyclooxigenase
DAG	diacylglycerol
DISC	death-inducing signal complex
DMSO	dimethylsulfoxide
DOC	deoxycholate
DTT	dithriotheritol
EC	endothelial cell
ED	endothelial dysfunction
EDFR	Endothelium-Derived Relaxing Factor
EDHF	Endothelium-Derived Hyperpolarizing Factor
EDTA	Ethylene-diamine-tetraacetic acid
EGF	epidermal growth factor
eNOs	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
ET	endothelin
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	fetal bovine serum
Gbr2	growth-factor-receptor-bound protein 2
GF	growth factor
GSK	glycogen synthase kinase
H_4B	tetrahydrobiopterin
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
HUVEC	human umbilical vein endothelial cell
IAP	inhibitor of apoptosis
ICAD	inhibitor of caspase-activated deoxyribonuclease
ICAM-1	intercellular adhesion molecule-1
IL-1	interleukin-1
IL-x1b	interleukin-x1b

iNOs	inducible nitric oxide synthase
IP_3	inositol-1,4,5-trisphosphate
JNK	c-Jun amino-terminal kinase
KLF_2	Kruppel-like factor 2
LDL	low density lipoprotein
LPL	lipoprotein lipase
LXR	liver X receptor
M199	medium 199
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MCP-1	monocyte chemoattractant protein-1
M-CSF	monocyte colony stimulating factor
MEM	minimum essential medium eagle
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	nuclear factor κB
nNOs	neuronal nitric oxide synthase
NO	nitric oxide
Ox-LDL	oxidized low density lipoprotein
PAF	platelet-activating factor
PAI-1	plasminogen activator inhibitor type 1
PARP	poly(ADP)ribose polymerase
PBS	phosphate-buffered saline solution
PDGF	platelet-derived growth factor
PGI_2	prostacyclin
PI	propidium iodide
PI3K	phosphatidylinositol 3 kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PIPES	2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid
PLCγ	phospholipase Cγ
PMSF	phenylmethanesulphonylfluoride
PS	phosphatidylserine
PTEN	PtdIns(3,4,5)P_3 3-phosphatase
PUFA	polyunsaturated fatty acid
PVDF	Polyvinylidene Difluoride
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SAPK	stress activated protein kinase
SDS	sodium dodecylsulfate
SMC	smooth muscle cell
SR	scavenger receptor

SRE	serum response element
SREBP	sterol regulatory element binding protein
STAT	Signal Transducers and Activator of Transcription
TCA	trichloroacetic acid
TNF-α	tumor necrosis factor-a
t-PA	tissue-type plasminogen activator
VCAM-1	vascular adhesion molecule-1
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor