SURVIVAL SIGNALS INDUCED BY CHOLESTEROL OXIDATION BY-PRODUCTS IN ATHEROSCLEROSIS

by

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SURVIVAL SIGNALS INDUCED BY CHOLESTEROL OXIDATION BY-PRODUCTS IN ATHEROSCLEROSIS

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ABSTRACT

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Biological Sciences and Bioengineering, Ph.D. Thesis, 2015

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Keywords: Oxysterols, 27-Hydroxycholesterol, Survival signaling, ROS, Nrf2

Atherosclerosis is the most prevalent cause of morbidity and mortality developed countries. Oxysterols are a family of 27-carbon molecules originated from cholesterol oxidation and the atherogenic potential of oxysterols is linked to their ability to induce apoptosis, vascular smooth muscle cell proliferation and monocyte migration. Apparently, these compounds are able to modulate not only pro-apoptotic but also anti-apoptotic signals in targeted cells; however, their anti-apoptotic effect has not been investigated in depth. Hence, we aimed to elucidate the molecular mechanisms underlying the survival signaling elicited by 27-hydroxycholesterol (27-OH) which is the most represented oxysterol in human blood. Using human promonocytic cells (U937) challenged with a relatively low (10 μM) concentration of 27-OH, a marked while transient increase of intracellular ROS level that enhanced both MEK-ERK and PI3K-Akt phosphorylation was observed between 6 and 24 hours, paralleled by Bad phosphorylation, resulting to be a crucial event in delaying apoptotic death. In turn, the knock down of ERK and Akt by means of selective inhibitors, increased ROS production at 12 h showing that ERK/Akt axis was responsible of a sustained quenching of ROS production. Involvement of antioxidant Nrf2 and its target genes, HO-1 and NQO-1 in this early survival response were shown. It thus appears that Nrf2 is responsible for the quenching of the oxidative imbalance exerted in 27-OH challenged cells that analyzed by confocal microscopy. The data obtained highlight oxysterols' ability to promote cell survival that might contribute to the pathogenesis of inflammation-driven chronic diseases such as atherosclerosis.

ÖZET

KOLESTEROL OKSİDASYON ÜRÜNLERİNİN ATEROSKLEROZDA İNDÜKLEDİĞİ SAĞKALIM SİNYALLERİ

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Anahtar kelimeler: Oksisteroller, 27-Hidroksikolesterol, Sağkalım sinyalleri, ROS, Nrf2

Dünyada ve özellikle gelişmiş ülkelerde damar sertliği (ateroskleroz) ölüm ve sakat kalmanın en yaygın sebebidir. 27-karbon ihtiva eden oksisteroller, kolesterolün oksidize türevleridir ve oksisterollerin aterojenik potansiyelleri; tetikledikleri apoptoz, vasküler düz kas hücre proliferasyonu ve monosit göçü ile ilgilidir. Elde edilen ön bulgulara göre, oksisteroller apoptotik sinyallerin yanında aynı zamanda anti-apoptotik sinyalleri de düzenlerler; ancak konuyla ilgili yeterince detaylı araştırma bulunmamaktadır. Bu nedenle, araştırmamızda, insan kanında en çok rastlanan oksisterol olan 27-hidroksikolesterol (27-OH), tarafından tetiklenen sağkalım mekanizmasının moleküler seviyede açıklanması hedeflenmiştir. Düşük dozda (10 μM) 27-OH uygulanan U937 monosit hücrelerinde , konfokal mikroskopu ile hücreiçi ROS seviyesinde gözlenen hızlı ancak geçici yükselme hem MEK-ERK hem de PI3K-Akt fosforilasyonunu 6. ve 24. saatler arasında artırmıştır, buna paralel olarak apoptotik ölümü ertelemede önemli rolü olan Bad fosforilasyonu tespit edilmiştir. Buna karşılık, Erk ve Akt ye özgü susturucuların uygulanması ROS üretiminin artışına neden olmuş ve ERK/Akt ekseninin süregelen ROS üretiminin baskılanmasından sorumlu olduğunu göstermiştir. Antioksidan Nrf2 defansının ve hedef genleri olan HO-1 ve NQO-1 nin bahsi geçen erken saatlerdeki sağkalım sinyalinde yeraldığı gösterilmiştir. Elde edilen bulgularda, Nrf2 nin konfokal mikroskopu ile analiz edilen 27-OH tarafından indüklenen oksidatif dengesizliği gidermekle sorumlu olduğu gösterilmiştir. Elde edilen sonuçlar, oksisterollerin düşük dozda hücre sağkalımını desteklemekte olduğunu ve ateroskleroz gibi enflamasyon güdümlü kronik hastalıkların patojenezine etki ettiğini vurgulamaktadır.

To my esteemed parents,

To my beloved husband...

"It always seems impossible until it's done. "

Nelson Mandela

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α	Alpha
β	Beta
μ	Micro
25-OH	25-hydroxycolesterol
27-OH	27-hydroxycholesterol
7K	7-ketocholesterol
7β-OH	7β-hydroxycholesterol
Akt	Protein Kinase B
ARE	Antioxidant response element
Bad	Bcl-2-associated death promoter protein
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2 protein
Bcl-xl	B-cell lymphoma extra-large protein
BH domain	Bcl-2 homology domain
Bim	Bcl-2 like protein 11
BSA	Bovine serum albumin
cDNA	complementary DNA
CYP27A1	27-hydroxylase
DAPI	4, 6-diamidino-2-phenylindole
DAPk	Death-associated protein kinase
DPI	Diphenyleneiodonium chloride
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
Erα	Estrogen receptor α
FACS	Fluorescence-activated cell sorting
FBS	Foetal Bovine Serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GPx	Glutathione peroxidase
GSR	Glutathione reductase
H_2O	Water
HO-1	Heme oxygenase-1
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
JC-1	5,50,6,60-Tetrachloro1,10,3,30-tetraethylbenzimidazolylcarbocyanine iodide
JNK	c-Jun NH_2-terminal kinase
K^+	Potassium ion

KCl	Potassium chloride
kDa	Kilo dalton
Keap1	Kelch-like ECH-associated proein 1
LDL	Low density lipoprotein
LOX-1	Lectin-like oxLDL scavenger receptor-1
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MEK	Mitogen-activated protein kinase ERK kinase
min	Minute
Na^+	Sodium ion
NaCl	Sodium chloride
NAC	N-acetylcysteine
NOX-2	NADPH oxidase type 2
NQO-1	NAD(P)H:quionone oxireductase
NRF2	Nuclear factor erythroid 2 p45- related factor 2
p38	Mitogen-activated protein kinase p38
PAGE	SDS-polyacrylamide gel electrophoresis
PBS	Phosphatase Buffered Saline
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein Kinase A
PKC	Protein kinase C
PM	Plasma membrane
PMA	Phorbol myristate acetate
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tryosine kinase;
RT-PCR	Reverse transcription–polymerase chain reaction
SERM	Selective estrogen modulator
VCAM-1	Vascular cell adhesion molecule-1;
VEFG	Vascular endothelial growth factor

1. INTRODUCTION

1.1 Oxysterols

1.1.1 Chemical structures and origins of oxysterols

Cholesterol is undoubtedly a molecule of key biological importance, being the structural core of estrogens and androgens, starting the synthesis of vitamin D and biliary acids and playing a primary role in stabilization and function of membrane lipid rafts, but its popularity" is biased by the fact that hypercholesterolemia actually represents a main risk factor of cardiovascular disease, neurodegeneration, inflammatory bowel disease and cancer.

Going a bit deeper in evaluating the pathophysiological impact of cholesterol, it appears clear that this powerful molecule exert a number of effects not simply *per se* but for a significant percentage through the biochemical properties exerted by its metabolites. Among the latter ones, an increasing attention is drawn by the family of cholesterol oxidation products termed oxysterols, 27-carbon molecules that, with respect to cholesterol, show an epoxide or ketone or an additional hydroxyl group in the sterol nucleus and/or a hydroxyl group in the side chain. Within this family of compounds there are components that are from 10 to 100 more chemically reactive than unoxidized cholesterol, thus suggesting their involvement in many of the biochemical and biological effects ascribed to cholesterol (Leonarduzzi et al., 2002; Schroepfer, 2000).

In oxysterols physico-chemical features studies, it has been clearly established that they contain a second oxygen atom as a carbonyl, hydroxyl or epoxide group and fit perfectly into the lipid bilayer of biological membranes. In Figure 1.1., chemical structures of principal oxysterols of biological interest are reported. Various oxysterols have been found in appreciable quantities in human tissues and biological fluids, including human plasma. Due to their elevated levels have been elected in foam cells and atherogenic lipoproteins it is suggested that oxysterols play active role in atherosclerotic plaque formation (Carpenter et al., 1995; Hodis et al., 1991). Moreover, oxidized cholesterol derivatives have been shown to have higher atherogenic potential than native cholesterol (Kumar and Singhal, 1991).

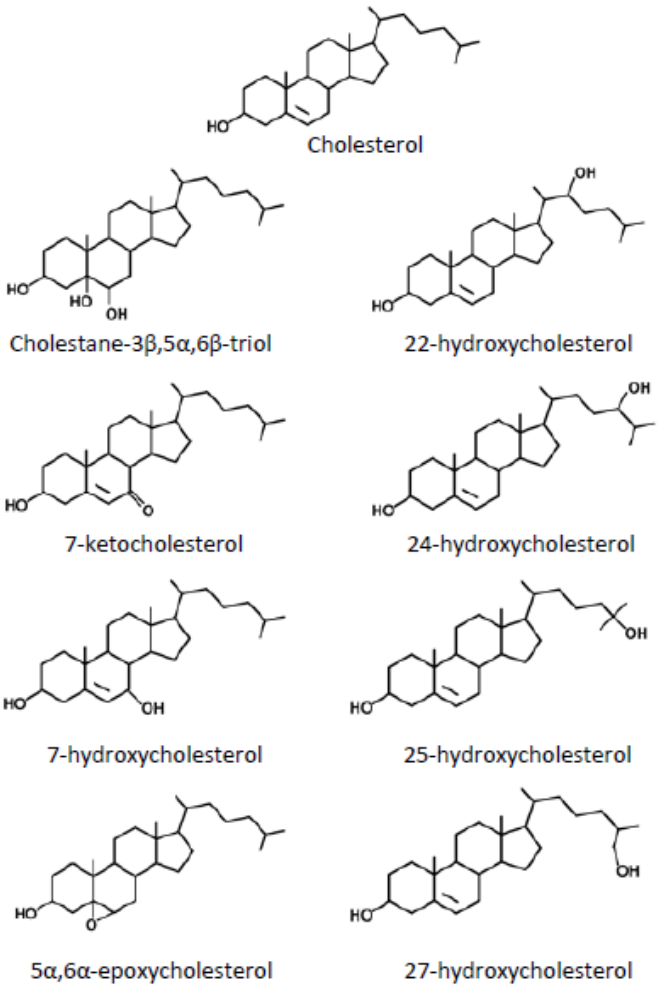

Figure 1.1. Chemical structures of some oxysterols. (Leonarduzzi et al., 2002)

The presence of oxysterols in human plasma can be explained in several different ways; from an exogenous source by absorption of dietary oxysterols or from endogenous sources that oxysterols formed by enzymatic or non-enzymatic oxidation (Otaegui-Arrazola et al., 2010). Dietary sources of oxysterols are cholesterol rich food products including eggs, milk powders, dairy products cheese, red meat, brain, liver, kidney, ham and stored fish (Leonarduzzi et al., 2002; Lordan et al., 2009). Among the oxysterols 25-hydroxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol and 5β, 6β-epoxycholesterol is the most commonly detected ones in processed food (van Reyk et al., 2006). In Table 1.1., both non enzymatic and enzymatic origin of the most representative oxysterols are reported.

Endogenous formation of oxysterols through the non-enzymatic oxidation of cholesterol mainly affects the sterol ring and in general mediated by radical mechanisms. On the other hand, enzymatic oxidation reacts in the side-chain of sterol structures. Some of the most abundant oxysterols such as 24-, 25- and 27-HCs found in vivo, are generated by enzymatic side-chain hydroxylation of cholesterol (Russell, 2000). Two key enzymes involved in cholesterol conversion to bile acids are sterol 27-hydroxylase (CYP27A1) and cholesterol 24-hydroxylase (CYP46A1) that are P450 enzymes and catalyze the hydroxylation reactions to form 27- and 24-HCs (Björkhem et al., 1994; Bretillon et al., 2007). The enzyme sterol 27-hydroxylase is a mitochondrial oxidase that is expressed in various tissues and cells, commonly in liver and macrophages (Brown et al., 2000; Russell, 2000). The oxysterol 27-hydroxycholesterol (27-OH) is one of the most common oxysterols in the peripheral blood of healthy volunteers as well as in atherosclerotic lesions (Honda et al., 2009; Riendeau and Garenc, 2009). Recently, this oxysterol has been reported as a selective estrogen receptor modulator (Umetani et al., 2007) and also a very good ligand of liver X receptors (LXRs) (Janowski et al., 1999, 1996), nuclear receptors that function as master transcription factors, in cell metabolism, macrophage survival, inflammation, and immunity (Bensinger and Tontonoz, 2008).

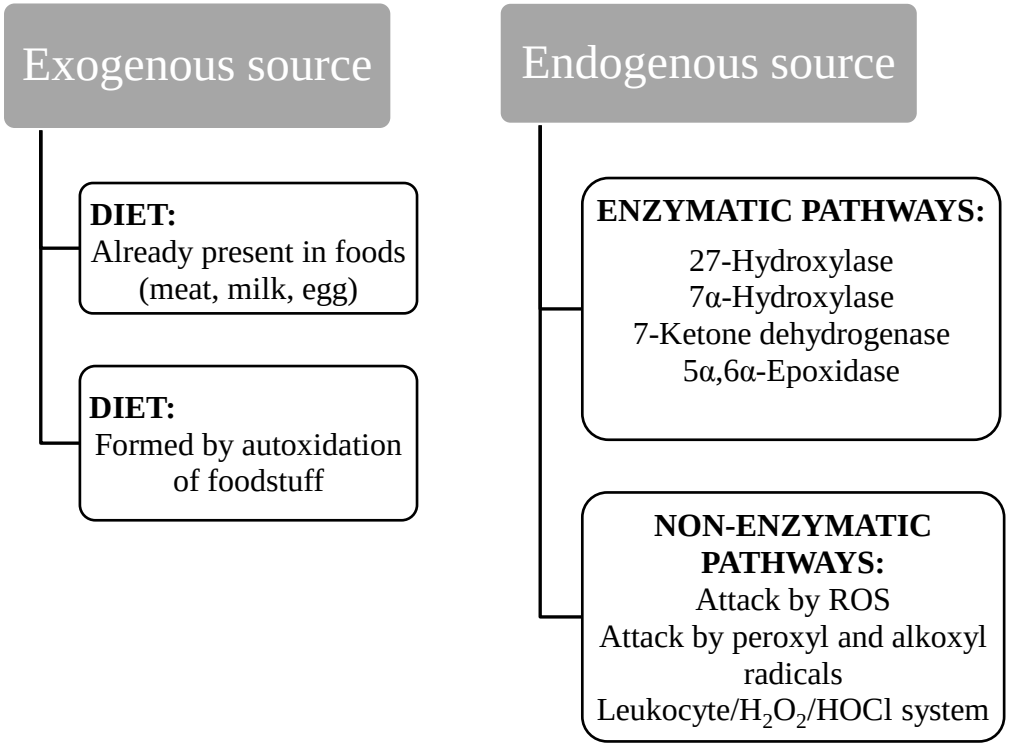

Table 1.1. Origin of oxysterols

It is well accepted that lipid oxidation products including oxysterols are able to modulate many biological activities, thus the biochemical effects of oxysterols are varied from modulation of lipid homeostasis as well as induction of cell death, fibrosis and chronic inflammation (Leonarduzzi et al., 2007).

Nowadays, new emphasis to the beneficial effects exerted by at least defined oxysterols, has been given by the vaste reconsideration that side-chain cholesterol oxides like 24-, 25- and 27-hydroxycholesterol (24-OH, 25-OH and 27-OH) are among the best ligands of a variety of physiologically important nuclear receptors (PPARs, LXRs) and by this way could modulate not only the inflammatory and immunological response but also cell viability, metabolism and function (Bensinger and Tontonoz, 2008; Janowski et al., 1999, 1996).

While the sterol ring-derived oxysterol, namely 7k-cholesterol (also named 7-oxo-cholesterol), definitely not binding to LXRs, would induce and sustain mainly pro-inflammatory reactions in human monocyte-derived macrophages (Buttari et al., 2013), 27-OH, good LXR ligand, should rather polarize human macrophages towards an anti-

inflammatory state. In any case, it becomes evident that various oxysterols are able to trigger and promote signal transduction pathways, which can be either dependent or independent from the binding to nuclear receptors.

Before trying to straighten out the actual knowledge about oxysterols and signal transduction, it is noteworthy to mention that 25-OH and 27-OH have been shown to exert a broad antiviral effect against a large number of viruses with or without lipid envelope, a highly promising beneficial property that is definitely mediated by a complex intracellular signaling, yet to be properly elucidated.

1.1.2 Oxysterols and signal transduction

There is not a unique way by which oxysterols can trigger cell signaling within cells and, as a consequence, the signaling pathways they can activate are quite a number. Definitely, uptake and cellular trafficking appear to significantly differ between sterol ring oxysterols and side chain oxysterols, even if the mechanisms underlying such events are far from being fully elucidated.

Because of their relative lower hydrophobic and higher amphipathic properties as to cholesterol, oxysterols diffuse much better through the lipid bilayer of biomembranes and the diffusion rate is concentration dependent, but, as in the case of cholesterol, a certain percent of both exogenous and endogenous oxysterols resides in the plasma membrane (PM), mainly localized in lipids rafts, i.e. small (10–200 nm) heterogeneous PM microdomains rich in cholesterol, sphingomyelin and phosphatidylcholine.

Of note, from 60 to 80% of total cell cholesterol is contained in the PM (Liscum and Munn, 1999) and lipid raft phosphatidylcholines are Phosphatidyl Inositol 4,5 Trisphosphate (PIP_2) and Phosphatidyl Inositol 3,4,5 Trisphosphate (PIP_3) (Wang and Richards, 2012), namely two key regulator of several signaling pathways, including the PIP3-Akt survival signaling cascade (Di Paolo and De Camilli, 2006). The effect of oxysterols on lipid rafts formation and stability is not homogeneous. While 27-OH and 25-OH seem to also favor raft physiological functions, 7-ketocholesterol (7K) and 7β-

hydroxycholesterol (7βOH) act rather as inhibitors and activate cytotoxic signals (Massey, 2006; Ragot et al., 2013). Up-regulation of the phospholipase c/PIP2 signaling cascade was proved to be exerted by a diet-compatible mixture of oxysterols, eventually leading to scavenger receptor CD36 overexpression in U937 promonocytic cells, and involving the PKC/MEK/ERK pathway (Leonarduzzi et al., 2010).

Another primary trigger of the PKC/MEK/ERK pathway, located as well as phospholipase c/PIP2 in caveolae and lipid rafts is represented by NADPH oxidase (NOX) (Jin et al., 2011), whose assembly and activation within plasma membrane has been investigated in details in phagocytic cells, but nowadays recognized to be present in various isoforms in most cell types. The NOX family of NADPH oxidases certainly is a predominant source or reactive oxygen species (ROS) under physiological conditions and oxysterols were shown able to upregulate at least some members of this family of enzymes, in particular NOX1 in colonic cells (Biasi et al., 2013) and neuronal cells (Gamba et al., 2011) and NOX2 in cells of the macrophage lineage (Leonarduzzi et al., 2004; Vurusaner et al., 2014). Oxysterol-mediated ROS signaling through PKC/MEK/ERK pathway was demonstrated to sustain the pro-inflammatory effects (Biasi et al., 2009) as well as CD36 induction (Leonarduzzi et al., 2010), but also the pro-survival stimuli exerted by oxysterols (Vurusaner et al., 2014).

Still on plasma membrane, at least defined oxysterols of pathophysiological relevance, like 25-OH and 27-OH, could activate the Hedgehog cell signaling (de Weille et al., 2013; Nedelcu et al., 2013), a transduction pathway based on two PM proteins, namely the receptor Patched (Ptc) and the transducer Smoothened (Smo), involved in the regulation of a number of cellular processes besides embryogenesis (Cohen, 2010). Apparently, oxysterols physically interact with Smo (Nedelcu et al., 2013) and the perturbation of this process is considered to play a significant role in carcinogenesis (de Weille et al., 2013). Smo function and Hedgehog signaling were shown as being strictly dependent on lipid raft integrity and function (Shi et al., 2013). Moreover, the internalization of oxidized low density lipoproteins (LDL) occurs at the level of lipid rafts and represents a further way of oxysterols' uptake by the cells. The latter process mainly depends on CD36 and related scavenger receptors (Kiyanagi et al., 2011; Rios et al., 2013), even if a receptor-independent entry of oxysterols within macrophagic cells was described as promoted by lipoprotein lipase (Makoveichuk et al.,

2004). In this relation, important appears that mentioned ability of a biologically relevant mixture of oxysterols to upregulate expression and synthesis of CD36 (Leonarduzzi et al., 2010, 2008).

The cell incorporation of lipoproteins containing also oxysterols leads to conclude that at least one way by which these molecules, besides the localization within lipid rafts, may move intracellularly is vesicular. But there is also a non vesicular way of oxysterols' transport within different cell compartments (Maxfield and Wüstner, 2002), possibly not only but certainly involving OSBPs. Oxysterol-binding proteins (OSBPs) are a group of cytoplasmic carrier proteins having oxysterols as major ligands that are involved in lipid homeostasis and sterol-dependent signal transduction (Olkkonen et al., 2012). With regard to the latter point and, in particular, the hereafter considered oxysterol-triggered survival signaling, OSBPs was displaying a key role in the modulation of ERK1/2 phosphorylation level, by forming an active oligomer with the serine/threonine phosphatase PP2A (Wang et al., 2005). OSPBs appear to play a major role in oxysterol-modulated signal transduction since allow at least part of the non vesicular transport of these cholesterol derivatives from the plasma membrane to intracellular organelles.

A further statement, even if the overall picture is far from being elucidated in full, is that vesicular and not vesicular transport of cholesterol and oxysterols, combined with their biomembrane crossing down a free-energy gradient or for passive diffusion, do operate the complex intracellular movements of these important molecules.

1.1.3 Oxysterol-mediated activation of transcription factors

Cell signaling induced and sustained by oxysterols of pathophysiological interest is combined with the activation of a number of transcription factors, that actually appear to be redox modulated and include sterol regulatory element binding proteins (SREBPs), nuclear factor kappa B (NF-kB), Toll Like Receptors (TLRs), nuclear factor erythroid 2-related factor 2 (Nrf2), Liver X Receptors (LXRs), RXR (Retinoid X Receptor), Peroxisome Proliferator-Activated Receptors (PPARs), Retinoic Acid Receptor-Related Orphan Receptors (RORs), estrogen receptors (ERs).

SREBPs are localized in a precursor form within the endoplasmic reticulum, complexed with SREBP cleavage activating protein (SCAP) that regulates its transport into the Golgi and consequent activation. Once activated, SREBPs translocate in the nucleus where bind to the sterol responsive elements of the genes involved in fatty acids and cholesterol synthesis and uptake (Yan and Olkkonen, 2008). Mainly side-chain oxysterols are good ligands and/or activators of SREBPs (Björkhem, 2009).

The widely recognized pro-inflammatory effect exhibited by a variety of oxysterols is definitely based, at least in part, on the strong activation and nuclear translocation of NF-kB, through the ERK-JNK pathway (Leonarduzzi et al., 2005; Umetani et al., 2014), with or without the involvement of estrogen receptor α (Erα) (Umetani et al., 2014). There is a strong experimental evidence that a variety of cholesterol oxidation products may upregulate a large number of inflammation-related genes whose expression is NF-kB-dependent, like those coding for interleukin-1β (IL-1β) and interleukin-6 (IL-6), interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Poli et al., 2013).

Moreover, enhanced NF-kB nuclear translocation and consequent gene transcription stem from the activation of Toll-like receptors (TLRs), a family of receptors primarily involved in the innate immunity and localized on the plasma membrane and/or in endosomes, that may also be induced by oxysterols such as 27-OH and 25-OH (Gargiulo et al., 2015).

Another redox-sensitive transcription factor that like NF-kB is kept in an inactive form within the cytoplasm but, once activated, translocates into the nucleus, namely Nrf2, has recently been considered a possible target of oxysterol-mediated cell signaling, as reported in more details in section 1.4.3.1.

A number of nuclear receptors playing a key role in a variety of physiological processes recognize oxysterols as primary ligands. This is especially the case of LXRα and β that form obligate heterodimers with RXR and then act as sensors of cholesterol and its oxidative metabolites, mainly side-chain oxysterols (Janowski et al., 1999, 1996). The two oxysterols mainly investigated for their LXR binding property are 24-

OH and 27-OH and their involvement in the physiological regulation of cholesterol and lipid metabolism strongly proposed (Björkhem, 2013, 2009). An additional interesting effect that has been ascribed to side-chain-oxysterols and triggered through the LXR-RXR pathway, is the stimulation of an anti-inflammatory phenotype in macrophages, i.e. an important process in the modulation of inflammatory and immunologic events (Töröcsik et al., 2009), which can lead to the survival of immune cells (Joseph et al., 2004) but also of foam cells (Sallam et al., 2014) and tumor cells (York and Bensinger, 2013). Still, the overall effect of oxysterols, usually present in mixture within human tissues and biological fluids, on the modulation of inflammation and immunity is far from being fully elucidated. Confirming the complexity of the subject is the report of a pro-inflammatory effect of the sterol ring oxysterol 7K on both human type I and type 2 differentiated macrophages.

Not only LXRs but also the PPARα, β/δ and γ form heterodimers with RXR, an example of integrated modulation of cell metabolism and inflammatory reactions (Hong and Tontonoz, 2008). There is not much evidence of an involvement of PPARs in signal transduction operated by oxysterols, but the very likely interconnection between the various nuclear receptor classes suggests not to exclude a priori while deeper investigate the possible modulation of the different PPAR isoforms by cholesterol oxides. At present, one study is available which proved the involvement of PPARγ isoform in the up-regulation of CD36 scavenger receptor in U937 promonocytic cells challenged with a biologically relevant mixture of oxysterols (Leonarduzzi et al., 2008).

A further class of nuclear receptor, namely RORα, β, γ, playing an important role in both development and functions of immune system, brain, retina and various other tissues (Burris et al., 2012) recognize several oxysterols as ligands. In this relation, the few data so far available indicate a significant inhibitory regulation of RORs as exerted by 7α-OH, 7β-OH, 7K (Wang et al., 2010b) and, with regard to side chain cholesterol oxides, by 24-OH (Wang et al., 2010a).

Finally and importantly, 27-OH was definitely demonstrated to act as competitive ligand for Erα and ERβ, by this way triggering intracellular signals potentially able to modulate cancer cell growth and atherosclerosis progression (Lee et al., 2014; Umetani et al., 2014, 2007). Consistently, a marked promotion of cell proliferation was observed

in human breast and ovarian cell lines, as well as in murine cardiomyocytes following treatment with 25-OH, which was shown to signal trough Erα (Lappano et al., 2011). Again, as in the case of oxysterol-mediated modulation of the activity of other nuclear receptors, because of the complex and yet largely unknown interaction between them, it is better not to draw any conclusion, for instance claiming that defined oxysterols can simply favor cancer proliferation and growth. In this regard, there is a line of evidence indicating that oxysterols could on the contrary counteract cancer progression, for instance in the case of tamoxifen and related drugs, by stimulating malignant cell differentiation and apoptotic (de Medina et al., 2011).

1.2 Programmed Cell Death

Programmed cell death occurs during embryonic development, preservation of tissue homeostasis, immune system regulation and morphogenesis throughout organism's life. Programmed cell death can be divided into three main types including apoptosis, necrosis and autophagy. Many reliable in vitro studies demonstrated the potential pro-apoptotic effect of oxysterols; thus we will especially focus on apoptotic cell death throughout this thesis.

1.2.1 Apoptosis

Apoptosis is one of the major types of programmed cell death which is genetically controlled and carried out in an ordered process in response to a wide range of exogenous and endogenous stimuli. Apoptosis is characterized by distinct morphological features including cell shrinkage, membrane blebbing, nuclear fragmentation, chromatin condensation and consequent phagocytosis by macrophages to for the deletion of damaged cells (Strasser et al., 2000).

Apoptosis can be divided into different stages including initiation, execution and removal of apoptotic bodies. There are two major pathways that trigger apoptosis: the death receptor-dependent (extrinsic) pathway or the mitochondrial (intrinsic pathway) (Fig. 1.2.). The extrinsic pathway can be initiated by external signals such as death

activators which bind to surface receptors. Binding of specific ligands like Fas ligand (FasL) or TNF-a to the death receptors on the target cells induces receptor multimerization leading to death inducing signaling complex (DISC) formation (Ashkenazi and Dixit, 1998). An adaptor protein, Fas associated death domain protein (FADD) acts as a bridge between DISC and caspase- 8, which is crucial for recruitment of caspase-8 to DISC, and also for activation of caspase-8 that results in caspase-3 activation to initiate degradation of the cell (Bodmer et al., 2000; Sprick et al., 2000).

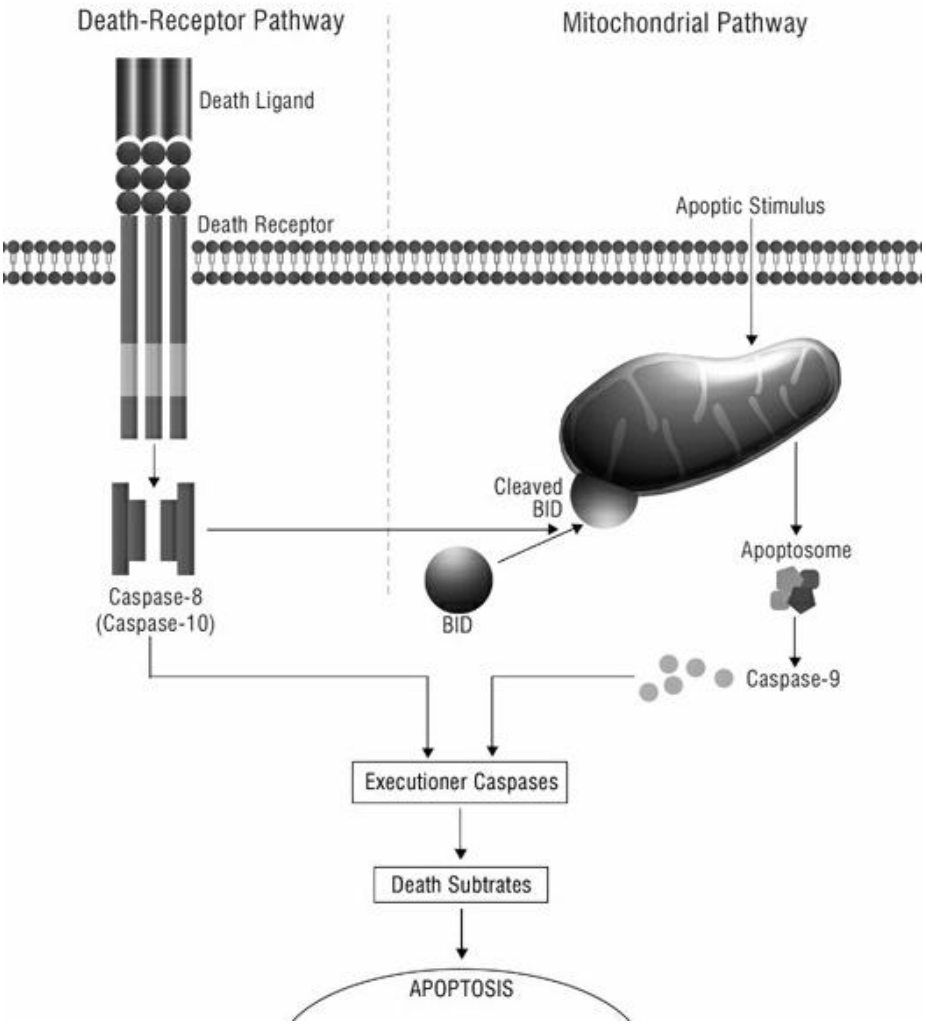

Figure 1.2. Extrinsic and intrinsic pathways of apoptosis. (Kutuk and Basaga, 2006)

The intrinsic pathway of apoptosis is centered on the mitochondrial outer membrane permeabilization (MMP) and initiated by DNA damaging agents, heat, radiation, hypoxia and infection (Moissac et al., 2000). Bcl-2 family members are the

main apoptotic sensors in intrinsic pathway that regulate the survival or death signals in the mitochondria. In response to apoptotic stimuli, MMP can be induced by pro-apoptotic members of Bcl-2 family proteins that lead to the release of small pro-apoptotic including cytochrome c, second mitochondrial activator of caspases/direct IAP-binding protein of low isoelectric point -pI (Smac/DIABLO), endonuclease G (Endo G) and apoptosis inducing factor (AIF) (Adrain et al., 2001). Once cytrochrome c released into the cytosol, it interacts with apoptotic protease-activating factor-1 (Apaf-1) and ATP/dATP. As a consequent of the complex formation, pro-caspase-9 is recruited to form apoptosome, which in turn cleaves and activates caspase-3, the effector protein that initiates degradation of nuclear material and proteases (van Gurp et al., 2003). Effector caspases including caspase-3, -6 and -7 cleaves various substrates such as poly (ADP-ribose) polymerase (PARP) that leads to the morphological and biochemical changes seen in apoptotic cells. In addition to cytochrome c release, Smac/DIABLO may promote caspase activation through competing with caspase-9 to bind to inhibitor of apoptosis proteins (IAPs) that result in apoptosis by eliminating IAP inhibition of caspases (Adrain et al., 2001).

In the early stages of mitochondrial pathway of apoptosis, regulation of intracellular Ca^{2+} stores appears central. In response to apoptotic stimuli, Ca^{2+} mobilizes from endoplasmic reticulum (ER) stores to the mitochondria. Then, the mitochondrial calcium uptake induces rupture of the subcellular organelle and modulate calcium-dependent enzymes that crosstalk with other apoptotic mechanisms. Moreover, the anti-apoptotic proteins of Bcl-2 family members which are also localized in the ER, have active role for Ca^{2+} homeostasis in apoptosis (Oakes et al., 2003).

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1.2.1.1 Bcl-2 family

Bcl-2 (B-cell lymphoma 2) family members are crucial regulators of intrinsic pathway of apoptosis. The balance between pro-apoptotic Bcl-2 family proteins such as Bad, Bax, Bim, Bok, Bcl-xs and anti-apoptotic proteins such as Bcl-2, Bcl-xl, Mcl-1 determine if a cell undergoes apoptosis. These family members share at least one of the four Bcl-2 homology domains; BH1, BH2, BH3, BH4 and based on the functional and structural properties, they can be classified into three groups (Cory and Adams, 2002;

Kutuk and Basaga, 2006) (Fig. 1.3.). Group I includes multidomain anti-apoptotic members (Bcl-2/Bcl-XL/Bcl-w/Mcl-1/A1/Bfl 1) that inhibit apoptosis by binding to and sequestering pro-apoptotic Bcl-2 family members. Proteins belongs to Group II involves Bax and Bak, multidomain pro-apoptotic members that acting as promoters of cell death, through inducing cytochrome c release from the mitochondria (Mikhailov et al., 2003). Group III pro-apoptotic proteins share only BH3 domain (Bid/Bad/Bik/Bim) that in response to apoptotic stimuli, they translocate from the cytosol to the mitochondria to inhibit Bcl-2/xl and activate Bax or Bak to induce the release of apoptotic proteins (Kutuk and Basaga, 2006). The function of BH3 domain only proteins can be characterized by two main pathways. In the first pathway, direct activators (Bim, Bid and Puma) interact with pro-apoptotic Bax and Bak to induce their activation while anti-apoptotic proteins may form complexes to inhibit this activation. The other pathway involves sensitizers, other BH3 only members (Bad) bind to anti-apoptotic Bcl-2 members and prevent them interacting and sequestering Bid and Bim, which can lead to dissociation of Bax and Bak to be active (Kim et al., 2006).

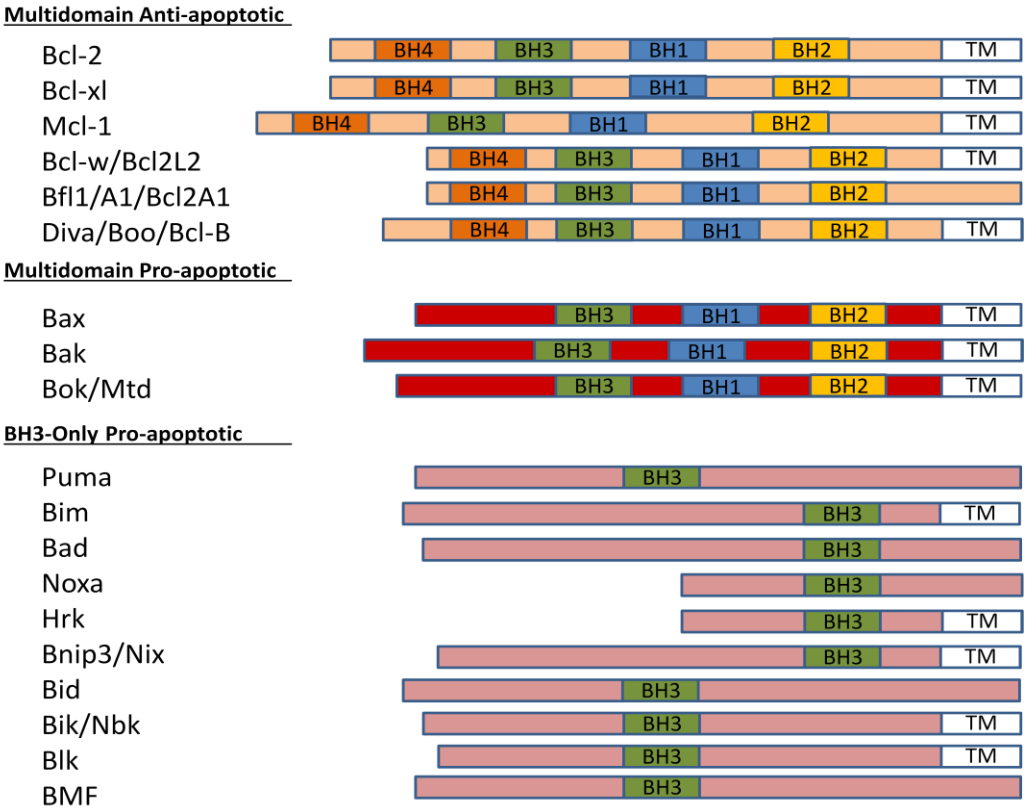

Figure 1.3. Bcl-2 protein family members. (Adapted from (Tezil and Basaga, 2013))

Under normal conditions, anti-apoptotic Bcl-2 and Bcl-xl are expressed in the outer membrane of the mitochondria that prevent the opening of mitochondrial permeability transition pore (Leonarduzzi et al., 2007). In the activation stages of the mitochondrial apoptosis, pro-apoptotic members translocate from the cytoplasm to the mitochondria where bind to anti-apoptotic Bcl-2 members to antagonize their function. In response to apoptotic stimuli, pro-apoptotic Bak and Bax undergoes series of structural changes that triggers insertion of these proteins into the mitochondrial membrane which leads to a lipidic pore formation and then increases mitochondrial membrane permeability that allows release of apoptogenic proteins such as cytochrome c (Antonsson et al., 2001; Mikhailov et al., 2003)

Pro-apoptotic Bad could be regulated by phosphorylation where survival signals induce its phosphorylation on serine residues (Ser-112, Ser-136 and Ser-155) that leads to the sequestration and inactivation of Bad by 14-3-3 proteins (Datta et al., 2002).

1.2.2 Oxysterols-induced apoptosis and associated signaling pathways

The increasing numbers of in vitro studies have described the potential pro-apoptotic effect of oxysterols in various cell types including smooth muscle cells, fibroblasts, endothelial cells and macrophages. Among the different oxysterols, 27-hydroxycholesterol 7-ketocholesterol, 7β-hydroxycholesterol and 25-hydroxycholesterol have shown to induce apoptosis on these given cell systems (Ares et al., 1997, 2000; Clare et al., 1995; Lemaire-Ewing et al., 2005).

With regard to extrinsic pathway of apoptosis, Lee and Chau showed that both 7β- hydroxycholesterol and 25- hydroxycholesterol induced apoptosis while up-regulating the levels of both Fas and Fas ligand (FasL) and their corresponding mRNAs in vascular smooth muscle cell (Lee and Chau, 2001). Moreover, Rho et al. demonstrated the effect of 7-ketocholesterol, using human aortic smooth muscle cells where the oxysterol predisposed cells to undergo cell death via Fas and TNF-α signaling pathway (Rho et al., 2005).

Numerous in vitro studies have demonstrated the involvement of mitochondrial pathway in oxysterols-induced cell death. In U937 human promonocytic cells, either treatment with 7β−OH or 7K induced apoptosis by activating caspase cascade, in association with loss of mitochondrial membrane potential. Luthra and colleagues confirmed the effect of 7K that activates caspases-3/7, -8, and -12 in human microvascular endothelial cells (Luthra et al., 2008). In a very recent study, Lizard et al. showed that U937 cells challenged with high concentrations of 7β−OH or 7K induced both extrinsic and intrinsic pathways of apoptosis in terms of activation of caspases and degradation of cytosolic Bid (Prunet et al., 2005). In other experiments using macrophage lineage, 25-OH or 7β−OH ,at final concentrations of 20-30 μM or above induced apoptosis (Aupeix et al., 1995). Moreover, 7K (30 μM) treatment induced pro-apototic effects in human promonocytic cells and murine J774A.1 macrophages (Biasi et al., 2004). In relation to Bcl-2 family proteins regulation, Seye et al. showed that the levels of Bax protein upregulated and translocated from cytosol to mitochondria to induce cytochrome c release, in rabbit aortic smooth muscle cells treated with 7K (Seye et al., 2004). Indeed, this oxysterol-induced transmembrane potential loss can be normalized upon removal of 7K after 16 h. Research carried out by Berthier et al. demonstrated the involvement of several signaling pathways in THP-1 cells in response to 7K-induced apoptosis (Berthier et al., 2005). Namely, Smac/DIABLO was released into the cytosol following oxysterol treatment with a subsequent depolarization of the mitochondria and cytochrome c release. Moreover, release of both Smac/DIABLO and cytochrome increased by inhibition of ERK1/2 in response to 7K treatment. Conversely, 7K treatment for 24 h induced apoptosis along with increased level of phosphorylation of ERK1/2, JNK and p38 MAPKs in THP-1 cells (Palozza et al., 2007). In addition MAPKs, some in vitro studies observed the phosphorylation status of Akt pathway along with oxysterol-induced apoptosis. In this relation, 25-OH was observed to induce apoptosis through inhibition of Akt in murine macrophage-like cell line (Rusiñol et al., 2004). More recently, Lordan et al demonstrated that 7β−OH induced apoptosis caused degradation of Akt in U937 cells (Lordan et al., 2008).

Taken together, these results suggest that oxysterols are able to active both intrinsic and extrinsic pathways of apoptosis with the involvement of MAPKs and other signaling pathways in various vascular cells.

1.3 Survival Signaling

Intercellular communication is a crucial process in all of life forms, especially in multicellular organisms. Growth factors, defined as polypeptides and act as signaling molecules that regulate diverse biological processes such as cellular growth, proliferation, differentiation, and migration through the binding to receptors on the surface of their target cells (Bafico and Aaronson, 2003). Many growth factors bind to and activate receptors with intrinsic protein kinase activity. These receptor tyrosine kinase (RTK) family receptors contain an extracellular ligand binding domain, a transmembrane region and an intracellular part that contains a catalytic domain with kinase activity and several regulatory tyrosines, which are modified through auto- or trans-phosphorylation (Bafico and Aaronson, 2003; Perona, 2006). There are many different RTK classes have been identified such as epidermal growth factor (EGF), vascular endothelial growth factor (VEFG), fibroblast growth factor (FGF) and platelet-derived growth (PDGF) which are important in pathological conditions including atherosclerosis and cancer (Raines and Ross, 1996; Witsch et al., 2010).

Kinase activation through the binding of growth factors to their receptors is mediated by receptor dimerization where ligand binding stabilizes interactions between adjacent cytoplasmic domains (Perona, 2006). This event results in autophosphorylation of tyrosine residues located at the cytoplasmic tail of the RTK and also phosphorylation of relay proteins that each can trigger a separate cellular response. Activation of receptor signaling constitutively initiates multiple signal transduction pathways. The three best characterized signaling pathways activated in response to RTKs are the mitogen-activated protein kinase cascades (MAPKs), the lipid kinase phosphatidylinositol 3 kinase (PI3K) and the phospholipaseC (PLC) pathway (Katz et al., 2007). Survival signaling pathways are summarized in Figure 1.4.

1.3.1 Mitogen-activated protein kinases

Cells can sense and respond to stress in various ways including initiation of cell death and promoting cell survival. There are many different types of response to stress that depends on the type, strength and duration of the stimuli and involves a complex network of signaling pathways. Several molecular pathways have been defined to

regulate the cell survival and cell death pathways. Among these pathways mitogen-activated protein kinases (MAPKs), a group of proline-directed serine/threonine kinases, are the best characterized signaling pathways (Arciuch et al., 2009). MAPKs regulate stress signals in a three layer cascade fashion with a MAP kinase kinase kinase (MAPKKK) phosphorylating and activating its substrate MAP kinase kinase (MAPKK) which are dual-specificity kinases and then phosphorylates serine and threonine residues in their substrate, a MAP kinase (MAPK) (Trachootham et al., 2008).

In mammals, three district cascades of MAPKs have been elucidated: extracellular signal-regulated kinases 1/2 (ERK 1/2), c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), and p38. These kinases are crucial for many biological processes and each pathway is regulated by distinct stimuli. Activation of JNK and p38 by oxidative stress and inflammatory cytokines are generally associated with initiation of apoptosis and cell cycle arrest. In contrast, ERK cascade is generally activated by G-protein coupled receptor ligands and growth factors, and regulates proliferation, survival, and differentiation signals (Matsuzawa and Ichijo, 2005).

The ERK1 and ERK2 are widely expressed in human tissues and have great research interest because of their critical involvement in broad array of cellular functions including cell cycle progression, proliferation, cytokinesis, transcription, differentiation, senescence, cell death, migration, learning and oncogenic transformation (Shaul and Seger, 2007). ERK1/2 signaling pathway is initiated by various-stress inducing stimuli including growth factors, viral infections, carcinogens and mitogens and this activation involves the Ras-Raf-ERK cascade. In depth, ligand binding of RTKs leads to GTP (guanosine triphosphate) loading and activates a small G protein, namely Ras which recruits the serine/threonine kinase, Raf (MAP-KKK) to the plasma membrane where it is activated, and sequentially phosphorylates and activates MEK1/2 and ERK1/2 (Ramos, 2008).

JNK is encoded by three genes, termed JNK1, JNK2 and JNK3 (also known as SAPKγ, SAPKα, and SAPKβ, respectively) and these genes alternatively spliced resulting in 10 or more JNK isoforms (Arciuch et al., 2009). JNK1 and JNK1 are ubiquitously expressed whereas JNK3 is present in the brain, heart and testis. JNK signaling cascade regulate cell death and the development of multiple cell types in the immune system, whereas JNK1 and JNK1 deficient mice are immunodeficient due to

severe defects in T cell function (Tournier et al., 2000). JNK activation is initiated by stress conditions such as ionizing radiation, heat shock, DNA damage and inflammatory cytokines. JNK phosphorylation is catalyzed by two protein kinases MKK4 (SEK1) and MKK7 which are dual specifity kinases and selectively phosphorylate JNK on Tyr and Thr, respectively (Davis, 2000). JNK translocates to the nucleus where it phosphorylates and upregulates several transcription factors, including c-Jun, JunA, JunB, activating transcription factor (ATF)-2 and Elk-1 (Katz et al., 2007).

The p38 kinase family consist of four members namely α, β, γ and δ and these enzymes activated by hormones, cytokines, G protein-coupled receptor ligands and cellular stress (Arciuch et al., 2009). Activation of p38 kinases is mediated by the MKK3 and MKK6 kinases, and following the activation, p38 phosphorylates its substrates including MAPK interacting kinases Mnk 1 and Mnk 2, and eukaryotic initiation factor 4e (eIF4e) (Roux and Blenis, 2004). Many studies have shown that p38 MAP kinases have critical role in signal transduction of immune and inflammatory responses. In addition, they are also involved in the regulation of angiogenesis, cytokine production, cell death and proliferation (Arciuch et al., 2009; Katz et al., 2007).

The crucial role of MAPKs in controlling gene expression, cell growth, differentiation and apoptosis has made them a priority for research whereas deregulation of these MAPKs activity can result in many diseases and cancer. Thus MAPKs including ERK, JNK, and p38 are all molecular targets for drug development, and pharmalogical manipulation of these kinases will likely help for the treatment of human disease related to disproportionate apoptosis.

1.3.2 PI3K-PKB/Akt

The PI3K/Akt pathway has been established as one of the most critical signaling pathway in regulating cell survival. PI3K is a heterodimeric enzyme composed of two subunits, namely the p85 regulatory subunit and the p110 catalytic subunit. PI3K activation can be stimulated by binding of its p85 regulatory subunit to an activated receptor (Katz et al., 2007). Alternatively, phosphorylation of RTKs can also stimulate the activation of PI3K cascade, resulting in recruitment of PI3K to the plasma membrane. Following the activation, PI3K converts the phosphatidylinositol (3,4)-bisphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-trisphosphate(PIP3) which is

a second messenger that recruits 3-phosphoinositide-dependent protein kinase 1 (PDK1 and Akt (also known as protein kinase B, PKB) to the plasmamembrane (Arciuch et al., 2009; Cantley, 2002). Consequently, the PDK1 phosphorylates and activates Akt that results in subsequent phosphorylation of various substrate proteins, including caspase-9, Mdm2, glycogen synthase kinase 3 (GSK3) and forkhead transcription factor (FKHRL1), which targets FasL, Bim, IGFBP1, and Puma. A large amount of evidence has suggested that BAD is one of the direct targets of Akt in promoting cell survival that phosphorylation of BAD on Ser^{136} by Akt prevents to exhibit pro-apoptotic activity of BAD in cells (Song et al., 2005; Trachootham et al., 2008).

Akt can also exert its anti-apoptotic functions by phosphorylating IKK and cyclic AMP response element–binding protein CREB that results in elevated transcription of genes encoding Bcl-2, Bcl-xl, and Mcl-1 anti-apoptotic proteins. ASK1 is also reported as target of Akt that Akt-mediated phosphorylation of ASK1 inhibited its ability to activate JNK/p38 and prevented stress-induced apoptosis. Thus, it can be suggested that there is a cross talk between the PI3K-Akt and ASK1-JNK pathways in the regulation of cell survival (Matsuzawa and Ichijo, 2005; Song et al., 2005; Trachootham et al., 2008). Akt is activated by site-specific phosphorylation at two regulatory sites, Thr^{308} in the activation loop and Ser^{473} in the carboxy-terminal (C-terminal) tail while phosphorylation of both sites is required for full activation (Arciuch et al., 2009).

1.3.3 Protein kinase C

Phospholipase C- γ (PLCγ) activation is stimulated by G protein coupled receptors (GPCRs) that interact with G proteins of the G_q family. Active PLCγ enzyme catalyzes the hydrolysis of PIP_2 to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Binding of IP_3 receptors on the membrane of the endoplasmic reticulum (ER) causes the release of intracellular Ca^{2+} which is followed by the activation of protein kinase C (PKC) family members (Katz et al., 2007; O'Gorman and Cotter, 2001).

The PKC is a ubiquitous family of serine/threonine kinases and has at least 10 members containing a highly conserved kinase core at the C-terminal and an amino-

terminal auto inhibitory pseudosubstrate peptide. PKC isoforms can be subdivided into three subfamilies according to their structural differences in isoenzyme regulatory domains. Conventional/classical PKC isoforms (α, β_1, β_2 and γ) are regulated by both Ca^{2+} and DAG; the novel isoforms (δ, ε, **θ** and η) contain DAG-sensitive C1 domains but Ca^{2+}-insensitive C2 domain while the atypical PKCs (ζ and ι) regulation both Ca^{2+} or DAG -independent. PKC isoforms play diverse role in signal transduction, mediating cell proliferation, differentiation, death, mitogenesis and stress responses (Arciuch et al., 2009; Guo et al., 2004). Most of the family members have been shown to contribute to cell survival whereas; novel members such as PKC α and PKC δ have been associated with apoptosis induction through inhibition of the PKB/Akt survival pathway and activation of p38 MAPK (Matsuzawa and Ichijo, 2005; Yang et al., 2008).

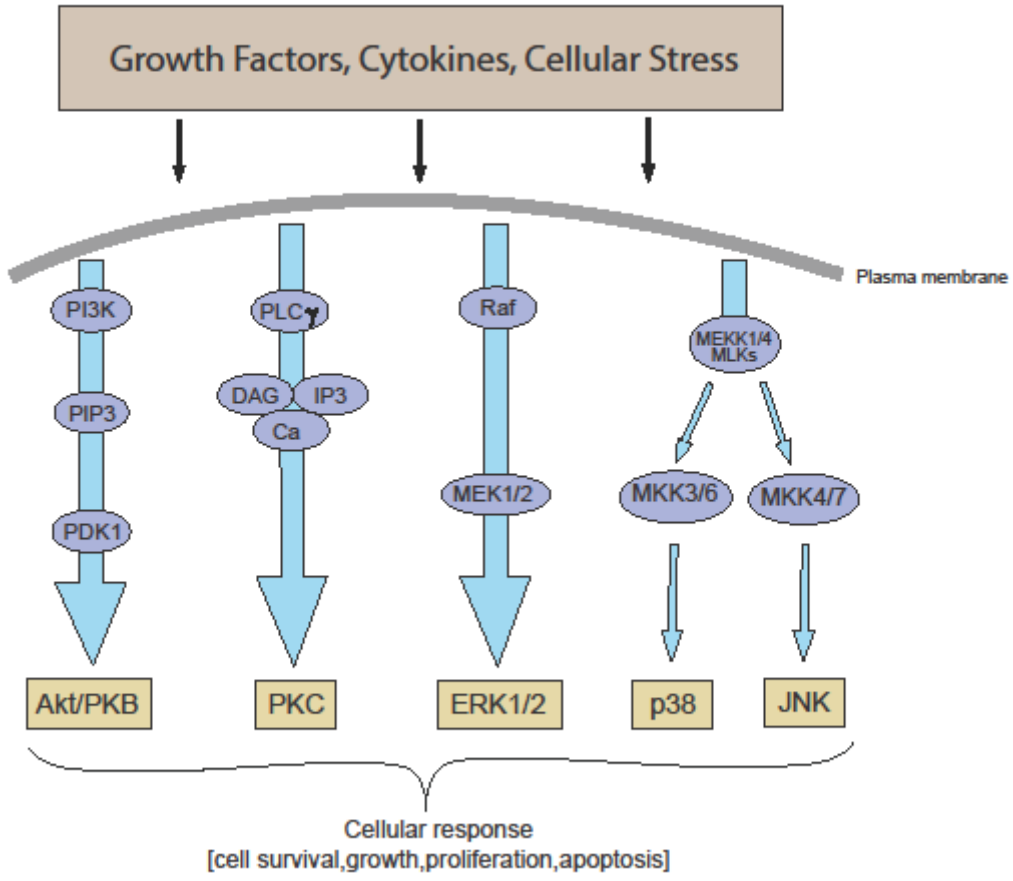

Figure 1.4. Survival signaling pathways.

1.4 Oxysterol -induced survival and associated pathways

Over the last decade, numerous in vitro studies have characterized the potential pro-apoptotic effect of oxysterols in variety of cells. It has been long accepted that apoptosis induced by oxysterols has been strongly related with the potential toxicity and pathogenic implication of these molecules in chronic diseases including atherosclerosis and common neurodegenerative diseases. Of note, an increasing bulk of studies is giving evidence of the involvement of the oxysterols in the modulation of cell survival signals.

Based on the presently available data, oxysterols have differences in the degree of cytotoxicity and ability to induce cell death, but these cellular effects of oxysterols have been mostly studied singularly. However, oxysterols are always present in oxLDL, foods and the core region of atherosclerotic plaques as a mixture and literature about the way in which oxysterols act collectively is limited. In this connection, a very interesting point has been shown by Biasi et al., namely the cytotoxicity of single oxysterol is quenched when cells challenged with the oxysterol mixture (Biasi et al., 2004). In particular, murine macrophages treated with 7-ketochosterol (7K) undergo apoptosis along with mitochondrial pathway, whereas the same cells are co-treated with equimolar concentrations of 7beta-hydroxycholesterol (7β-OH), the pro-apoptotic effect of single oxysterol was markedly attenuated. Notably, 7K-induced intracellular ROS rise through NADPH oxidase activation had been inhibited by the oxysterol mixture, suggesting that a substrate-based competition among oxysterols at the level of NADPH oxidase, (7β-OH binds to NADPH oxidase less efficiently than 7K, may reduced the concentration of free enzyme available for 7K binding) attenuated ROS production and direct toxicity (Biasi et al., 2004; Leonarduzzi et al.). In agreement with this, Aupex et al. showed that the challenge of U937 human promonocytic cell line with 7β-OH (30-40 μM) alone was exerting pro-apoptotic effect, significantly diminished with the addition of identical amount of 25-hydroxycolesterol (25-OH) (Aupeix et al., 1995).

1.4.1 Regulation of cell survival at the signal-transduction level

Growing evidence points to activation of survival signaling pathways such as MAP kinases, PKC, PI3K/Akt by oxysterols, depending on their concentration and the exposure time. The first evidence of the involvement of oxysterols in cell survival was demonstrated by Berthier and colleagues, in an in vitro study challenging THP-1 human monocytic cells with a high concentration of 7K (100 μM) which is one of the most abundant oxysterol with a strong pro-apoptotic effect (Berthier et al., 2005). Challenging that cell line with7K leading to the activation of MEK/ERK signaling pathway followed by inactivation of pro-apoptotic protein Bad, thereby delaying the apoptotic mechanism initiated by 7K itself. More recently, another group performed experiments on other human promonocytic cell line (U937), with low micromolar concentration of 27-hydroxycholesterol (27-OH), produced results showing a significant induction of cell viability through triggering the phosphorylation of Akt at residue Thr308 that delayed apoptotic death whereas high concentrations of 27-OH triggered lysosomal-independent apoptosis (Riendeau and Garenc, 2009). Moreover, Akt-dependent survival signaling induced by 27-OH is impaired when higher concentrations of the same oxysterol is applied suggesting that the effect of the oxysterol on macrophagic cells appeared to depend on the concentrations used. Again using low doses of an oxysterol, this time treating human cholangiocyte MMNK-1 cells with cholestan-3β,5α,6β-triol (Triol), Jusakul et al. showed that activation of pro-survival signaling including ERK1/2 and p38 α phosphorylation were found in Triol-exposed cells (Jusakul et al., 2013). In agreement with these results, another oxysterol, 7β-OH has been shown as anti-apoptotic and induced cell proliferation when added at low concentrations (below 20 μg/ml) to human umbilical-vein endothelial cells (HUVEC); this effect is dependent on the activation of MEK/ERK cascade, but independent of ROS production (Trevisi et al., 2009). However, at higher concentrations 7β-OH induces HUVEC's apoptosis suggesting that oxysterol treatment had a dual effect on endothelial cell viability, depending on the concentration.

Most likely, oxidized low density lipoprotein (oxLDL) has been shown to exert similar effects with oxysterols that oxLDLs have a dual effect on cell viability, proliferation or inducing apoptosis in endothelial cells (Galle et al., 2001). This dual effect is dependent on the concentrations of the oxLDL; at low concentrations (5-10

μg/mL) they induce a proliferative effect, while at high concentrations (50-300 μg/mL) they induce cell death. Moreover, NADPH oxidase dependent ROS increase is involved in both effects (Galle et al., 2001; Heinloth et al., 2000).

In the context of oxLDL induced proliferation, another research group challenged cultured bone marrow derived macrophages with oxLDL in the 1.56-200 μg/ml concentration range and showed the activation of both ERK1/2 and PKB kinases and subsequent phosphorylation of Bad and IkBa which are the pro-survival targets of PKB (Hundal et al., 2001). Indeed, only PI3K/PKB survival pathway is involved in oxLDL's anti-apoptotic effect against macrophage colony-stimulating factor (M-CSF) with drawal where prevention of MEK pathway by PD98059 and U0126 inhibitors did not diminish cytokine-independent macrophage survival. Conversely, in another study, THP-1 monocytic cell line was challenged with an oxLDL final concentration of 50 μg/ml, it was reported that oxLDL attenuates staurosporine-induced apoptosis by activating ERK signaling pathway whereas PI3K/Akt activation was not involved in cell protection by the compound (Namgaladze et al., 2008). Similarly, in a very recent study the neuroprotective effect of 27- and 24-hydroxycholesterol have been shown in human neuroblastoma SH-SY5Y cells against staurosporine-mediated apoptotic events (Emanuelsson and Norlin, 2012).

Since oxLDL induced macrophage proliferation and survival was linked to activation of pro-survival signaling pathways; however, little is known regarding the upstream signaling events including the pattern recognition receptors. In this relation, Riazy et al. have recently demonstrated that oxLDL-mediated survival of bone marrow derived macrophages involves PI3K signaling pathway whereas none of the pattern recognition receptors including endocytic pattern recognition receptors (PRRs), scavenger receptor A (SR-A) and CD36 are essential for activating the anti-apoptotic effect of oxLDL which is not dependent on the uptake of oxLDL (Riazy et al., 2011).
It thus appears that both MEK/ERK and PI3K/Akt signaling pathways have critical role in the pro-survival effect of modified lipoproteins whereby the balance between anti-apoptotic pathways (ERK, Akt) and stress-activated pro-apoptotic pathways (JNK,p38) would determine the final effect: cell survival or apoptosis. In this relation, Anticoli et al. demonstrated that liver-derived cells challenged with physiological concentrations of 7K and 5,6-secosterol (5,6-S), a recently discovered oxysterol, elicits low

concentrations of 5,6-S had a pro-proliferative effect inducing ERK1/2 activation not counterbalanced by p38 MAPK and/or JNK pathways (Anticoli et al., 2010). Indeed, Nox-4 mediated 7KC-induced apoptosis in human aortic smooth muscle cells (SMCs) involved JNK/AP-1 signaling pathway (Pedruzzi et al., 2004). However, Ares and colleagues showed that the effects induced by 7β-OH involved ERK1/2 signaling but not JNK in human aortic smooth muscle cells (Ares et al., 2000). Interestingly, a significant increase of cytosolic Ca^{2+} as induced by oxysterol itself was observed in smooth muscle cells with 24.8μM of 7β-OH. In addition to this, several lines of evidence also suggest that increase of cytosolic free Ca^{2+} involved in pro- and anti-apoptotic signaling triggered by oxysterols. Firstly, Lizard and colleagues demonstrated that 7K-induced THP-1 cell apoptosis is triggered by a sustained influx of extracellular Ca^{2+} which elicits the activation of calcineurin (PP2B) leading to apoptosis through dephosphorylation of the pro-apoptotic Bad protein (Berthier et al., 2004). Moreover, the activation of calcium-dependent calcineurin is induced by translocation of transient receptor potential calcium channel 1 (Trp-1), a component of the store-operated Ca^{2+} entry channel, into lipid raft domains of the plasma membrane. Indeed, the same group also showed the involvement of calcium-dependent activation of MEK/ERK survival pathway via calcium-sensitive proline rich tyrosine kinase (PYK-2) during 7K-induced apoptosis, as mentioned in the first paragraph (Berthier et al., 2005). In the context of oxLDL induced macrophage proliferation, it has been reported that an increase in intracellular $Ca^{2+}$is required for the pro-survival effect of oxLDLs (Matsumura et al., 1997). More recently, to clarify the downstream pathways that are activated by the increase Ca^{2+}, bone marrow derived macrophages was challenged with oxLDL; it was observed that oxLDL can prevent macrophage apoptosis by mobilizing calcium which is followed by the activation of the Ca^{2+}-sensitive eukaryotic elongation factor-2 (eEF2) kinase through inhibiting its negative regulation by blocking p38 MAPK phosphorylation (Chen et al., 2009).

According to the available literature on oxysterol-induced pro-survival signals it can be suggested that, in general terms, this is a complex phenomenon depending on the cell type, environmental factors, cell senescence, oxysterol concentration and exposure time. Notably, when relatively low and not directly toxic concentrations of oxysterols are applied or administered in a biologically representive mixture; this is, incidentally, a more realistic approach, they exert a "Trojan-horse" like behaviour (Biasi et al., 2004;

Vurusaner et al., 2014). Namely, instead of killing the cell directly, oxysterols might delay its irreversible damage, in the meantime initiating pro-inflammatory and pro-fibrogenic pathways while relatively higher amounts induce earlier and direct cell death. Be this it may, through the uptake of modified LDL, toxic levels may exist even in LDL that is taken up by normal LDL receptors, then they may cause damage, once properly concentrated and metabolized (Leonarduzzi et al., 2002). Moreover, it can be suggested that delayed macrophage apoptosis would favor growth and destabilization of advanced atherosclerotic plaques (Martinet et al., 2012). Thus, understanding the molecular mechanism underlying the relationship between signal transduction pathways including protein kinases and oxysterols-induced pro- and anti-apoptotic signaling pathways might contribute to a better understanding of several oxysterol-associated diseases.

1.4.2 Regulation of cell survival at the transcription level

Increasing evidence indicated the existence of a redox regulation for many signaling proteins such as MAP kinases, PKC, PI3K/Akt and several transcription factors that their modulation leads to cell cycle changes and integration of pro- and anti-apoptotic signals. These redox-sensitive transcription factors include Nrf2, NF-kB, Jun/AP-1 and p53 that have key roles in cell survival or death decision (Trachootham et al., 2008). AP-1 and p53 are involved in both the induction and prevention of cell death according to circumstances whereas Nrf2 and NF-kB activation mostly lead to cell proliferation and survival. Moreover, p53 affects cell survival at all three levels including transcription, signal transduction and execution through crosstalk among them. In general terms, they regulate cell survival through altering transcription of multiple genes whereby activation of these transcription factors by stimuli such as cytokines or oxidative stress lead increased expression of pro- or anti-apoptotic proteins.

At the moment, little is known on the molecular mechanisms involved in oxysterol-induced survival pathways at the transcriptional level while information on the involvement of protein kinases have increased greatly. In section 1.3, the oxysterol-mediated activation of transcription factors was reported in detail. In addition to this,

throughout this thesis we focused on the redox sensitive Nrf2 pathway which plays a critical role in protection against oxidative/electrophilic stress generated from exposure to exogenous and endogenous chemicals including lipid peroxidation products and promotes cell survival. Although Nrf2 appears to be one of the major cellular defense pathway, the studies on Nrf2 regulated downstream pathways remain limited, thus understanding the regulatory mechanisms may contribute to drug resistance induced by Nrf2 to immoderate protection from anti-cancer treatments (Niture et al., 2010).

1.4.3 Nrf2 signaling pathway

Nrf2 (nuclear factor E2-related factor 2), a member of the Cap'n'Collar family of b-Zip transcription factors and p45 NF-E2–related proteins, has been identified as a key mediator of the antioxidant genes activation through the antioxidant response element (ARE) (Nguyen et al., 2003). Under normal conditions, Nrf2 is sequestered in the cytoplasm through interacting with Kelch-like ECH-associated protein 1 (Keap1), an actin-binding cytoskeletal protein (Motohashi and Yamamoto, 2004). Under oxidative stress Nrf2 dissociates from Keap1, then also from cytoskeleton, and migrates into the nucleus where it heteromerizes with Mafs and binds to ARE sequences, codifying for antioxidant enzymes including aldoketoreductase, glutathioneperoxidase (GPx), glutathione reductase (GSR), heme oxygenase-1 (HO-1), and NAD(P)H:quinone oxyreductase (NQO-1) (Dinkova-Kostova, 2002; Dinkova-Kostova et al., 2001; Iqbal et al., 2003; Nguyen et al., 2009) (Fig.1.5.). Although growing evidence showed that the protective adaptive response to ROS/RNS is mediated by enhanced expression of these cytoprotective enzymes, the specific upstream signal transduction pathways used to activate transcription of these phase II genes are poorly defined (Lee and Choi, 2003; Mann et al., 2007). In this relation, recent studies demonstrated the clear involvement of several protein kinase pathways including the ERK 1/2, JNK,p38 and PKC in the phosphorylation and stabilization of Nrf2 to facilitate its nuclear translocation and binding to ARE sequences of target genes (Anwar et al., 2005; Yu et al., 2000).

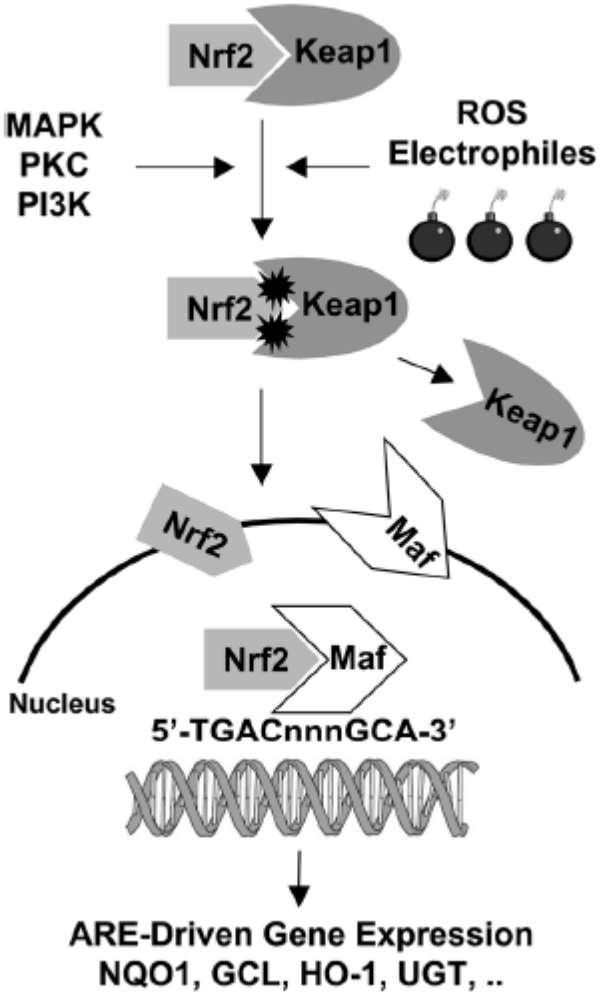

Figure 1.5. General scheme for the induction of Nrf2-ARE signaling pathway. (Lee and Johnson, 2004)

1.4.3.1 Nrf2 antioxidant pathway and lipid oxidation products

Recent studies in oxLDL-treated vascular cells demonstrated the activation of the transcription factor Nrf2 by components of oxLDL that points to the involvement of this antioxidant response element in the signaling pathway sustaining atherosclerosis progression (Ishii et al., 2004). In the same study, they provided the first evidence that, oxLDL activates PPAR-γ and controls expression of scavenger receptor CD36 in macrophages suggesting that Nrf2 is a key regulator in the oxLDL uptake by the vascular wall and induction of antioxidant stress genes in atherosclerosis. Moreover, oxLDLs were shown to activate Nrf2 much strongly in murine macrophages than in smooth muscle cells, while 4-hydroxynonenal (HNE), a major product of oxidized lipids was equally stimulating Nrf2 translocation to the nucleus in both cell types, by

this way suggesting that modulation of Nrf2 response could much depend on the type of chemical inducer and the type of cell. In a very recent study, C6 glioma cells challenged with 10 or 20 μM 27-OH to observe the role of this oxysterol in the modulation of Nrf2 in neurodegenerative diseases including AD, Alzheimer's disease (Ma et al., 2015). Observations showed that the down regulated expression of Nrf2 and its down-stream antioxidant genes HO-1, NQO-1 and γ -GCS at both of gene and protein levels in response to 27-OH which is neurotoxic and pro-oxidant in astrocyte cells.

A number of reports have addressed Nrf2-target genes such as HO-1 and NQO-1 that are critical in the cellular response against pro-oxidative stimuli including oxidized LDL and oxidized phospholipids, result to be expressed in all main cell types present in mouse and human atherosclerotic lesions, such as macrophages, endothelial cells, and smooth muscle cells (Araujo et al., 2012). In this relation, the oxidized phospholipid ox-PAPC was shown to induce HO-1,NQO-1 and GCLM expression in endothelial cells in a Nrf2-dependent manner (H. K. Jyrkkänen et al., 2008). Similarly, HO-1 induction via Nrf2 signaling pathway was demonstrated in vascular smooth muscle cells in response to moderately oxidized LDL (Anwar et al., 2005). Moreover, induction of this cytoprotective response includes HO-1 up-regulation implied the activation of PKC and MAPK pathways including p38, JNK and ERK1/2. Besides activation of MAPKs by lipid oxidation products modulates smooth muscle cell proliferation, evidence relating the involvement of these signaling pathways in the induction of Nrf2 antioxidant response is limited (Velarde et al., 2001; Yang et al., 2001).

Recent studies have implicated that Nrf2 translocation to the nucleus is modulated by ERK1/2, p38 MAPK, PKC and PI3K/Akt pathways, however the intensity of these upstream signals that control Nrf2 nuclear transposition varies with the different inducers in the different cell types. Regarding this, Papaiahgari and colleagues showed that ROS-dependent anti-apoptotic ERK and Akt activation regulates Nrf2-mediated transcription by favoring translocation of Nrf2 from cytoplasm to the nucleus in response to hyperoxia in pulmonary epithelial cells (Papaiahgari et al., 2006, 2004). Collectively, these studies indicate that Nrf2 transcription factor has an important role as downstream effectors of PI3K signaling pathway to regulate transcription against hyperoxia that contribute to cell survival. Another study has also shown that survival signaling pathway PI3K/Akt-dependent Nrf2 protein increase and

HO-1 expression up-regulation in PC12 pheochromocytoma cells (Martin et al., 2004). HNE-mediated induction of HO-1 mRNA and protein have been postulated in macrophages and epithelial cells involving the activation of ERK survival pathway suggesting that HO-1 induction is an adaptive response to oxidative stress (Iles et al., 2005; Li et al., 1996). Contrary results were reported in hepatoma cells challenged with defined metabolites of the prostaglandin J(2) series in which activation of HO-1synthesis was not quenched by the ERK1/2 inhibitor PD98059, suggesting that up-regulation of this heme catabolism-related enzyme might be obtained through more than a single signaling pathway (Liu et al., 2004). More recently, the anti-apoptotic effect of the same compound was demonstrated in rat pheochromocytoma cells showing that Nrf2 activation and subsequent HO-1 induction involving survival ERK and Akt pathways to protect cells against oxidative-stress induced cell death (Kim et al., 2008). Therefore, based on the presently available data, the switch on or off of Nrf2 by different types of inducers including lipid oxidation products leads to cell survival and protection against oxidative stress whereas reduced apoptosis may result in tumorigenesis and drug resistance (Niture and Jaiswal, 2012).

1.5 Oxysterols in the pathogenesis of major chronic diseases

In the years of predominant biochemistry approach to the role played by various members of the oxycholesterol family in humans, oxysterols have been mainly investigated for their physiological role played in the synthesis of bile acids and steroid hormones, in the sterol transport and metabolism, in gene regulation. But evaluating the biochemical effects of cholesterol oxidation products it appeared quite evident the strong pro-inflammatory, pro-apoptotic and pro-fibrogenic properties of some of them (Sottero et al., 2009). Over the last few years, effectively supported by a molecular biology-based technology, the molecular aspect of such a pro-inflammatory effect of oxysterols has been well deepened, and a still growing bulk of experimental findings strongly points to a significant contribution paid by these cholesterol derivatives to the progression of inflammatory-based chronic pathologies.

Among the very common human diseases that recognize hypercholesterolemia as primary risk factor and are associated with inflammation are vascular aging, atherosclerosis and cardiovascular accidents, Alzheimer’s disease as well as multiple

sclerosis, inflammatory bowel disease and colorectal cancer, non alcoholic liver disease and retinopathies, diabetes mellitus, etc. (Biasi et al., 2013; Gamba et al., 2011; Poli et al., 2013). Cholesterol oxidation products have been shown to be involved in various key stages of atherosclerosis where promising therapeutic strategies targets oxysterol elimination in different stages of this disease; we will especially focus on atherosclerosis throughout this thesis.

1.5.1. Atherosclerosis

The World Health Organization emphasized the importance of scientific research into atherosclerosis which is the leading cause of mortality in developing countries. Atherosclerosis is the principal contributor to some serious diseases including heart diseases, myocardial infarction and stroke affecting numerous people (McLaren et al., 2011). Atherosclerosis is a multifactor chronic inflammatory disease characterized by the accumulation of lipid and cholesterol within the intima of large and medium sized arteries (Lusis, 2000). Atherosclerotic early lesions develop from an initial fatty streak, which progress by endothelial dysfunction, expression of adhesion molecules, further inflammatory processes including infiltration of the monocytes under the endothelium where these monocytes differentiate into macrophages and consequent engulfment by macrophages leads to formation foam cells which is the most characteristic hallmark of the atherosclerosis (Crowther, 2005; Kutuk and Basaga, 2003). Many biological processes including migration, cell proliferation and apoptosis contribute to the progression of atherosclerotic lesion. Migration of vascular smooth muscle cells from the media results in synthesis of collagen to surround the lipid laden macrophages formed lipid cores with a protective fibrous cap. In advanced lesions with a large lipid core which contains both the necrotic and apoptotic cells thus become highly instable, are prone to rupture. The rupture of the fibrous plaque usually occurs at the shoulder region of the lesion, which is followed by thrombus formation that cause symptoms of acute coronary syndromes and stroke (Martinet and Kockx, 2001; Shibata and Glass, 2010). The pattern of atherosclerotic lesions development can be described into two main phases. The first phase includes perturbations in endothelial functions and inflammation stimuli that provoke the release of cytokines and proliferative response for smooth muscle cells, subsequent intimal migration and intima formation. It has been suggested that apoptosis has a role in development of lesions through initiating

extensive endothelial cell turnover leading to endothelial dysfunction (Endemann and Schiffrin, 2004). Thus, it seems that apoptosis contribute to the first phase of atherosclerotic lesions formation targeting two cell types including proliferative stimulation for vascular smooth muscle cells. In the second phase of lesion progression the formation of oxidized low density lipoproteins occurs in the vascular wall while inflammatory response increases. It also includes the accumulation of free radicals with alteration of redox balance and modification of lipids, proteins and DNA which results in plaque development and stabilization by extracellular matrix (Robbesyn et al., 2004; Tabas, 2009). In the following, advanced lesions with extensive apoptosis of cells leads to fibrous cap thinning thus plaque destabilization, rupture and thrombosis (Dimmeler and Zeiher, 2004; Stoneman and Bennett, 2004). In Figure 1.6., a schematic representation of the main events involved in the remodeling of arterial vessels in atherosclerotic process and the contribution made by cholesterol oxidation products in each step of this process was illustrated.

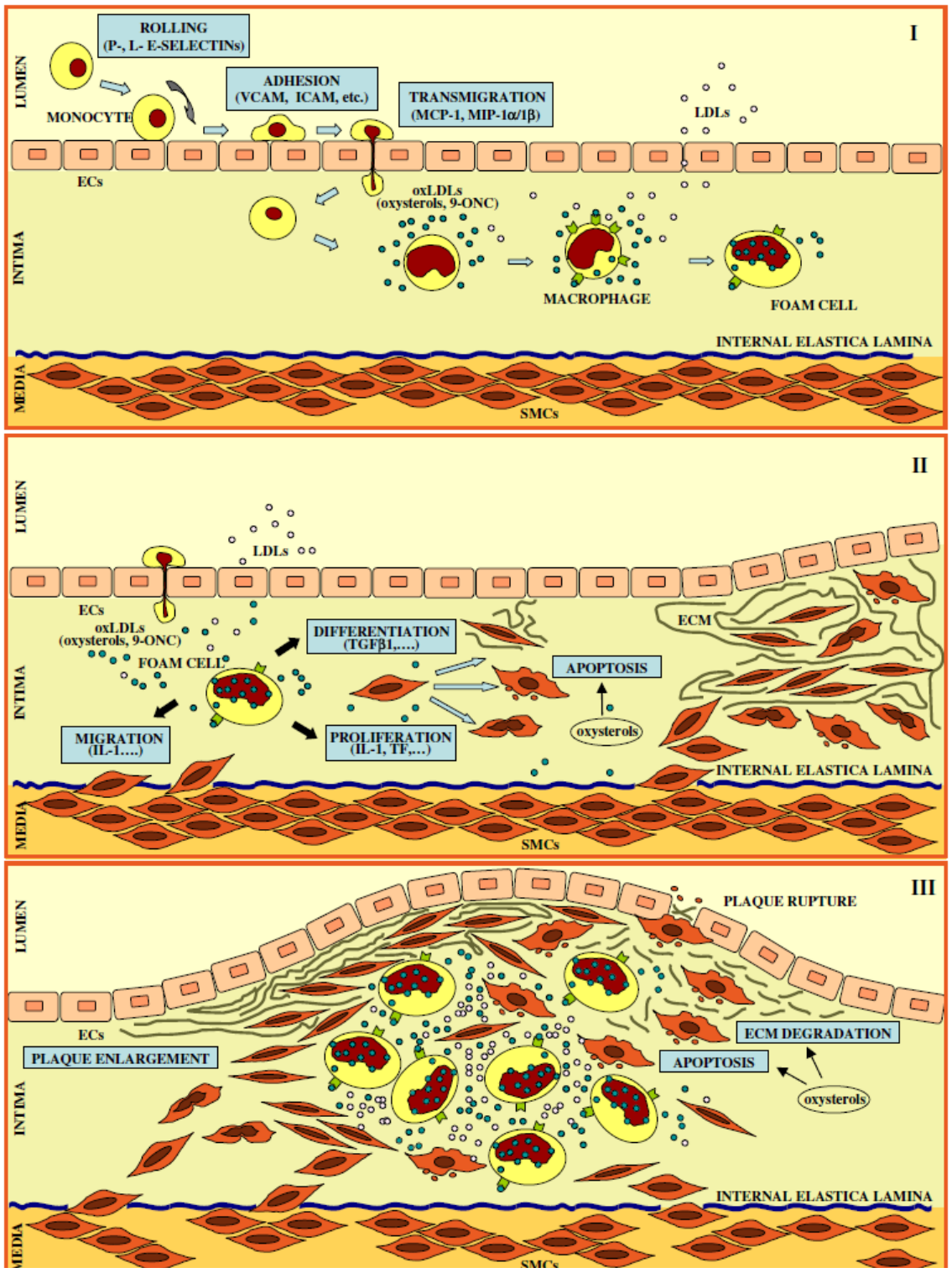

Figure 1.6. Schematic diagram of the vascular remodeling due to atherosclerosis. (Poli et al., 2009)

1.5.2. Oxysterols and Atherosclerosis

Increasing number of experimental studies have revealed that oxysterols are major compounds of favoring the development of atherosclerosis and play crucial role in various stages of the atherosclerotic process including endothelial dysfunction, smooth muscle cell proliferation, leucocyte adhesion and platelet aggregation.

Among side-chain cholesterol oxidation products, 27-OH is the major oxysterol implicated in advanced atherosclerotic lesions. In addition, 7K and 7β-OH are thought to have high pro-atherogenic potential which comprise 75% of all oxysterols found in atherosclerotic plaques from different sites (Khatib and Vaya, 2014; Zarrouk et al., 2014). It has been demonstrated that oxysterols can contribute endothelial dysfunction where 7K has upregulated the expression of proatherogenic actin-binding protein profilin-1 in aortic endothelial cells through activation of signal transducer and activator of transcription 3 (STAT3) (Romeo and Kazlauskas, 2008).

Another studies showed that oxysterols can contribute to monocyte differentiation and also foam cell formation which is the key process during the development of atherosclerotic lesions. In this relation, Hayden et al. demonstrated that 7K favors monocyte differentiation through a sterol-mediated regulatory pathway and promotes foam cell formation in THP-1 cells (Hayden et al., 2002). Moreover, 7K could also contribute to fatty streak formation and induces production of pro-inflammatory molecules by M1 and M2 macrophage subsets (Buttari et al., 2013). Moreover, 7K as well as 7β-OH, and 5β,6β-epoxycholesterol can induce expression of adhesion molecules that contribute to monocyte accumulation at the sub-endothelial level (Vejux et al., 2008, 2007). These oxysterols can also induce endolysosomal phospholipidosis by triggering the formation of myelin figures with high levels of phospholipids. Regarding to this, an in vitro study showed that 7K-induced phospholipidosis involves downregulation of PI3K/Akt signaling pathway which is inhitable by vitamin E in U937 cells (Vejux et al., 2009).

Increasing evidence is accumulating of the pro-inflammatory effects of oxysterols on vascular cells that contribute to their pro-atherogenic role. Various oxysterols including 7β-OH, 7K, 25-OH, 24-OH, and TRIOL have been shown to

induce the expression of chemokines such as as monocyte chemotactic protein-1 (MCP-1), inflammatory cytokines such as interleukin-1 (IL-1) and interleukin-8 (IL-8) in vascular cells including monocytic, endothelial and smooth muscle cells (Erridge et al., 2009; Lemaire-Ewing et al., 2009; Rydberg et al., 2003). Lemaire-Ewing and colleagues demonstrated that in THP-1 cells IL-8 is upregulated either by 7β-OH or by 25-OH, was shown to be associated with Ca^{2+} influx and leading to of transcription factor activator protein-1 (AP-1) activation (Lemaire-Ewing et al., 2009). In recent studies, the crucial role of PKC and ERK1/2 signaling pathways in oxsyterol-induced cytokine inflammation was also reported (Poli et al., 2009). Induction of MCP-1 over-expression has been reported in promonocytic U937 cells challenged with a biologically relevant mixture of oxysterols, involving activation of ERK1/2 pathway and increased nuclear translocation of NF-κB. Furthermore, 7α-OH was shown to upregulate both expression and synthesis of MCP-1 in macrophage cells (Leonarduzzi et al., 2005). The pro-fibrogenic activity of oxysterols was also studied in terms of modulation of transforming growth factor-β1 (TGFβ1) while mixture of oxysterols was shown to induce both expression and synthesis of TGFβ1 in murine macrophagic cells (Leonarduzzi et al., 2001).

Based on the studies, it can be suggested that in early phases of atherosclerotic plaque development, the increase of oxidized cholesterol mixture might provoke pro-inflammatory, proliferative and pro-fibrogenic stimuli rather than pro-apoptotic, thus leading a Trojan horse like effect (Hajjar and Haberland, 1997). Moreover, in advanced lesions, the pro-apoptotic effects exerted by oxysterol leads to their excessive accumulation and favors cap destruction.

2. AIM OF THE STUDY

Oxysterols are a family of 27-carbon molecules and generated from cholesterol oxidation by both enzymatic and non-enzymatic mechanisms. A number of studies demonstrated the pro-apoptotic, pro-oxidant and pro-inflammatory effects of these compounds which likely contribute the development of variety pathophysiological processes. Accumulating evidence suggested that oxysterols are able to modulate not only death pathways but also anti-apoptotic signals in targeted cells; however, the latter matter has not yet been investigated in deep. The lack of published studies addressing the molecular mechanism underlying the survival response and adaptation stage against the low concentrations of these compounds has prompted our study. Thus, we have identified the aim of this thesis as such; to identify the main genes and related products involved in the transduction of survival signals and elucidate the relevant molecular mechanisms in human promonocytic cells (U937) challenged with low concentration of 27-hydroxycholesterol (27-OH) by far the most represented oxysterol in human blood.
Specifically, the aim was;

i. to elucidate molecular mechanism of low concentration (10µM) of 27-hydroxycholesterol induced early response (<24 h) survival/apoptosis delay,
ii. to investigate the impact of both mitogen-activated protein kinase ERK kinase (MEK)/ERK and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways on this survival mechanism,
iii. to understand role of Bcl-2 family proteins whereby 27-OH may generate survival signals in cells of the macrophage lineage,
iv. to clarify whether the pro-oxidant effect of 27-OH is involved in that survival response,

v. to identify the possible involvement of Nrf2 and antioxidant response in the prolonged cell survival in 27-OH treated cells.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

All chemicals used in this study are listed in Appendix A.

3.1.2. Antibodies and enzymes

All antibodies and enzymes used in this study are listed in Appendix B.

3.1.3. Growth Media

RPMI: U937 cell lines was maintained in filter-sterilized RPMI1640 medium (PanBiotechGmbH, Aidenbach,Germany) that is supplemented with 10% fetal bovine serum, 2mM L-Glutamine, 100 U/ml penicillin/streptomycin at 37ºC with 5% CO_2.

Freezing medium: U937 cell lines were frozen in heat-inactivated fetal bovine serum containing 10% DMSO (v/v).

3.1.4. Mammalian Cell Lines

U937: Human promonocytic cell line (ATCC® CRL1593.2 ™)

3.1.5. Molecular Biology Kits and Reagents

Molecular biology kits and reagents that are used for apoptosis screening and protein analysis are listed in Appendix C.

3.1.6. Buffers and Solutions

All buffers and solutions used in this study are listed in Appendix D.

3.1.7. Primers

The primers used in this study are listed in Table 3.1.

PRIMER NAME	SEQUENCE	COMPANY/CATALOG #
Hs_NFE2L2	AAC TTG ATT GAC ATC CTT TGG AGG	Qiagen/ QT00027384
Hs_NQO1	CTC GCC TCA TGC GTT TTTG	Qiagen/ QT00050281
Hs_HMOX1	CGT TTC TGC TCA ACA TCC AGC	Qiagen/ QT00092645

Table 3.1. The list of the primers used in this thesis. Primer names, their sequences, supplier company and their catalog numbers are given.

3.1.8. Protein Molecular Weight Marker

Protein molecular weight marker used in this study is listed in Appendix E.

3.1.9. Equipments

Equipments used in this study are listed in Appendix F.

3.2. Methods

3.2.1. Mammalian Cell Culture and Treatments

The human promonocytic cell line U937 was obtained from Professor Giuseppe Poli, cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin/streptomycin and maintained at 37 °C with 5% CO_2 in a humidified incubator. The cells were dispensed at 1×10^6 /mL in culture plates 96-well, 6-well, 12-well, 60 mm or 100 mm depending on the experiment and made quiescent through overnight incubation in serum-free medium; they were then placed in RPMI-1640 medium with 2% fetal bovine serum and treated with 27-OH dissolved in ethanol. In some experiments, cells were pretreated (45min) with N-acetylcysteine

(NAC), an antioxidant compound, or with PD98059, a selective inhibitor of MEK1/2, or with diphenyleneiodonium chloride (DPI) , an inhibitor of nitric oxide synthase, or with LY294002, a selective inhibitor of PI3K.

3.2.2. Cell death, viability and proliferation assays

In order to detect cell viability or cell proliferation, exposure to 27-OH was determined by WST-1 (Cell Proliferation Reagent) following the manufacturer's instruction. Briefly, U937 cells seeded in 96-well plates were treated as indicated and 10 μl of WST-1 reagent was added to each well, after which the plates were incubated for 4h at 37 °C. Absorbance was measured with a microtiter plate reader at a reference wavelength of 655nm and a test wavelength of 450 nm. Results were expressed as percentage of cell viability, proliferation versus controls, taken as 100%. Apoptotic cell death response was determined by an AnnexinV affinity assay. U937 cells seeded in 12-well plates were treated as indicated, transferred to flow cytometry tubes, and harvested by centrifugation at 300g for 5min. The cells were then resuspended in 1ml of cold PBS and again centrifuged at 300g for 5min. After removal of supernatant, the cells were incubated in AnnexinV buffer (see Appendix D) containing 1% (v/v) AnnexinV (FITC) for 15 min in the dark. Cells were analyzed by FACS on FlowJo software. The rate of apoptosis was evaluated through 4,6-diamidino-2-phenylindole (DAPI) staining. To identify apoptotic nuclei, cells were fixed and permeabilized with 95% cold ethanol for 5 min at room temperature, and then washed twice with 0.1 M PBS. Slides were then incubated for 15 min at room temperature in DAPI solution. After rinsing in PBS, cells were observed and photographed under a Zeiss Axiovert 200 M fluorescence microscope.

3.2.3. Cleaved caspase 3 staining

Cells were seeded in 12-well plates and subjected to 3% formaldehyde (w/v) fixation for 10 min. Fixed cells were incubated with Methanol for 15 min at room temperature for permeabilization and washed with % 0.5 BSA (w/v) containing FACS incubation buffer (see Appendix D). Cells were incubated for 1 hour with 1:400 anti-cleaved caspase 3 monoclonal antibody and then with 1:800 FITC-anti-rabbit secondary

antibody for 30 min. After two washes with PBS (pH: 7, 4), cells were subjected to FACS analysis on FlowJo software.

3.2.4. Quantification of 27-OH in U937 cells by mass spectrometry

Cells were incubated with 10 μM 27-OH up to 72h. At different incubation times, cells were centrifuged, washed with PBS, and resuspended in 1ml NaCl 0.9%. The deuterium labeled 27-OH-d7 was added as internal standard and lipids were extracted with chloroform–methanol (2/1, v/v). The intracellular concentrations of the oxysterol were quantified by isotope dilution mass spectrometry essentially as previously described (Iuliano et al., 2003). The mass spectrometer was set to the selected ion monitoring mode; the ions used for analysis were as follows: [$^{2}H_{7}$] 27-OH 463 m/z, 27-OH 456 m/z. Quantification of the oxysterol was made by the internal standard ratio method.

3.2.5. Protein extraction and immunoblotting

Cells were treated as indicated and harvested by centrifugation at 300g for 30 s. Following resuspension in 1 ml of ice-cold PBS and transfer to 1.5 ml microfuge tubes, cells were spun at 2640g for 30 s. The pellet was lysed by incubation for 30 min in 200 μl of cold cell lysis buffer. After centrifugation at 2640g for 10 min, the supernatant containing the total protein extract was removed and stored at -80°C. For nuclear and cytoplasmic extracts, following treatments and cold centrifugation, cell pellets were resuspended in hypotonic T1 buffer (see Appendix D) and incubated on ice for 20 min. After the cold incubation, tubes were centrifuged at max speed for 1 minute and supernatants containing cytoplasmic proteins were stored at -80°C. Nuclear protein isolation was carried out by incubation for 20 min on ice in a cold saline T2 buffer (see Appendix D). After cold centrifugation at 13,000g for 20 min, supernatants containing nuclear proteins were removed and stored at -80°C. Proteins (30 μg) were mixed with loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris/HCl pH:6.8) and separated on 10-15% SDS–polyacrylamide gel electrophoresis (PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% blocking reagent in PBS-Tween 20 and

incubated with appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies in 5% blocking reagent. After the required washes with PBS 1X-Tween 20, proteins were finally analyzed using an enhanced chemiluminescence detection system and exposed to Hyperfilm-ECL. All critical immunoblotting experiments were repeated at least three times.

3.2.6. Measurement of protein concentration

Protein concentrations of protein extracts were determined by Bradford solution. Bovine serum albumin (BSA, stock solution: 1mg/ml) was used as the standard protein. To constitute a standard graph, starting with 5μg BSA/well in 96-well plates, BSA was 1:1 diluted. Samples were diluted 1:100 with Bradford solution which were added to the wells. Absorbance was spectrophotometrically measured at 595nm by a microtiter plate reader with the first standard set as blank. Protein concentrations in the samples were then determined by extrapolating their absorbance values against the standard curve. For every new assay a new standard curve was plotted.

3.2.7. RNA extraction and cDNA preparation

Total RNA was extracted from cells using TRIzol reagent, following the manufacturer's instructions. RNA was dissolved in RNase-free water with RNase inhibitors. The amount and purity (A_{260}/A_{280} ratio) of the extracted RNA were assessed spectrophotometrically. cDNA was synthesized by reverse transcription from 2 μg RNA with a commercial kit and random primers, following the manufacturer's instructions.

3.2.8. Real-time RT-PCR

Singleplex real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed on 30 ng of cDNA using TaqMan gene expression assay kits prepared for human NFE2L2 (Nrf2), HO-1, NQO-1 and β-actin, and TaqMan Fast Universal PCR master mix, and analyzed by a 7500 Fast real-time PCR system. The oligonucleotide sequences are not revealed by the manufacturer because of proprietary interests. The cycling parameters were as follows: 20 s at 95 °C for AmpErase UNG

activation, 3 s at 95 °C for AmpliTaq Gold DNA polymerase activation, and 40 cycles of 3 s each at 95 °C (melting) and 30 s at 60 °C (annealing/extension). The fractional cycle number at which fluorescence passes the threshold in the amplification plot of fluorescence signal vs. cycle number was determined for each gene considered. The results were then normalized to the expression of β-actin, as housekeeping gene. Target gene expression was quantified relatively with a mathematical method proposed by Livak & Schmittgen (Livak and Schmittgen, 2001).

3.2.9. siRNA transfection

Small interfering RNA (siRNA) was used for transient gene knockdown studies. The siRNAs used were NFE2L2 s9493 (Nrf2) and siRNA #1 for the negative control (scramble siRNA). Negative control corresponds to a siRNA with nonspecific sequence. Transfection of Nrf-2-specific and control siRNAs was performed following the manufacturer's instructions. Briefly, 50 nM of siRNAs was mixed with 25 μL of transfection reagent solution and left at room temperature for 10 min in RPMI medium with 1% fetal bovine serum and without antibiotics. After 24 h of reverse transfection, the cells (4×10^4 500 μL^{-1}) were incubated with 27-OH for 24 h. For gene expression analysis, total RNA was isolated from the cells and used for quantitative RT-PCR as described above. The transfection efficiency, validated by quantitative RT-PCR, was approximately 87%.

3.2.10. Measurement of intracellular reactive oxygen species

The overproduction of reactive oxygen species (ROS), mainly superoxide anion (O_2^-), was detected by didihydroethidium (DHE) fluorescence staining. After treatment with 27-OH, in the presence or absence of selective inhibitors, the cells were washed and resuspended with RPMI-1640 medium (+2% fetal bovine serum) and incubated for 30 min in the dark with 5 μM DHE at 37 °C. Fluorescence was immediately detected on glass base dishes by a laser scanning confocal microscope (planneofluar lens 40x/0.75) setting the exciting laser band to 543nm, and using a 560–615nm band pass emission filter. All images were processed using LSM510 Image Examiner software.

3.2.11. Measurement of intracellular hydrogen peroxide

To measure hydrogen peroxide (H_2O_2) production, cell aliquots were lysed for cytosolic protein analysis in ice-cold buffer containing 20 mM Hepes, pH 7.9, 0.35 M NaCl, 20% glycerol, 1% Igepal CA-630, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, and protease inhibitors. Levels of H_2O_2 were determined in the cytosolic fractions by monitoring the HRP-dependent oxidation of acetylated ferrocytochrome c, as described by Zoccarato et al (Zoccarato et al., 1993). Ferrocytochrome c (0.8 ml of 50 μM), HRP (2 μl of 40 μg/ml), and p-hydroxyphenylacetic acid (100 μl of 50μM) were added to 100 μl cytosolic samples. The oxidation of acetylated ferrocytochrome c was monitored spectrophotometrically at 550 minus 540 nm, after 1, 2, and 3 min of incubation, using an absorption coefficient of 19.9 mM^{-1} cm^{-1}.

3.2.12. Measurement of transmembrane mitochondrial potential

5,50,6,60-Tetrachloro1,10,3,30-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) is a cationic dye that exhibits membrane-potential-dependent accumulation in mitochondria as J-aggregates that, in depolarized mitochondria, are converted to JC-1 monomers; this is indicated by the fluorescence emission shift from red to green. Before incubation with oxysterols and specific inhibitors, U937 cells were incubated for 10min with 10 mg/ml JC-1 in RPMI 1640 medium with 1% fetal bovine serum. After treatments, cells were observed through a LSM510 confocal laser microscopy system. The images were processed using LSM510 Image Examiner software.

3.2.13. Statistical Analysis

All the illustrated results represent one of at least three independent experiments with similar outcomes. All numerical data are presented as means ± standard deviation (SD). Statistical significance of the results was analyzed by the Student's t-tail test and *P<0.05, **P<0.01 and ***P<0.001 were considered statistically significant.

3.2.14. Densitometric analysis

Densitometric values for immunoblotting results were calculated using Image J software.

3.2.15. Illustrations

All illustrations in all sections including “Introduction”, “Results”, “Discussion” and “Conclusion” were designed using Adobe Photoshop CS5 & CS6, IrfanView and MS Office 2007 and 2010 softwares.

4. RESULTS

4.1. Effect of 27-OH treatment on cell viability and cell death in U937 promonocytic cells

4.1.1. Dose-dependent pro-apoptotic effect of 27-OH

In order to determine the pro-apoptotic effect of 27-OH, aliquots of U937 cells were incubated with different final concentrations (10, 50 and 100 µM) of 27-OH for up to 72 h. Cell death was then assessed by the Annexin V test and present results show that 27-OH-induced apoptosis in U937 promonocytic cells depending on its final concentration (Fig. 4.1). In particular, concentrations above 50 µM of 27-OH doubled the number of Annexin V-positive cells present versus the control after 24 h cell treatment indicating the cells undergoing cell death, while at low concentrations of 27-OH (10 µM), significant induction of apoptosis only occurred after 72 h.

To investigate the type of cell death in high concentrations of 27-OH treated U937 cells, after 24 h incubation with 10 and 100 µM, cells were fixed, permeabilized and labeled with cleaved caspase 3 monoclonal antibody and evaluated by FACS analysis (Figure 4.2). While at a final concentration of 100 µM showed active caspase 3 positive cells about 30%, cells treated with low concentration (10 µM) did not show a significant change in caspase 3 cleavage after 27-OH treatment. These results indicate that cell death induced by high concentration of 27-OH is mainly apoptotic The anticancer drug cisplatin (30 µM) was used a positive control for this experiment and showed active caspase 3 positive cells as expected.

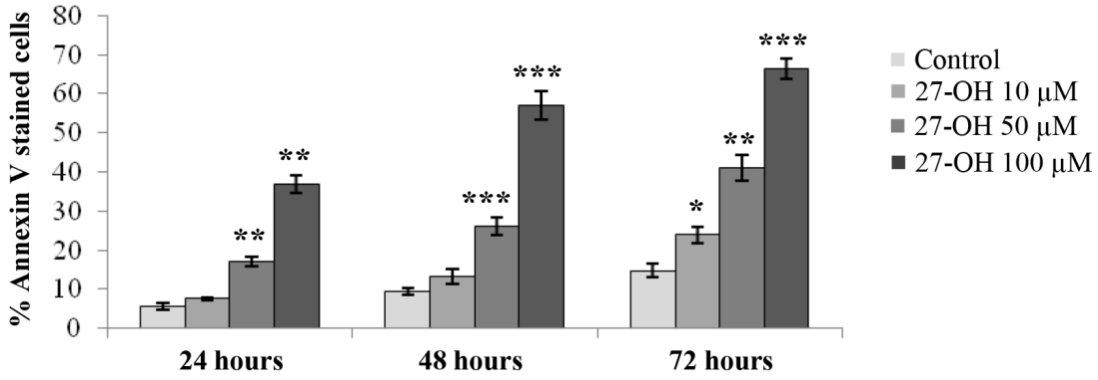

Figure 4.1. The pro-apoptotic effect of 27-hydroxycholesterol (27-OH) is dose dependent. Different kinetics of 27-OH-induced apoptosis in U937 cells was determined by flow cytometry. U937 cells were treated with increasing concentrations of 27-OH (10, 50 and 100 µM) for 24, 48, and 72h, stained with FITC– AnnexinV and subjected to FACS analysis. Untreated cells (Control) were used as controls. Relevant histograms represent the mean values ±SD of all four independent experiments; *P<0.01, **P<0.005, and ***P<0.001 vs control at each time point.

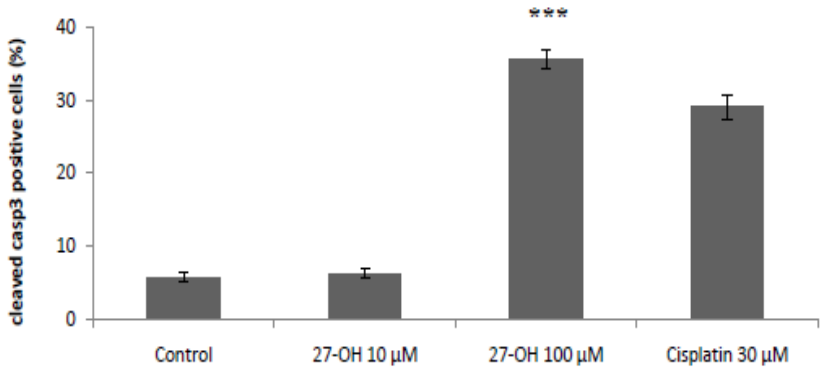

Figure 4.2. High micromolar concentration of 27-hydroxycholesterol (27-OH) induces apoptosis. U937 cells were treated with 27-OH (10 µM and 100 µM) for 24 h or cisplatin 30 µM for positive control and then incubated with a specific monoclonal antibody against cleaved caspase 3. Following required washes, cells were subjected to FITC anti-rabbit antibody incubation. Stained cells were then analyzed by flow cytometry. Untreated cells (Control) were used as controls. Relevant histograms represent the mean values ±SD of all three independent experiments; ****P*<0.001 vs. control at each time point.

4.1.2. Pro-apoptotic effect of low micromolar concentration of 27-OH

Physiological level of oxysterols in plasma is very low and it has been shown that the concentration of 27-OH in human serum is about 117±35 ng/ml (Honda et al., 2009). Previous studies have shown that concentrations of oxysterols above 50 µg/ml could be pro-apoptotic (Trevisi et al., 2009). Thereby, we have used the lowest oxysterol concentration, i.e., 10 µM of 27-OH in further experiments for the evaluation of molecular and cellular mechanisms of survival effects of oxysterol.

Focusing on the low concentration of 27-OH (10 µM), cell viability and proliferation was measured in terms of WST-1 tetrazolium salt reduction by cellular dehydrogenases (Fig. 4.3. A). WST-1 assay which was performed for the interval considered (0–72h), showed that 10 µM 27-OH caused a slight though significant reduction of cell viability and proliferation at 72 h. However, it induced a slight increase in cell proliferation after 6-12 h treatment of 27-OH suggesting that a survival adaptation response was induced.

In addition to WST-1 assay, we also performed an Annexin V staining to confirm assay results and also to determine a time course of its pro-apoptotic effect in response to low concentration of 27-OH (Fig. 4.3 B). U937 cells were treated with 27-OH up to 7 days and time course of pro-apoptotic effect was evaluated by FACS analysis. Under the experimental conditions applied here, the number of apoptotic cells was about 20% of the total after 3 days, 40% after 5 days, and about 60% after 7 days of treatment. A significant induction of apoptosis was obtained after long term treatment of low concentration of 27-OH.

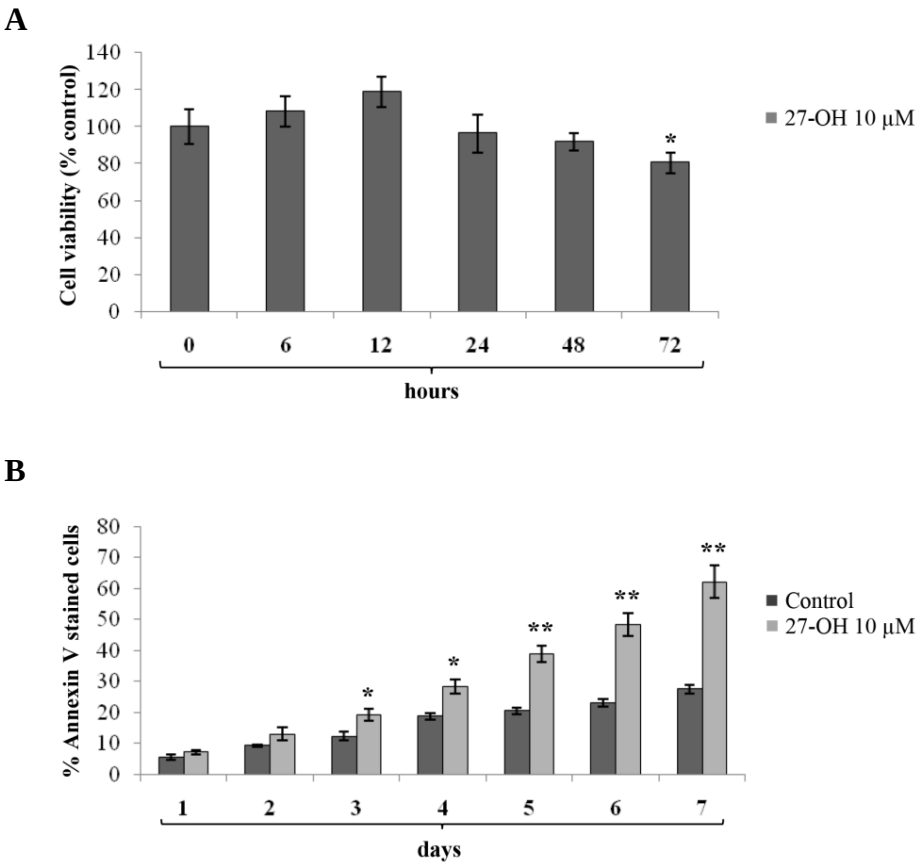

Figure 4.3. The pro-apoptotic effect of 27-hydroxycholesterol (27-OH) is time dependent. (A) Effect of 27-OH (10 µM) on U937 cell viability. Viability of U937 cells was analyzed by the WST-1 assay, following treatment with 10 µM 27-OH for times between 6 and 72 h. Untreated cells (Control) were taken as controls. Average absorption values versus untreated cells are displayed after multiplication by 100. Assay and results represent the mean values ±SD of all three independent experiments with six repeats; *P<0.05 vs. control. (B) Time course of the pro-apoptotic effect of a low micromolar amount (10 µM) of 27-OH. Cells were treated for 1 to 7 days with 27-OH at a final concentration of 10 µM and cell death was analyzed by flow cytometry. Untreated cells (Control) were taken as controls. Histograms represent the mean values ±SD of all four independent experiments; *P<0.01 and **P<0.001 vs. control at each time point.

4.2. Determination of 27-OH actual concentrations in 27-OH treated U937 promonocytic cells

As discussed above, the physiological level of oxysterols in plasma is very low. Since 10 µM dose is much higher than given the physiological concentration, we measured 27-OH concentrations in 27-OH treated U937 promonocytic cells to show that the levels do not exceed the pathophysiological range. Thus, in order to quantify the 27-OH amount actually present overtime within promonocytic cells, U937 cells were incubated with a single dose of 10 µM 27-OH up to 72 h. After different incubation times (5min-72h), cell aliquots were taken from the same cell suspension and 27-OH intracellular amount was quantified by mass spectrometry as described under Materials and Methods. As shown in Fig.4.4, from the very beginning (1.5 h) the amount of 27-OH ranged between 18 and 26% of the total oxysterol amount added to the cells, showing a steady concentration over the experimental time (up to 72 h) with non significant and minor variations.

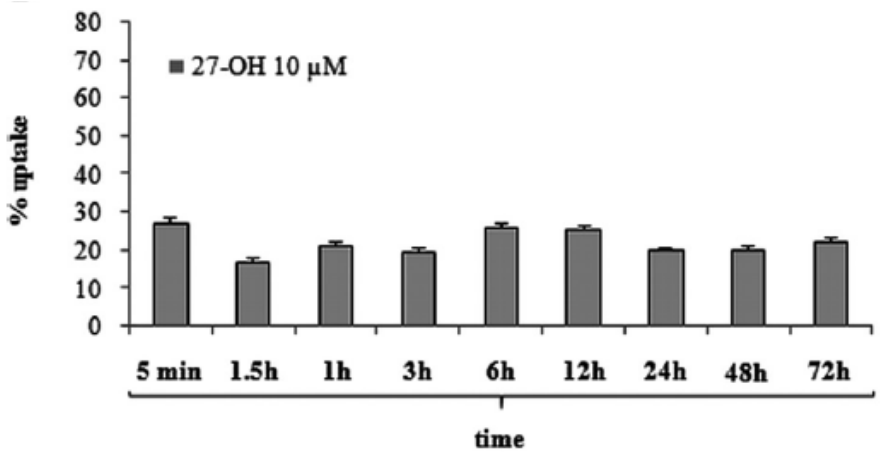

Figure 4.4. Measurement of 27-hydroxycholesterol (27-OH) amount within U937 cells. Cells were treated up to 72 h with a single dose of 10 µM 27-OH and the oxysterol concentrations were measured by mass spectrometry. Histograms represent the percentage of 27-OH present in the cells compared to the total oxysterol administered (100%) at each time point.

4.3. 27-OH induced modulation of ERK1/2 and PI3K/Akt survival pathways

4.3.1. Low micromolar concentration of 27-OH produces stimulation of ERK1/2 and Akt phosphorylation

After determination oxysterol actual amount in cells and selection final concentration of 27-OH, the molecular mechanism of the survival signal in response to low concentration of 27-OH was questioned. In order to get more detailed insight on molecular mechanism at the signal transduction level, a possible involvement of two well-known cell survival signals, ERK1/2 and Akt was investigated. Since the well-known apoptotic effect of 27-OH was actually delayed by almost 3 days when U937 cells were incubated in the presence of a 10 μM concentration, the possible prevalence of survival signals over death signals during this early time interval (up to 72 h) was investigated. As reported in Fig.4.5, low micromolar concentration of 27-OH produces a transient stimulation of ERK1/2 and Akt survival pathways. The results demonstrated that a significant increase of ERK1/2 and Akt (Thr308) phosphorylation was observed in U937 cells treated with 10 μM 27-OH. Moreover, this up-regulation marked at maximum between 6 and 24 h incubation with regard to ERK, and between 6 and 48 h incubation in the case of Akt.

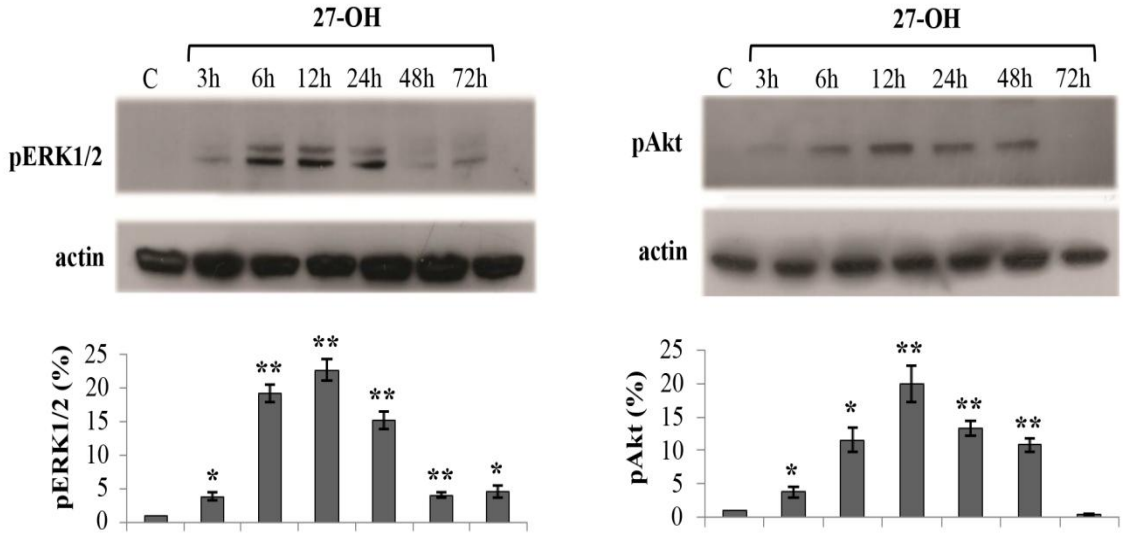

Figure 4.5. Phosphorylation of ERK1/2 and Akt induced by low micromolar concentration of 27-hydroxycholesterol (27-OH). U937 cells were treated with 10 μM 27-OH for 3 to 72 h. Untreated cells (Control) were taken as controls. Levels of phosphorylated ERK1/2 and phosphorylated Akt proteins were analyzed by Western blotting. Actin was used as loading control. One blot representative of three experiments is shown for each protein. Histograms represent the mean values ±SD of all three independent experiments; densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control values; *P<0.01, **P<0.005, and ***P<0.001 vs. control.

4.3.2. Effect of high micromolar concentration of 27-OH on ERK1/2 and Akt phosphorylation

Although, low molecular concentration of 27-OH may induce survival response including ERK1/2 and Akt signaling pathways, it raised the question whether if pro-apoptotic, high concentration of the oxysterol inhibited these survival pathways or had any negative effect on the given response. Thus, immunoblotting experiments were performed up to 72h with U937 cells treated with 100 μM 27-OH. As shown in Fig.4.6, high micromolar concentration of 27-OH does not induce any survival response including ERK1/2 and Akt signaling pathways. A significant down-regulation was observed in U937 cells which occurred at 6h and between 48-72 h incubation with regard to ERK, and between 24 and 48 h incubation in the case of Akt. According to

these results, it can be suggested that 27-OH induced survival response in promonocytic cells is treated concentration and time specific.

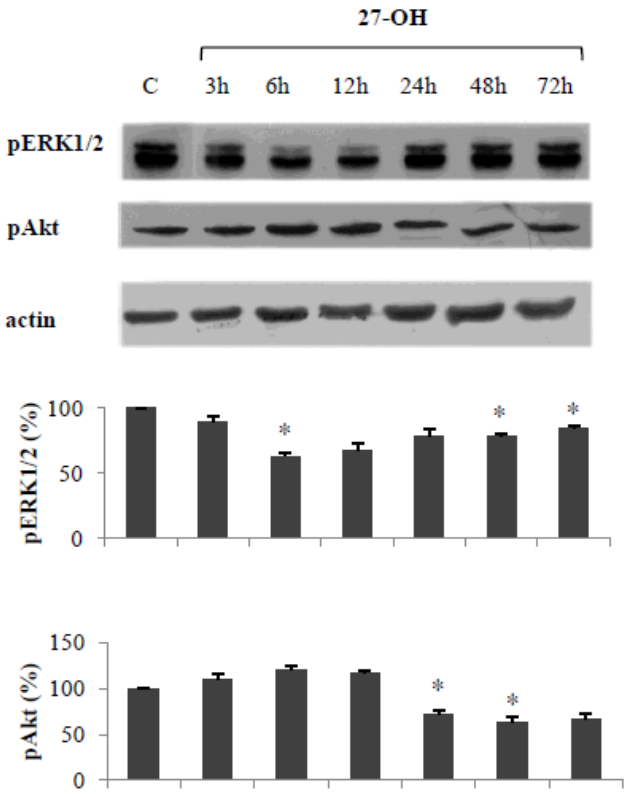

Figure 4.6. High micromolar concentration of 27-hydroxycholesterol (27-OH) does not induce ERK1/2 and Akt phosphorylation. U937 cells were treated with 100 μM 27-OH for 3 to 72 h. Untreated cells (Control) were taken as controls. Levels of phosphorylated ERK1/2 and phosphorylated Akt proteins were analyzed by Western blotting. Actin was used as loading control. One blot representative of three experiments is shown for each protein. Histograms represent the mean values ±SD of all three independent experiments; densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control values; *P<0.01 vs. control.

4.4. Expression of Bcl-2 family proteins in response to low micromolar concentration of 27-OH

After signal transduction level of the survival response mechanism was elucidated, the role of B-cell lymphoma (Bcl)-2 family proteins was investigated, which are the important apoptosis regulators and placed at the helm of the execution phase of apoptosis. For this purpose, to determine the expression pattern of anti and pro-apoptotic members of Bcl-2 family, U937 cells were treated with low concentration of 27-OH then total cell lysates were subjected to immunoblotting using specific antibodies.

4.4.1. Increased phosphorylation of Bad at Ser75 and Ser99, in U937 cells challenged with a low micromolar concentration of 27-OH

Based on the finding that activation of the PI3K/Akt signaling pathway culminates in phosphorylation of the Bcl-2 family member pro-apoptotic Bad protein, and it thus promotes cell survival through inhibiting Bad's pro-apoptotic effect (Datta et al., 1997), phosphorylation of Bad protein was examined by immunoblotting, in U937 cells treated with 10 μM 27-OH up to 72 h. As illustrated in Fig.4.7, Bad was phosphorylated at the level of Ser75 at some point between 3 and 72 h cell incubation, likely in connection with MEK/ERK phosphorylation. The maximum increase in Bad phosphorylation in response to 27-OH treatment was observed by 12 h. Moreover, Bad phosphorylation was also observed at its Ser99 residues at some point between 3 and 48 h cell incubation which reached a maximum at 12 h, likely depending on 27-OH induced Akt phosphorylation.

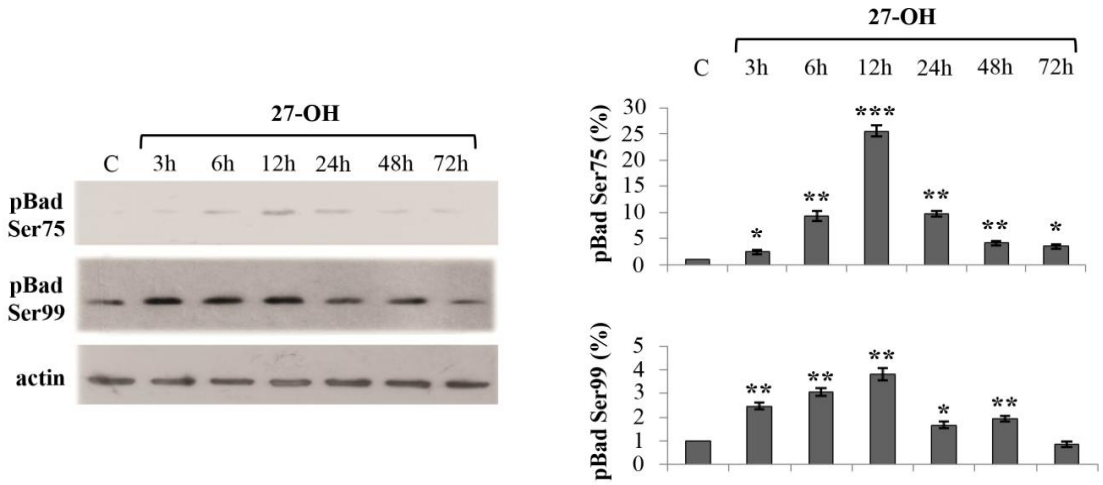

Figure 4.7. Phosphorylation of pro-apoptotic Bad protein induced by 27-hydroxycholesterol (27-OH). U937 cells were treated with 10 μM 27-OH for 3 to 72 h. Untreated cells (Control) were taken as controls. Levels of phosphorylated Bad (Ser 75 and Ser 99) proteins were analyzed by Western blotting. Actin was used as loading control. One blot representative of three experiments is shown for each protein. Histograms represent the mean values ±SD of all three independent experiments; densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control values; *P<0.01, **P<0.005, and ***P<0.001 vs. control.

4.4.2. Bim and Bcl-xl proteins are not involved in survival response induced by low micromolar concentration of 27-OH

In addition to Bad protein, to elucidate the expression pattern of Bcl-2 family proteins in detail, the expression of two important apoptosis/survival regulators, pro-apoptotic Bim and anti-apoptotic Bcl-xl proteins was analyzed by immunoblotting. For this purpose, U937 cells were treated with 10 μM 27-OH up to 48 h and then total cell lysates were subjected to immunoblotting using antibodies specific for Bcl-xl and Bim. As shown in Fig.4.8, while Bcl-xl expression very slightly increased at 12 h in response to low concentration of 27-OH, Bim expression did not indicate a significance change.

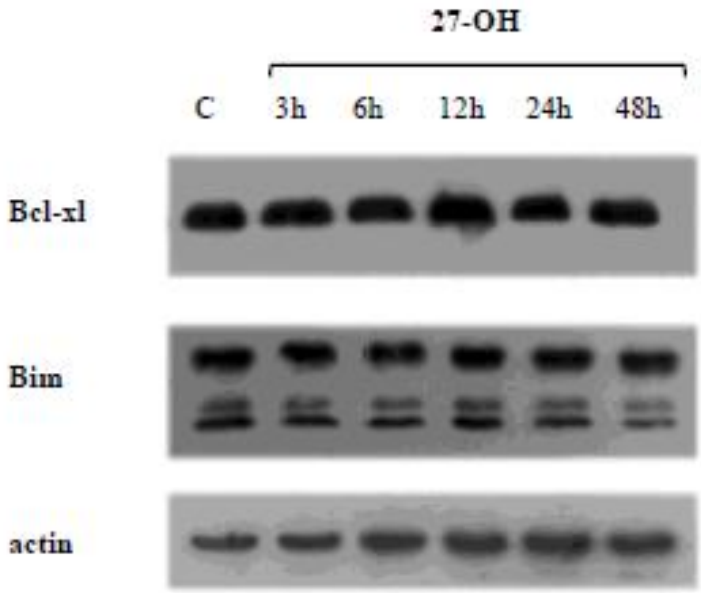

Figure 4.8. Modulation of anti-apoptotic Bcl-xl and pro-apoptotic Bim proteins by 27-hydroxycholesterol (27-OH). U937 cells were treated with 10 μM 27-OH for 3 to 48 h. Untreated cells (Control) were taken as controls. Levels of Bcl-xl and Bim proteins were analyzed by Western blotting. Actin was used as loading control. One blot representative of three experiments is shown for each protein. Histograms represent the mean values ±SD of all three independent experiments.

4.5. Effect of MEK/ERK and PI3K/Akt signaling pathways inhibition on the pro-apoptotic effect of 27-OH

In order to determine the noncytotoxic concentrations of two metabolic inhibitors, namely the MEK/ERK inhibitor, PD98059 and the PI3K/Akt inhibitor, LY294002, FACS analysis was utilized. Therefore, U937 cells were treated with different concentrations of inhibitors and incubated for 24 h, and then cells were subjected to FITC–AnnexinV staining to analyze the cell death by flow cytometry. The FACS analysis results showed that 10, 20, 40 μM final concentrations for PD98059 (Fig.4.9 A) and 10 and 25 μM final concentrations for LY294002 (Fig.4.9 B) are noncytotoxic for U937 promonocytic cells. Depending on the results obtained and previously published papers, 40 μM PD98059 and 25 μM LY294002 were used for further molecular experiments.

To investigate the involvement of ERK and Akt signaling pathways in low concentration of 27-OH induced survival response, U937 cell suspensions preincubated (45 min) with either ERK or Akt inhibitors, PD98059 and LY294002 respectively. The number of Annexin V positive cells was measured by FACS analysis and the percentage of apoptotic cells was already about 20%, after 24 h challenge with 10 μM 27-OH (Fig. 4.9 C) which is approximately the percentage of apoptotic cells induced by this low concentration of 27-OH when cell incubation was continued to 72 h in the absence of the ERK/ Akt inhibitors. In particular, the importance of ERK and Akt-dependent survival signaling inhibition on cell survival was of course more evident after 48 and 72 h oxysterol incubation of U937 cells; the percentage of apoptosis was measured as 30% after 48 h and about 40% after 72 h treatment of the oxysterol. These results support that prevention of ERK-Akt-dependent survival signaling anticipated the pro-apoptotic effect of the low micromolar concentration of the oxysterol.

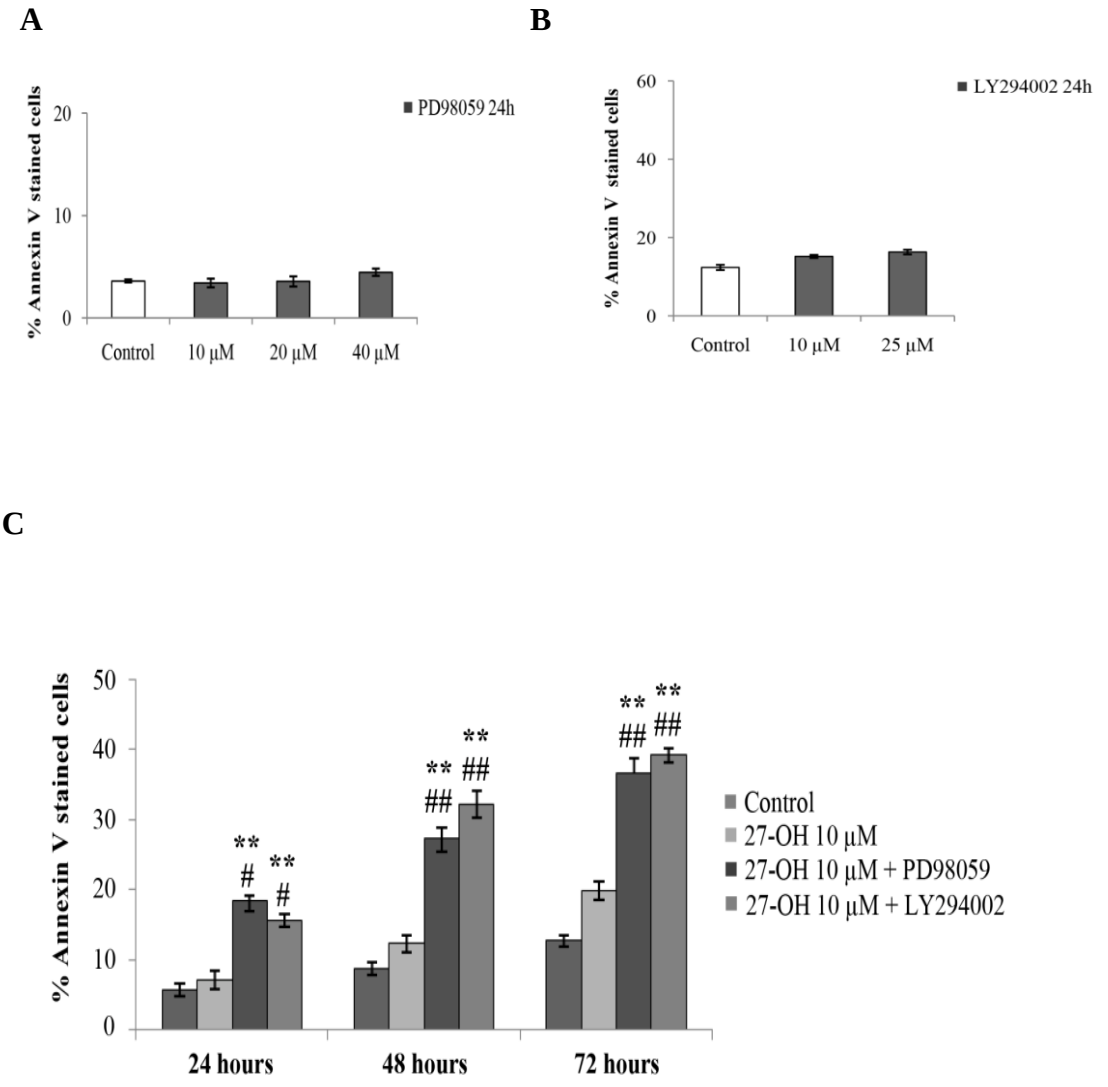

Figure 4.9. Inhibition of MEK/ERK and PI3K/Akt signaling pathways anticipates the apoptotic effect of 10 µM 27-OH. Untreated cells were taken as controls. Cells were treated with indicated concentrations of inhibitors for 24 h. Cytotoxicity of inhibitors (A) PD98059, a selective MEK1/2 inhibitor, and (B) LY294002, a selective PI3K inhibitor was determined by flow cytometry. (C) U937 cells were either treated with 27-OH (10 µM) alone or preincubated with PD98059 (40 µM) or LY294002 (25 µM) 45 min before 27-OH treatment. FACS analysis was performed by harvesting and FITC–AnnexinV staining the cells at 24, 48, and 72 h, to analyze the effect of inhibitors on cell death response. Histograms represent the mean values ±SD of all four independent experiments; *P<0.01 and **P<0.001 vs. control at each time point; #P<0.005 and ##P<0.001 vs. 27-OH at each time point.

4.6. Effect of MEK/ERK and PI3K/Akt signaling pathways inhibition on the 27-OH induced Bad phosphorylation

To examine the dependence of Bad phosphorylation and of the consequent loss of its pro-apoptotic potential, on the activation of either ERK (Bad phosphorylation at Ser75) or Akt (Bad phosphorylation at Ser99) a series of immunoblotting experiments were performed employing two metabolic inhibitors, PD98059 and LY294002. Therefore, U937 cell aliquots were pretreated (45 min) with either 40 μM PD98059 or 25 μM LY294002. The results showed that MEK/ERK inhibitor PD98059 dramatically reduced ERK1/2 phosphorylation after 6 and 12 h treatment with 10 μM 27-OH (Fig. 4.10 A). Moreover, it significantly prevented Bad phosphorylation at Ser75. As expected, cell pretreatment with PI3K/Akt inhibitor LY294002 fully prevented Akt phosphorylation and Bad phosphorylation at Ser99 after 6 and 12 h treatment with 10 μM of the oxysterol (Fig. 4.10 B). These results confirmed the dependence of Bad phosphorylation to ERK-Akt signaling pathways that results in marked anticipation of the pro-apoptotic effect of 27-OH.

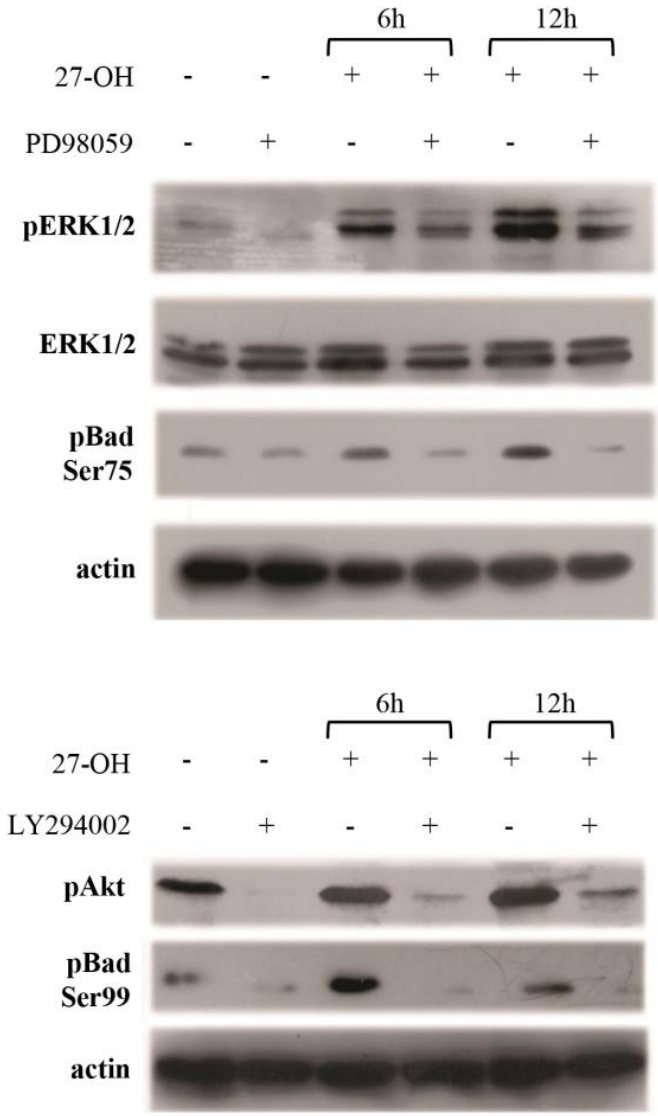

Figure 4.10. Erk- and Akt-dependent Bad phosphorylation: effect of selective inhibitors. U937 cells were either treated with 27-OH (10 μM) alone (6 and 12 h) or preincubated with PD98059 (40 μM) or LY294002 (25 μM) 45 min before 27-OH treatment. Untreated cells were taken as controls. (A) Levels of phosphorylated and nonphosphorylated ERK1/2, phosphorylated Bad (Ser75), and (B) phosphorylated Akt and phosphorylated Bad (Ser99) proteins were analyzed by Western blotting. Actin was used as loading control. One blot representative of three experiments is shown for each protein.

4.7. Determination of intracellular ROS levels in U937 cells treated with 27-OH

The pro-oxidant effect of low dose of 27-OH in U937 promonocytic cells was determined with the fluorescent probe DHE as shown in Fig.4.11. Challenging U937 cells with a single 10 μM dose of 27-OH for a time interval between 30min-96h, confirmed the pro-oxidant effect of this oxysterol in which a biphasic increase of ROS steady-state levels was observed. As shown in microscopy results, in DHE-stained cells with red fluorescent, intracellular ROS showed a peculiar trend; a marked increase of ROS levels was evident until 4–5 h of cell treatment. However, ROS intracellular levels disappeared between 6 and 24/48 h of cell treatment, and interestingly reappeared at longer incubation times such as 72 and 96 h. This peculiar trend suggested that ROS disappearance correlates with maximum phosphorylation times (6-12 h) of ERK and Akt signaling pathways. Thus, the first oxidative burst was demonstrated after 30 min-3h incubation, to be responsible for the transient up- regulation of ERK1/2 and Akt phosphorylation.

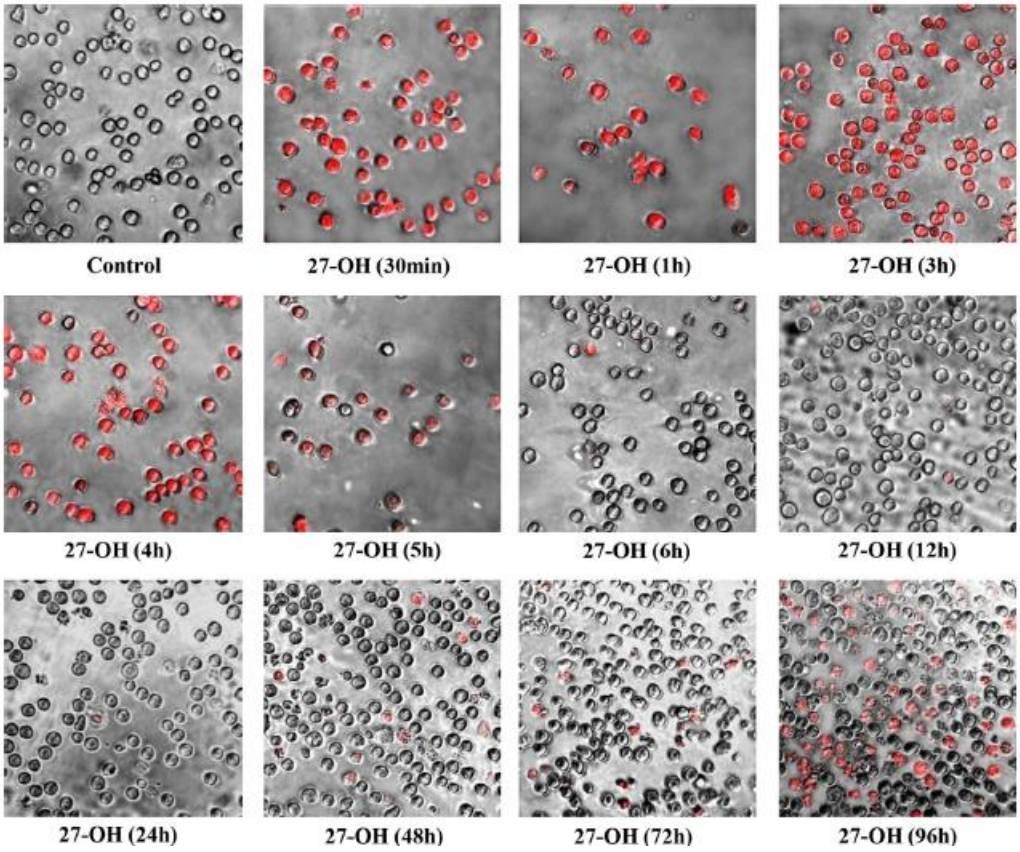

Figure 4.11. Pro-oxidant effect of low micromolar concentration of 27-hydroxycholesterol (27-OH). Intracellular generation of ROS was run with dihydroethidium (DHE) in U937 cells incubated with the oxysterol for 1 to 72 h. Untreated cells (Control) were taken as controls.

To confirm DHE staining results for intracellular ROS generation, another technique was also used. Therefore, U937 promonocytic cells treated with a single dose of 27-OH (10 μM) and then incubated up to 72 h at 37°C, the pro-oxidant effect of the oxysterol was confirmed by monitoring H_2O_2 production. As shown in Fig. 4.12, a significant increase in ROS intracellular levels was evident until 6 h incubation; however it disappeared at longer incubation times, i.e. between 12 and 72 h. These results confirmed the remarkable but transient pro-oxidant effect of the low dose of the oxysterol. As shown by DHE staining previously, in the time frame corresponding to the up-regulation of phosphorylation of ERK and PI3K/Akt induced by 27-OH, i.e. between 6 and 24 h after cell treatment, ROS generation resulted to be quenched. This finding suggested the ability of ERK and Akt signaling pathways to modulate the steady-state of cellular ROS.

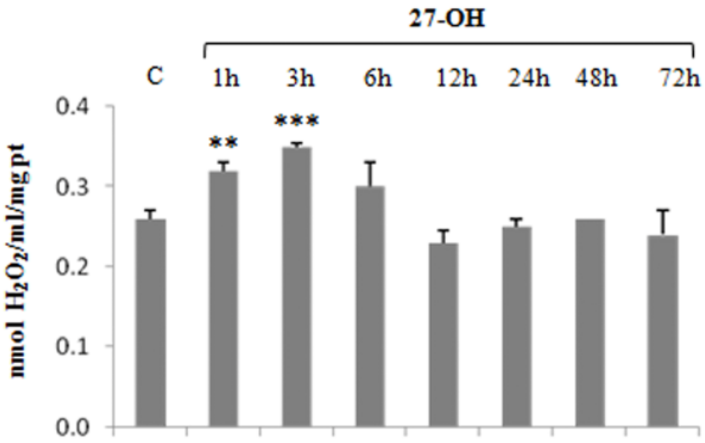

Figure 4.12. Effect of 27-hydroxycholesterol (27-OH) on H_2O_2 production. U937 cells were incubated with 10 μM 27-OH for 1 to 72 h. H_2O_2 production was measured spectrophotometrically. Histograms represent the mean values ±SD of all three independent experiments. **p<0.005 and ***p<0.001 vs. control group.

4.7.1. Effect of high micromolar concentration of 27-OH on ROS generation

To observe any difference between low, 10 μM and high, 100 μM concentrations of 27-OH on ROS generation, U937 cells treated with high concentrations of 27-OH and incubated between 1 and 24 h. The pro-oxidant effect of 100 μM 27-OH was determined with the fluorescent probe DHE as shown in Fig.4.13. DHE red fluorescence increased up to 24 h compared to untreated control cells and a lasting ROS production was observed while the marked quenching of ROS generation induced by low concentration of oxysterol was not any more observed in U937 cells. Moreover, the morphological change such as cell shrinkage was observed in U937 cells challenged with 27-OH for 24 h that confirmed the apoptotic effect of high concentration of oxysterol's.

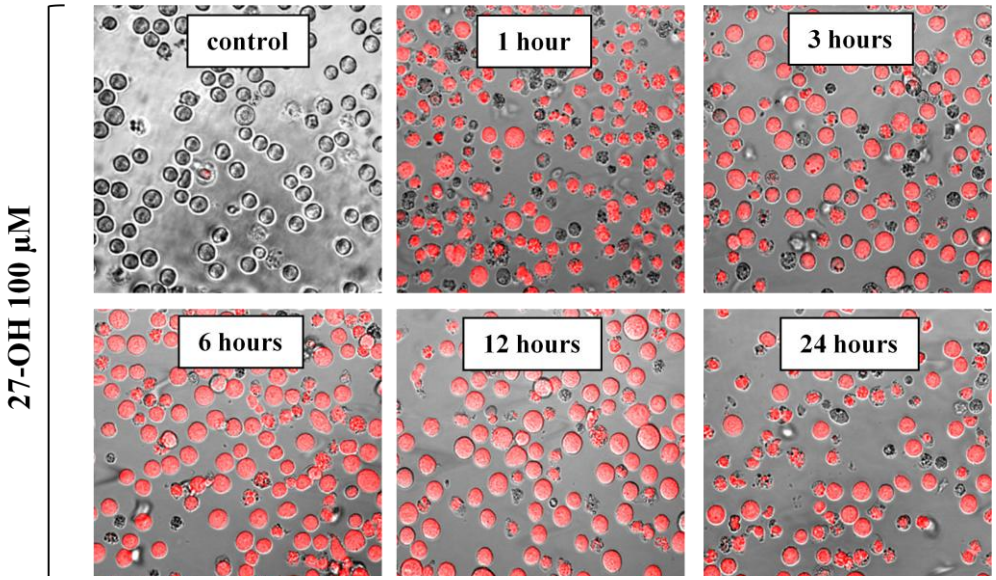

Figure 4.13. Pro-oxidant effect of 100 µM 27-Hydroxycholesterol (27-OH). Intracellular ROS generation was measured by dihydroethidium (DHE) florescence staining in U937 cells incubated with the oxysterol from 1-24 h. Untreated cells (Control) were taken as controls.

4.8. Dependence of 27-OH induced ERK and Akt phosphorylation on the ROS increase

In order to determine the noncytotoxic concentrations of antioxidant NAC, FACS analysis was utilized. Therefore, U937 cells were treated with different concentrations of NAC and incubated for 24 h, and then cells were subjected to FITC–AnnexinV staining to analyze the cell death by flow cytometry. The FACS analysis results showed that 0.1, 1 and 10 mM final concentrations for NAC (Fig.4.14 A) are noncytotoxic for U937 promonocytic cells. Depending on the results obtained and previously published studies, 100 µM NAC was used for further experiments.

To confirm antioxidant potential of NAC in U937 cells, DHE staining was performed using NAC, at a noncytotoxic concentration (100 µM). Challenging U937 cells with a single 10 µM dose of 27-OH for 3h showed that cell pretreatment with NAC (45 min) able to impede the intracellular rise of ROS (Fig. 4.14 B).

After determining the regulatory role of NAC on the pro-oxidant effect of 27-OH, immunoblotting experiments were performed to examine whether NAC pretreatment has a similar effect on ERK and Akt phosphorylation. As shown in Fig. 4.14 C, challenging cells with NAC fully prevented 27-OH-dependent up-regulation of both ERK1/2 and Akt phosphorylation at 3 and 6h. Based on these findings, it can be suggested that 27-OH induced ERK1/2 and Akt phosphorylation is ROS-dependent in U937 promonocytic cells.

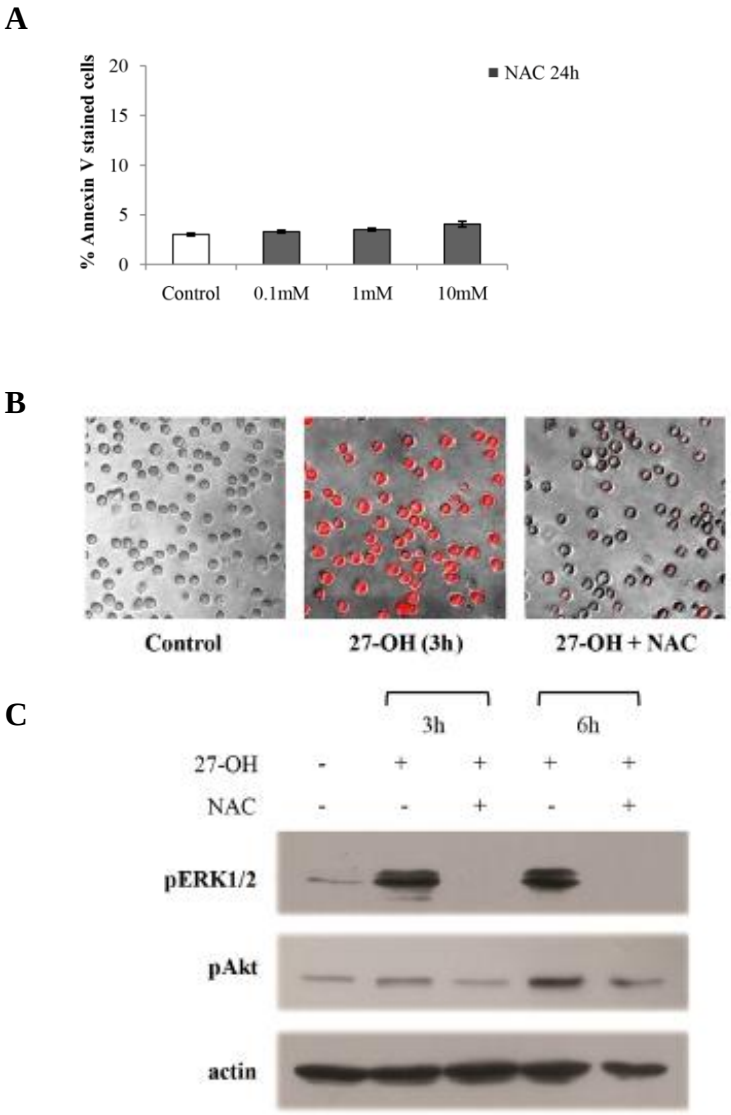

Figure 4.14. Modulation of 27-hydroxycholesterol's (27-OH) pro-oxidant effect by N-acetylcysteine (NAC). (A) Cytotoxicity of NAC was determined by flow cytometry. Cells were treated with indicated concentrations of NAC for 24 h, stained with FITC–AnnexinV and subjected to FACS analysis. (B) Protection exerted by NAC pretreatment on ROS generation. U937cells were treated for 3h with 10 μM 27-OH. Untreated cells (Control) were taken as controls. Other cells were preincubated with 100 μM NAC for 1h and then treated with 27-OH for 3h. (C) Effect of NAC pretreatment on 27-OH-dependent ERK and Akt phosphorylation. Untreated cells were taken as controls. Other cells were preincubated (1h) with 100 μM NAC and then treated for 3 and 6h with 27-OH at a final concentration of 10 μM. The levels of pERK1/2 and pAkt were analyzed by Western blotting. One blot representative of three experiments is shown for each protein.

4.9. Effect of MEK/ERK and PI3K/Akt signaling pathways inhibition on intracellular ROS levels

Given the observation that the time interval of the transient quenching of 27-OH's pro-oxidant effect corresponded to the transient prevalence of ERK- and Akt-dependent survival signaling, it was decided to examine the potential interference of such survival signals with ROS generation. For this purpose, cells were pretreated with either PD98059 or LY294002 and subjected to DHE staining to determine intracellular ROS levels. As shown in Fig. 4.15, the inhibition of ERK1/2 and Akt phosphorylation, by cell pretreatment with selective inhibitors, indeed abolished the transient disappearance of 27-OH's pro-oxidant effect. A high level of intracellular ROS was also observed after 12 h challenge with 10 μM 27-OH, provided that cells were pretreated with MEK/ERK or PI3K/Akt inhibitors while cells were treated only with PD98059 or LY294002 did not induce any pro-oxidant effect. In addition, the sustained elevation of the ROS steady state was proven both by DHE staining and by H_2O_2 measurement. Data shown that pretreated cells with MEK/ERK or PI3K/Akt inhibitors significantly increased ROS production at 12 h incubation with low micromolar concentration of 27-OH.

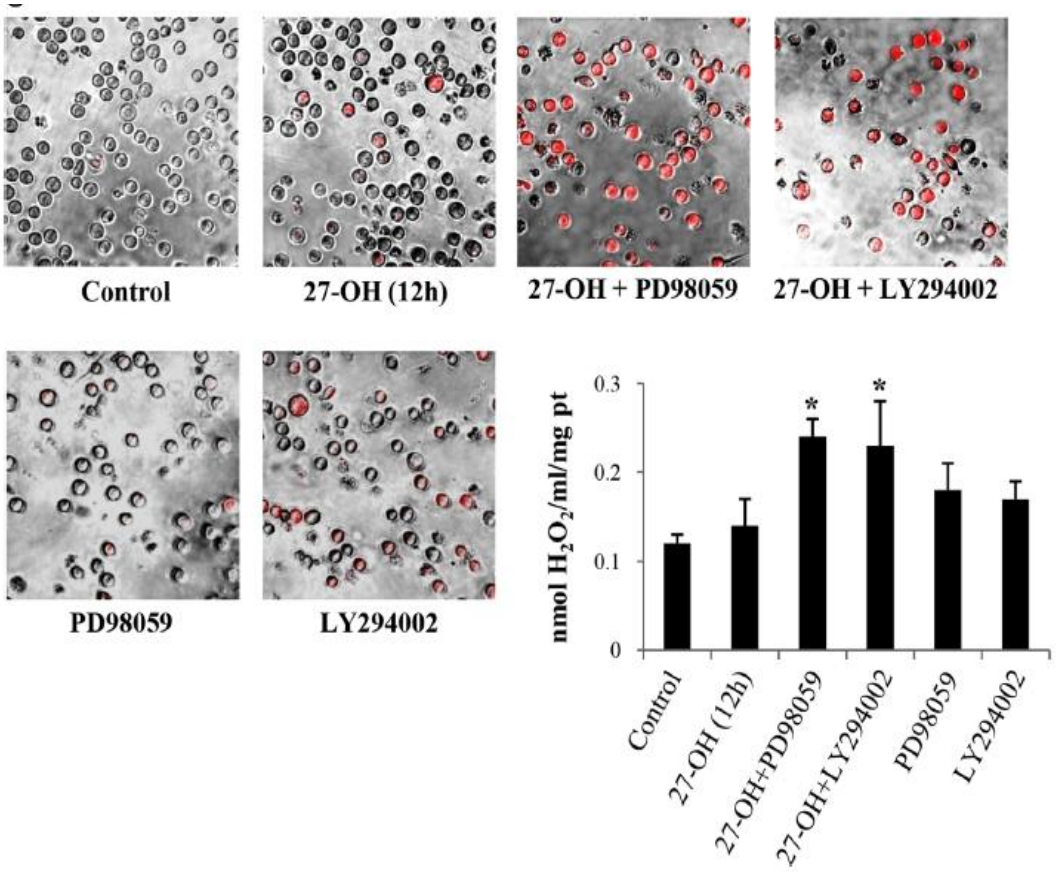

Figure 4.15. Modulation of 27-hydroxycholesterol's (27-OH) pro-oxidant effect by pERK1/2 and pAkt selective inhibitors. U937 cells were incubated with l0 μM 27-OH for 12 h. Untreated cells (Control) were taken as controls. Some cells were preincubated with PD98059 (40 μM) or LY294002 (25 μM) for 45 min and then treated with 27-OH for 12 h. Other cells were treated only with PD98059 or LY294002 as internal controls. The overproduction of ROS was detected by dihydroethidium (DHE) fluorescence staining, while H2O2 production was measured spectrophotometrically. Histograms represent the mean values ±SD of three experiments; *P<0.05 vs. 27-OH.

4.10. Determination cellular source of ROS increased in 27-OH treated U937 cells

Previous studies pointing to oxysterols' ability to impair mitochondrial membrane potential (Δψm) and upregulate Nox-2, thus increasing the intracellular ROS production (Biasi et al., 2009; Gargiulo et al., 2011). According to these findings, it was decided to check derangement of Δψm and activation of Nox-2 in 27-OH-treated cells, after 12 h incubation, in the presence or absence of pharmacological inhibitors PD98059 and LY294002. As reported in Fig. 4.15, 12 h incubation of U937 cells with 27-OH

alone did not cause any significant ROS increase versus control cells, unless the contemporary upregulation of ERK and Akt phosphorylation was inhibited. In addition to this, cells were subjected to JC-1 staining which is a cationic dye that only enters into mitochondria with high $\Delta\psi m$, when its color changes from green to red, displayed an intense red fluorescence only in 27-OH-treated and control cells. However, it showed diffuse green fluorescence, indicating $\Delta\psi m$ depression, when cells were challenged with 27-OH in the presence of selective inhibitors, PD98059 and LY294002 (Fig. 4.16 A). Moreover, the expression of Nox-2 which is an additional source of ROS was analyzed by immunoblotting in 27-OH-treated U937 cells. According to results, Nox-2 activation was observed by a significant increase of membrane translocation of its component $p47^{phox}$ after 12 h incubation with 27-OH, only when MEK/ERK and PI3K/Akt survival signaling was inhibited by pharmacological inhibitors (Fig. 4.16 B).

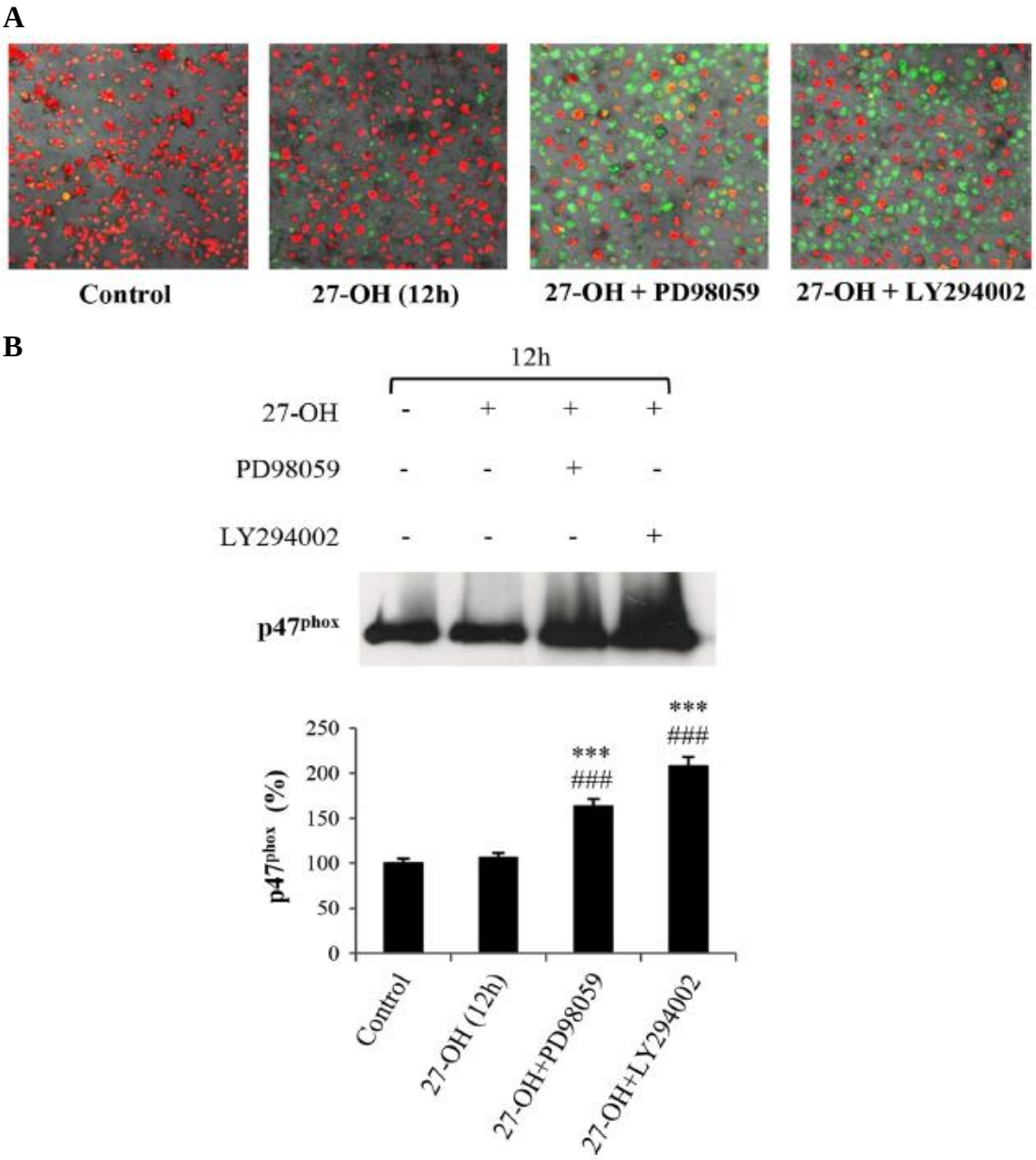

Figure 4.16. Both mitochondrial depolarization and Nox-2 activity contribute to the pro-oxidant effect of 27-hydroxycholesterol (27-OH). U937 cells were treated for 12 h with l0 μM 27-OH. Untreated cells (Control) were taken as controls. Other cells were preincubated with PD98059 (40 μM) or LY294002 (25 μM) for 45 min and then treated with 27-OH for 12h. (A) The transmembrane mitochondrial potential was detected by JC-1 staining, and (B) Nox-2 activation by Western blotting. One blot representative of three experiments is shown for each protein. Histograms represent the mean values ±SD of all three independent experiments; densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control values; ***P<0.001 vs. control; ###P<0.001 vs. 27-OH.

4.11. Nrf2 pathway in response to 27-OH in U937 promonocytic cells

With regard to the quenching of ROS intracellular levels by ERK and Akt survival signaling induction, of the pro-oxidant effect exerted in 27-OH challenged promonocytic cells, one likely explanation for this effect is the induction of antioxidant defense systems. While activation of these survival signaling pathways has repeatedly been demonstrated to induce Nrf2 and its antioxidant response (Kim et al., 2008; Papaiahgari et al., 2006; Wang et al., 2012, 2011), it was decided to investigate the effect of 27-OH treatment on Nrf2 pathway.

4.11.1. Induction of Nrf2 expression, total cellular levels and nuclear translocation by low micromolar concentration of 27-OH

To examine the induction of Nrf2 gene expression, cells were treated with low concentration of 27-OH for 1 and 3 h and mRNA levels were analyzed by RT-PCR. As reported in Fig. 4.17 A, a significant up-regulation of Nrf2 mRNA was detectable in U937 cells since the first few hours after addition of 10 μM 27-OH to the incubation medium. In particular, after 3 h incubation with 27-OH, Nrf2 mRNA level was maximum and almost 2 fold induced. In order to elaborate Nrf2 antioxidant response induction, cells were challenged with 10 μM 27-OH and total proteins were analyzed by immunoblotting (Fig. 4.17. B). In consistent with gene expression results, a net increase of Nrf2 total cellular levels was observed between 1 and 3 h in U937 cells. The transcription activity of Nrf2 mainly depends on nuclear shuttling in response to a stimulus. Thus, to investigate the localization of the transcription factor, cytoplasmic and nuclear protein fractions were obtained separately at 1 and 3 h and evaluated by Western blotting. Immunoblotting results demonstrated a marked stimulation of Nrf2 nuclear translocation already after 1 h cell challenge with the low concentration of 27-OH (Fig. 4.17. C). On the other hand, Nrf2 activation by 27-OH appeared to be transient, whereas after 3 h challenge, the nuclear level of the transcription factor in the oxysterol treated cells showed a decreasing trend.

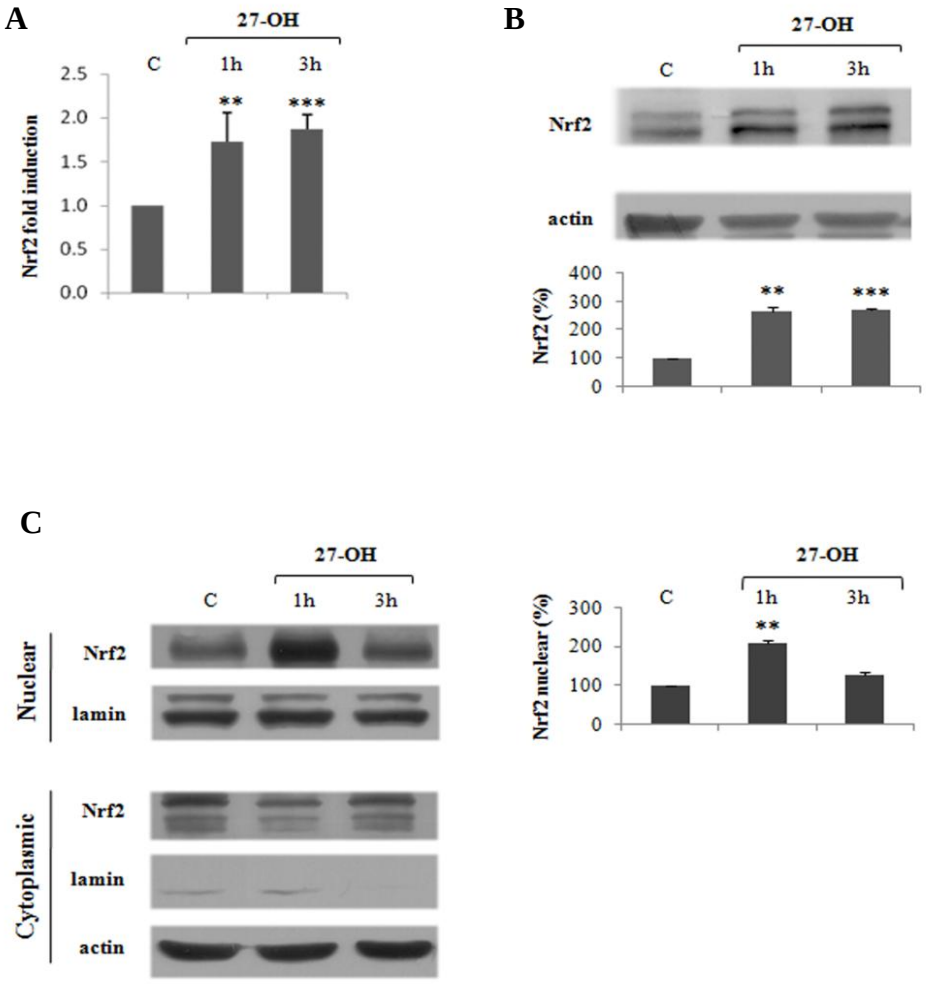

Figure 4.17. 27-hydroxycholesterol (27-OH) induces gene expression, synthesis and nuclear translocation of Nrf2 in U937 cells. (A) Effect of 27-OH on the expression of Nrf2. Gene expression was quantified by real-time RT-PCR in U937 cells treated for 1 and 3 h with 10 μM 27-OH. Untreated cells were used as controls. Data, normalized to actin, are expressed as mean values ± S.D. of three different experiments. ***P < 0.001, and **P < 0.005 vs. control group. (B) Nrf2 protein level in U937 cells treated with 10 μM 27-OH for 1 and 3 h was analyzed by Western blotting. Untreated cells (c) were taken as controls. Actin was used as loading control. (C) Nrf2 expression in nuclear and cytoplasmic lysates was determined by Western blotting. Untreated cells (c) were taken as controls. Lamin A/C and actin were used as loading controls. One blot representative of three experiments is shown. Histograms represent the mean values ± S.D. of all three independent experiments; densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control values; *P<0.05, **P<0.01 and ***P<0.001 vs. control group.

4.11.2. High micromolar concentration of 27-OH does not induce Nrf2 total cellular levels and nuclear translocation

To examine whether Nrf2 antioxidant response induction is oxysterol' concentration dependent, induction of Nrf2 pathway was evaluated in cells challenged with high concentration of 27-OH. U937 cells treated with 100 μM 27-OH for 1 and 3h and total cell lysates were analyzed by immunoblotting (Fig. 4.18 A). Results clearly showed that Nrf2 total cellular levels did not changed significantly compared to untreated control after oxysterol treatment. In order to determine the effect of high dose treatment on Nrf2 nuclear localization, Western blotting experiments was performed to evaluate this transcription factor levels in nuclear and cytoplasmic fractions. As shown in Fig. 4.18 B, 100 μM 27-OH treatment did not significantly changed the localization of Nrf2 in U937 cells. After 1 and 3 h incubation with the oxysterol, nor cytoplasmic neither nuclear levels of transcription factor did not changed compared to untreated control. It was apparent that induction of Nrf2 antioxidant response was modulated only by low concentration of 27-OH in promonocytic cells.

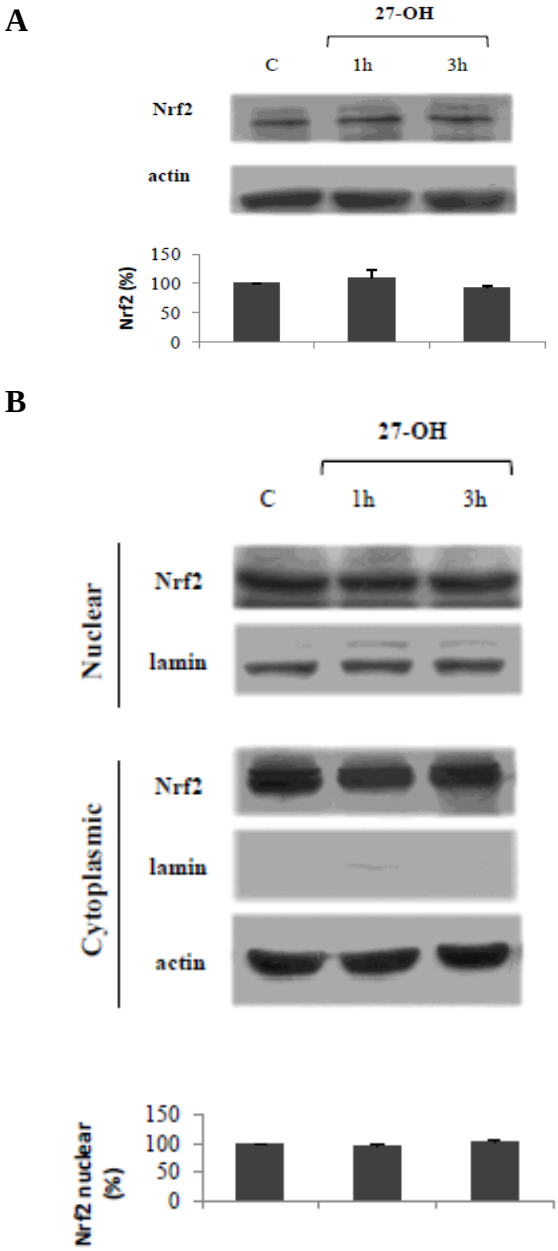

Figure 4.18. Nrf2 synthesis and nuclear translocation not induced by high micromolar concentration of 27-hydroxycholesterol (27-OH). (A) Nrf2 protein level in U937 cells treated with 100 μM 27-OH for 1 and 3 h was analyzed by Western blotting. Untreated cells (c) were taken as controls. Actin was used as loading control. (B) Nrf2 expression in nuclear and cytoplasmic lysates was determined by Western blotting. Untreated cells (c) were taken as controls. Lamin A/C and actin were used as loading controls. One blot representative of three experiments is shown. Histograms represent the mean values ± S.D. of all three independent experiments; densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control values.

4.12. HO-1 and NQO-1 induction by 27-OH in U937 promonocytic cells

It has been clearly demonstrated that Nrf2 plays a central role in controlling the expression of detoxyfying genes such as HO-1 and NQO-1 whose protein products are involved in the regulation of oxidative imbalance (Nguyen et al., 2009). For the purpose of demonstrating HO-1 and NQO-1 induction by 27-OH in promonocytic human cells, HO-1 and NQO-1 mRNA and protein levels were measured, following incubation with the oxysterol at the concentration of 10 μM in U937 cells. The effect of 27-OH on HO-1 and NQO-1 mRNA expression was checked by quantitative RT-PCR. As reported in Fig. 4.19 A, real-time RT-PCR results showed a significant upregulation of the expression of both antioxidant genes. In particular, HO-1 gene was increased after 3 h oxysterol treatment and the maximum increase was observed by 6 h, but it decreased thereafter. However, NQO-1 gene was rapidly increased with a maximal response after 3 h treatment and then with declining trend mRNA level returned to the basal value after 24 h treatment of the oxysterol. Furthermore, a net increase of both HO-1 and NQO-1 protein levels in U937 cells treated with low concentration of 27-OH was clearly presented by immunoblotting (Fig. 4.19 B). As demonstrated in Fig. 4.19 B, 27-OH increased HO-1 protein level between 1 and 72 h treatment with a maximal response at 12 h, thereafter protein level slightly decreased up to 72 h. With regard to NQO-1, in U937 cell suspension challenged with 10 μM 27-OH a marked up-regulation was observed for its protein levels between 3 and 72 h treatment (Fig. 4.19 B). Statistical analysis of all data obtained for 1-72 h time interval demonstrated that HO-1 and NQO-1 synthesis was significantly increased in 27-OH-treated cells compared with untreated control cells.

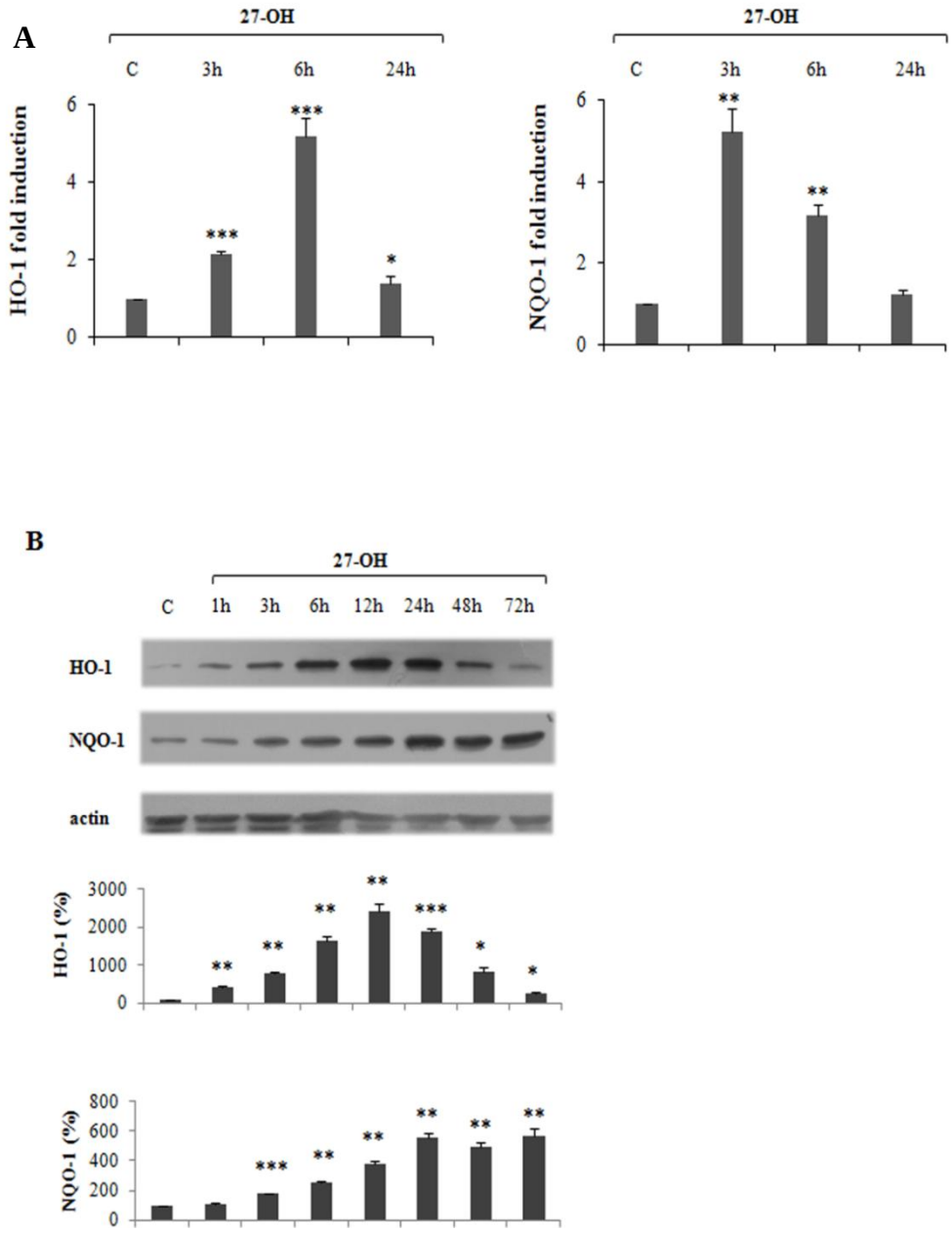

Figure 4.19. Effect of 27-hydroxycholesterol (27-OH) on HO-1 and NQO-1 expression and protein levels. (A) Expression of HO-1 and NQO-1 genes was quantified by real-time RT-PCR in U937 cells treated up to 24 h with 10 μM 27-OH. Data, normalized to actin, are expressed as mean values ± S.D. of four different experiments. (B) Protein levels of HO-1 and NQO-1 in U937 cells treated with 10 μM 27-OH for 1 to 72 h, were analyzed by Western blotting. Untreated cells (c) were taken as controls. Actin was used as loading control. One blot representative of three experiments is shown for each protein. Histograms represent the mean values ± S.D. of all three independent experiments; densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control values; *$p < 0.05$ and **$p < 0.01$ and ***$p < 0.001$ vs. control group.

4.13. Effect of 27-OH induced PI3K/Akt and ERK signaling pathways on Nrf2 induction

After induction of Nrf2 antioxidant signaling in response to 27-OH was deciphered, the impact of survival signaling pathways including PI3K/Akt and ERK on Nrf2 induction was investigated. With this aim, to determine the dependence of the upregulation of Nrf2 protein level and nuclear translocation in U937 cells challenged with 10 μM 27-OH upon the phosphorylation of either ERK or Akt by this oxysterol was proved by a series of immunoblotting experiments employing pharmacological inhibitors of these kinases. Pretreatment of U937 cells with PD98059 (40 μM), the MEK/ERK inhibitor or with LY294002 (25 μM), the PI3K/Akt inhibitor significantly prevented the 27-OH-mediated upregulation of Nrf2 protein level, as observed after 1 and 3 h oxysterol treatment (Fig. 4.20 A). In addition, as shown in Fig. 4.20 B, the inhibition of ERK and Akt survival signaling with PD98059 or LY294002 respectively resulted a significant downregulation of the 27-OH-induced nuclear translocation of Nrf2 observed after 1 h treatment with the oxysterol. Based on these findings it can be suggested that ERK1/2 and PI3K/Akt are upstream regulators of Nrf2 pathway and modulate the nuclear localization of Nrf2 transcription factor in U937 promonocytic cells incubated in the presence of low micromolar amount of the oxysterol.

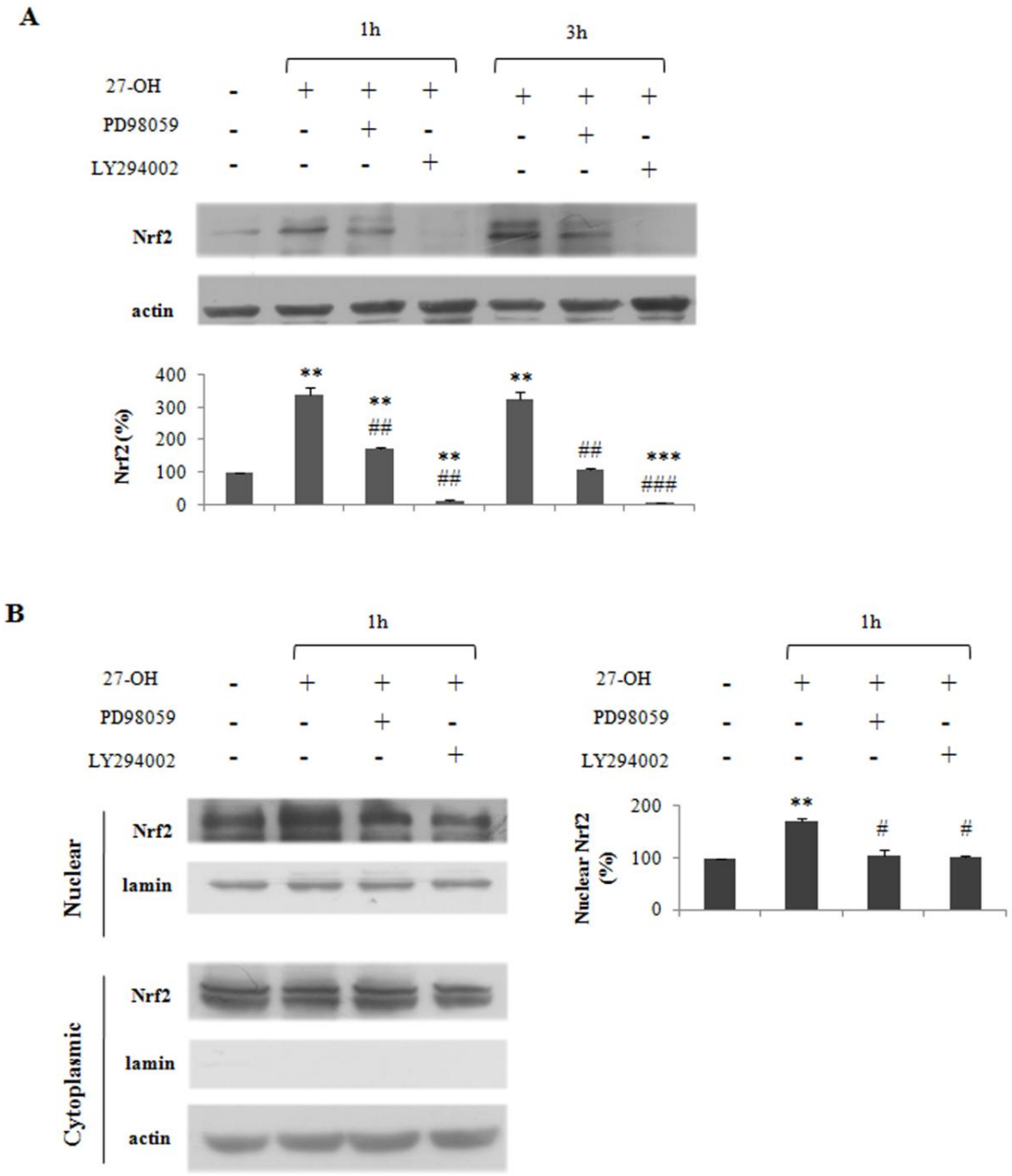

Figure 4.20. Inhibition of MEK/ERK and PI3K/Akt signaling pathways downregulates Nrf2 induction. U937 cells were either treated with 27-OH (10 μM) alone or pre-incubated with PD98059 (40 μM) or LY294002 (25 μM) 45 min before 27-OH treatment. Untreated cells were taken as controls. (A) Nrf2 protein levels were analyzed by Western blotting. Actin was used as loading control. (B) Nrf2 levels in nuclear and cytoplasmic lysates were determined by Western blotting. Lamin A/C and actin are used as loading controls. One blot representative of three experiments is shown. Histograms represent the mean values ± S.D. of all three independent experiments; **$p < 0.01$ and ***$p < 0.001$ vs. control group; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. 27-OH.

4.14. Effect of 27-OH induced PI3K/Akt and ERK signaling pathways on HO-1 and NQO-1 induction

To examine the effect of 27-OH induced PI3K/Akt and ERK signaling pathways on HO-1 and NQO-1 induction, protein level of these detoxyfying enzymes were analyzed by Western blotting. For this purpose, U937 cell aliquots were preincubated (45 min) with either 40 μM PD98059 or 25 μM LY294002 then challenged with low dose of oxysterol. Immunoblotting results showed that inhibition ERK signaling by PD98059 dramatically reduced HO-1 protein levels after 6 and 12 h treatment with 10 μM 27-OH (Fig 4.21 A). Moreover, cell pre-incubation with PI3K/Akt inhibitor, LY294002 fully prevented HO-1 expression after 6 and 12 h treatment of the oxysterol. Furthermore, the effect of these metabolic inhibitors was also examined for NQO-1 protein after 24 and 48 h treatment with 27-OH (Fig 4.21 B). It has been clearly demonstrated that pretreatment with Erk and Akt inhibitors remarkably prevented 27-OH-induced NQO-1 upregulation in U937 cells. This effect was observed more significantly after 24 h treatment of the oxysterol. In the light of these results, it can be suggested that ERK and PI3K/Akt survival signaling pathways are involved in 27-OH-induced upregulation of HO-1 and NQO-1 detoxyfying genes in U937 cells.

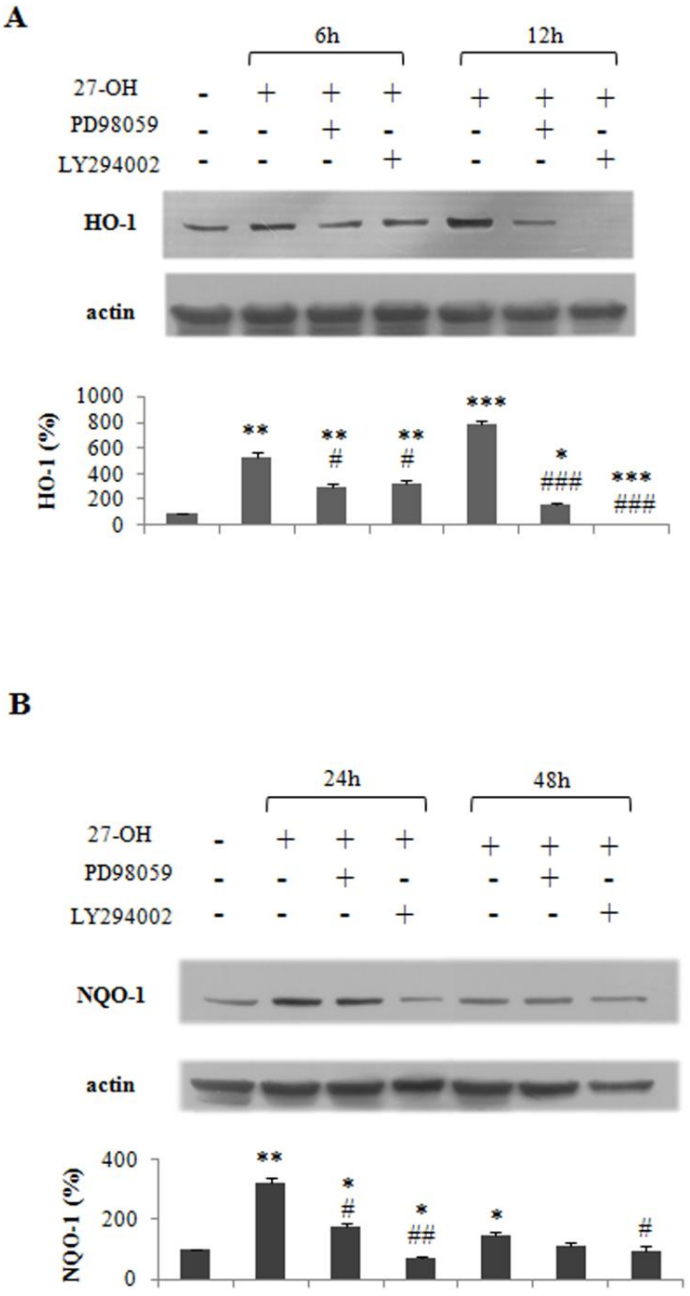

Figure 4.21. Effects of MEK/ERK or PI3K/Akt inhibition on HO-1 and NQO-1 induction. U937 cells were either treated with 27-OH (10 μM) alone or pre-incubated with PD98059 (40 μM) or LY294002 (25 μM) 45 min before 27-OH treatment. Untreated cells were taken as controls. (A) Protein levels of HO-1 and (B) NQO-1 were analyzed by Western blotting. Actin was used as loading control. One blot representative of three experiments is shown for each protein. Histograms represent the mean values ± S.D. of all three independent experiments; *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control group; #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. 27-OH.

4.15. Effect of ROS up-regulation on 27-OH induced Nrf2 expression

It has been clearly established that mitochondrial ROS play a critical role in activation of downstream antioxidant mechanisms, including Nrf2/ARE pathway (Cheng et al., 2011). We have showed above that ROS were generated through the derangement of mitochondrial membrane potential and activation of Nox-2 in response to low dose of 27-OH in human promonocytic cells. Based on these findings, we determined to investigate whether mitochondrial and Nox-2 sourced ROS could play a critical role in mediating 27-OH-induced Nrf2 up-regulation. Therefore, U937 cell suspensions were pretreated with or without the antioxidant NAC prior to 3 h incubation with 10 μM 27-OH, and Nrf2 total cellular levels were analyzed by Western blotting (Fig. 4.22 A). Interestingly, it was observed that the 45 min cell pretreatment with 100 μM NAC significantly prevented 27-OH-dependent upregulation of Nrf2 cell protein level. Moreover, statistical analysis reported that as a result of quenching ROS by antioxidant NAC, Nrf2 protein content nearly halved in NAC + 27-OH treated cells in comparison to cells treated with the oxysterol alone (Fig. 4.22 A). Notably, cell pretreatment with NADPH oxidase inhibitor, DPI, gave a similar result. In particular, as shown in Fig. 4.22 B, pre-incubation of U937 cells with 50 μM DPI significantly prevented the induction of Nrf2 after 3 h oxysterol treatment. These findings indicated that ROS upregulation has a modulatory role in 27-OH induced Nrf2 expression.

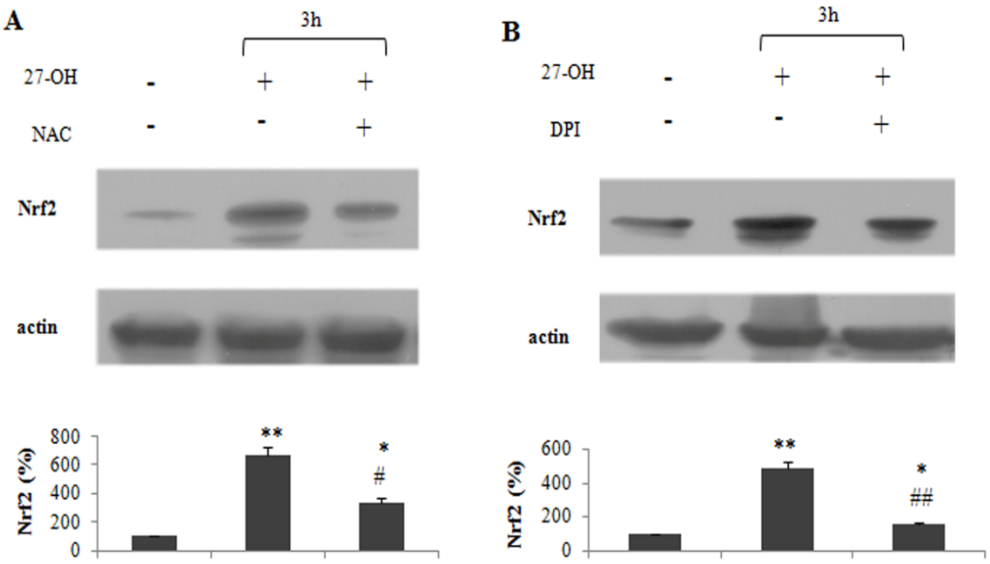

Figure 4.22. Modulation of Nrf2 induction by N-acetyl cysteine (NAC) and diphenyleneiodonium chloride (DPI). (A) Effect of NAC (B) DPI on 27-OH-dependent Nrf2 induction. Cells were treated for 3 h with 27-OH at the final concentration of 10 μM. Untreated cells were taken as controls. Other cells were pre-incubated (1 h) with (A) 100 μM NAC or (B) 50 μM DPI and then treated with 27-OH for 3 h. Protein level of Nrf2 was analyzed by Western blotting. Actin was used as loading control. One blot representative of three experiments is shown. Histograms represent the mean values ± S.D. of all three independent experiments; *$p < 0.05$ and **$p < 0.01$ vs. control group; #$p < 0.05$ and ##$p < 0.01$ vs. 27-OH.

4.16. Involvement of Nrf2 in 27-OH induced survival response in U937 promonocytic cells

Once demonstrated that 27-OH-induced phosphorylation of ERK and Akt pathways was in turn involving the activation of Nrf2, the possible role of the latter event in delaying the oxysterol's pro-apoptotic effect was investigated. For this purpose, cells were transfected with specific Nrf2 siRNA and subjected to DAPI staining to analyze cell death. As shown in Fig. 4.23, molecular inhibition of Nrf2 caused a marked increase in the number of apoptotic cells already after 24 h incubation with 10 μM 27-OH, while the cell incubation with the oxysterol alone did not show any difference as to

untreated control cells. According to these results, the actual role of Nrf2 in low concentration of 27-OH induced survival response was validated.

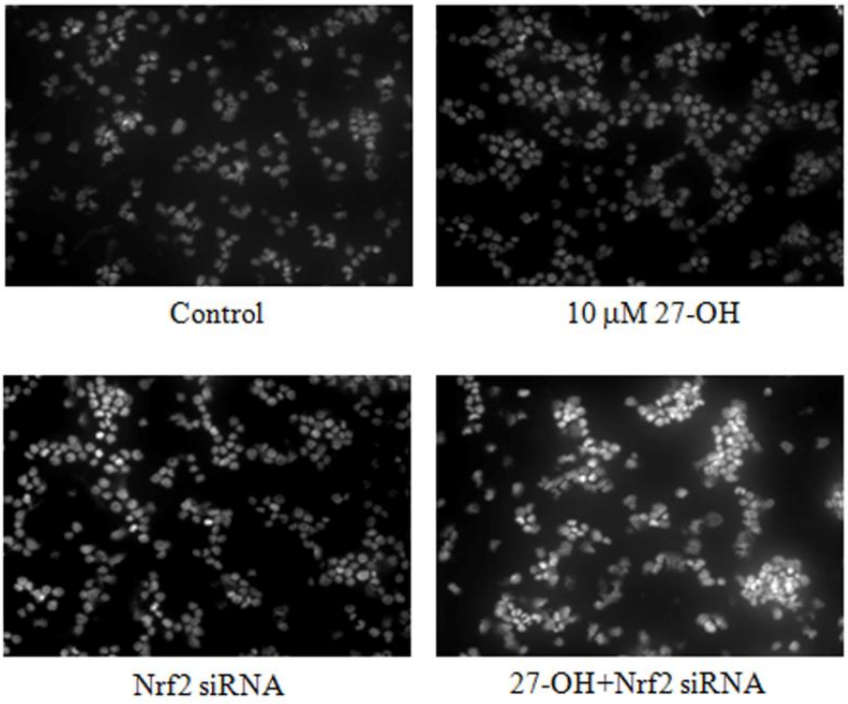

Figure 4.23. Evaluation of apoptosis by DAPI staining. U937 cells were transfected with a specific Nrf2 siRNA for 24 h and then incubated with 10 μM 27-OH for 24 h. Apoptotic cells were evaluated in terms of DAPI staining and examined using a fluorescence microscope (Zeiss Axiovert 200 M) with an ultraviolet filter and a 20x/0.30 lens.

5. DISCUSSION AND CONCLUSION

5.1 Redox modulated 27-hydoroxycholesterol-induced survival signaling

Oxidative modification of low-density lipoproteins (LDL) has been implicated to play a role in the pathogenesis of several oxidative-stress related chronic diseases that include atherosclerosis, preeclampsia, diabetes, and also in the acceleration and worsening of the aging process. Oxysterols are quantitatively relevant components of oxidized LDL (oxLDL) and their pro-inflammatory, pro-fibrogenic and pro-apoptotic effects were well-characterized in vascular cells. 27-hydroxycholesterol (27-OH) is one of the most represented oxysterols in the human circulation and also in atherosclerotic lesions (Khatib and Vaya, 2014). On behalf of its important role in pathophysiology, 27-OH has been shown to induce LXR-dependent pathways and increase the risk of breast cancer by acting as a selective estrogen receptor modulator (Janowski et al., 1999, 1996). In this relation, anti-apoptotic signals induced by 27-OH should be investigated in depth.

In the present thesis, it was demonstrated that survival signaling inducible by low concentrations of 27-OH involves the MEK/ERK and PI3K/Akt phosphorylation pathways in cells of monocyte lineage. Namely, enhanced MEK-ERK/PI3K-Akt phosphorylation followed by a net phosphorylation of pro-apoptotic Bad protein, respectively at Ser75 and at Ser99, results in macrophagic apoptotic death delay. Moreover, up-regulation of both kinases depends on oxysterol's pro-oxidant effect which likely to be the key mechanism in this survival response.

Among the previously published studies focusing on the survival signaling induced by oxysterols, Berthier et al. reported that THP-1 monocytic cells treated with 7K (100 μM) upregulated ERK 1/2 phosphorylation (between 1 and 6 h), consequent phosphorylation of Bad at residue Ser75 (Berthier et al., 2005). Unlike our data which is shown in Fig.4.5. , Akt phosphorylation at Thr 308 was downregulated in response to 7K. It can be suggested that usage of different cell lines and the relatively greater cytotoxicity of 7K than 27-OH caused this discrepancy. Because of 27-OH is very good while 7K is very poor ligands of LXRs both oxysterols induce MEK/ERK-dependent survival signaling rules out involvement of LXRs in this kinase phosphorylation. A recent study using another oxysterol demonstrated that the human cholangiocyte MMNK-1 cells challenged with cholestan-3β,5α,6β-triol (Triol) activated anti-apoptotic signaling via ERK phosphorylation (Jusakul et al., 2013). However, triol is considered as a poor LXR ligand while its concentration is negligible in human blood.

In an interesting study, the same cell line with our study, U937 monocytes, after phorbol myristate acetate (PMA) differentiation, was treated with 28 μM final concentration of 27-OH, authors observed a significant induction of cell viability as well as enhancement of Akt phosphorylation at residue Thr308 (Riendeau and Garenc, 2009). The oxysterol incubation time frame of that study was limited to 24 h, whereas we challenged monocytes with oxysterol till 72 h in most cases. On the other hand, neither modulation of Akt phosphorylation, nor pro-survival signaling, was detectable when the same 27-OH concentration was added to cultured human aortic smooth muscle cells suggesting that pro-survival response induced by oxysterol is cell specific.

Accumulating evidence including our present findings showed that 27-OH modulate survival signaling through MEK/ERK and PI3K/Akt survival signaling pathways in cells of the macrophage lineage. As macrophages have been shown to induce CYP27A1 expression, the enzyme that catalyses 27-OH production, understanding this enzymatic process in detail became a trend topic among immunology studies (Westman et al., 1998). Moreover, our data indicate that 27-OH trigger the MEK/ERK - PI3K/Akt pro-survival axis which is a well known contributor to the carcinogenesis, thus targeting this intracellular axis is a promising therapy against cancer resistance.

In Fig.4.4., the 27-OH amount actually present in promonocytic cells was quantified showing that only 20% amount of 10 μM oxysterol was recovered throughout the experiment whereas the actual amount of oxysterols recovered was reported as above 1-2 x 10^{-6} M in atherosclerotic lesions (Carpenter et al., 1995).

Regarding the impact of ROS in this survival response, the pro-oxidant activity of 27-OH was confirmed with several experiments as shown in Fig. 4.11, 4.12. and 4.13. In addition to this, pretreatment of cells with the antioxidant NAC decreased ERK1/2 and Akt phosphorylation clarified the mechanism underlying marked pERK and pAkt levels in which ROS has an active role in the enhanced kinase phosphorylation and in the related survival response (Fig. 4.14.). With regard to cellular sources of ROS, we demonstrated a Nox-2 dependent ROS increase as well as a derangement of the mitochondrial membrane potential of U937 cells. In parallel to our data, in macrophage colon-stimulating factors (M-CSF)-stimulated human monocytes/macrophages, upregulation of Nox-2 dependent ROS and the ROS-dependent enhancement of pAkt have been demonstrated where knocking-out $p47^{phox}$ and pretreatment with NAC inhibited these two events (Wang et al., 2007).

Besides demonstrating the survival effect of low concentrations of 27-OH, we also observed the high micromolar amount of the oxysterol. As depicted in Fig 4.6., 27-OH did not stimulate the survival signaling regarding the Erk/Akt axis enhancement. On the other hand, 100 μM of 27-OH exerted a constant pro-oxidant effect and also stimulated apoptosis of human promonocytic cells at an earlier time period.

As we discussed above the ROS-dependent ERK and Akt phosphorylation in low concentrations of 27-OH-treated promonocytic cells was demonstrated. In addition to this, a very interesting data showed that upregulation of the ERK/Akt axis was able to quench ROS increase induced by 27-OH itself. As shown in 4.11., in cells treated with 27-OH between 6 and 48 h incubations, ROS production was decreased coinciding with the time period of phosphorylation of ERK and Akt was upregulated. Furthermore, a biphasic pattern was observed for the oxidative stress provoked by 27-OH when up-regulation of the ERK/Akt axis was downregulated by pretreatment of selective inhibitors (Fig 4.15.). According to these findings, it can be suggested that an induction of antioxidant defense system may modulate the quenching of ROS by enhancement of

ERK-Akt phosphorylation (Papaiahgari et al., 2006; Wang et al., 2012). Thus, we further investigate the role of Nrf2 and its antioxidant response in the quenching of ROS levels which modulated by the activated survival signaling.

5.2 Involvement of Nrf2 antioxidant defense in 27-hydoroxycholesterol-induced survival signaling

In the first part of this thesis, we demonstrated that low concentrations of 27-OH stimulated survival signaling in a ROS-dependent manner that upregulated MEK-ERK/PI3K-Akt pathways with consequent quenching effect on ROS intracellular level (Vurusaner et al., 2014). To elucidate the complex mechanism that define the link between survival signaling pathways and down-regulation of ROS production, in the second part of the study, induction of Nrf2 antioxidant pathway was investigated.

Based on the findings, it can be discussed that in U937 promonocytic cells, low micromolar concentration of 27-OH induced the activation of Nrf2 redox-sensitive transcription factor and its target genes including HO-1 and NQO-1 (Vurusaner et al., 2016). Notably, activation of Nrf2-dependent antioxidant response by the oxysterol was demonstrated to dependent upon the up-regulation of MEK/ERK and PI3K/Akt signaling pathways. Moreover, it was observed that Nrf2 plays a key role in the quenching of intracellular oxidative stress and also in the 27-OH-induced survival signaling.

In a very recent study, Kim et al. demonstrated the enhancement of ERK/Akt phosphorylation in response to 27-OH in THP-1 monocytic cells, however induction of Nrf2 by low micromolar concentrations of this oxysterol has not been previously shown (Kim et al., 2014). In another study, vascular cells lines treated with oxLDLs were shown to activate Nrf2, therefore suggesting that this redox-sensitive transcription factor involved in the signaling pathways such as CD36 regulation that sustain atherosclerosis progression (Ishii et al., 2004). In the same study, it was suggested that modulation of Nrf2 antioxidant response could depend onthe type of cell and the type of chemical inducer where oxLDLs induced Nrf2 activation more strongly in macrophages than in smooth muscle cells, but 4-hydroxynonenal (HNE) equally stimulated Nrf2

translocation in both cell lines. In contrast to our findings (Fig. 4.17. and 4.19), in C6 glioma cells challenged with 10-20 μM 27-OH, both expression and synthesis of Nrf2 and its targets genes HO-1, NQO-1 and γ-GCS were downregulated in response to oxysterol (Ma et al., 2015). These few relevant reports together with our findings supported the cell specificity for the given response.

As shown in Fig. 4.19, in promonocytic cells challenged with 10 μM of 27-OH, protein levels of HO-1 and NQO-1 upregulated between 1 and 12 h of incubation that coinciding with phosphorylation of Erk and Akt time period. Interestingly, after 48 h oxysterol treatment HO-1 synthesis was started to decrease that consistent with ROS reappearance period at 72 and 96 h incubation times. On the other hand, the other cytoprotective enzyme, NQO-1 remained increased at 48 and 72 h of cell incubation. Thus, it can be suggested that HO-1 seems more involved than NQO-1 in quenching the pro-oxidant effect of 27-OH. It has been well reported that HO-1 and NQO-1 antioxidant enzymes expressed in all main cell types present in human and mouse atherosclerotic lesions, such as smooth muscle cells, macrophages and endothelial cells (Araujo et al., 2012). In parallel to our findings, moderately oxidized LDL were shown to upregulate HO-1 expression in a Nrf2-dependent manner in vascular smooth muscle cells (Anwar et al., 2005). Another study reported that in endothelial cells challenged with the oxidized phospholipid oxPAPC, Nrf2-dependent HO-1 and NQO-1 induction was observed (Jyrkkänen et al., 2008).

In the “Introduction” section we mentioned the modulation of Nrf2 nuclear translocation by ERK1/2, p38 MAPK, PKC and PI3K/Akt signaling pathways. Based on the previous reports and our data, it can be suggested that the intensity of these upstream signaling pathways that modulate Nrf2 nuclear translocation varies with the different inducers in the different cell types. In this relation, in pulmonary epithelial cells ROS-dependent ERK and Akt activation was shown to induce translocation of Nrf2 to the nucleus (Papaiahgari et al., 2004). Similarly, in PC12 pheochromocytoma cells Nrf2 protein increased was observed in a PI3K/Akt-dependent manner (Martin et al., 2004). Similar to these results, we demonstrated ERK and Akt-dependent Nrf2 and its target genes, HO-1 and NQO-1 induction in response to low micromolar amounts of 27-OH (Fig. 4.20 and 4.21.) where using selective inhibitors, both expression and synthesis of Nrf2 was inhibited. In consistent with these findings, the effect of Nrf2 in

modulation of survival signaling was evaluated by using Nrf2 siRNA and a Nrf2-dependent survival response was observed in DAPI staining experiments (Fig. 4.23.).

Another interesting data was that up-regulation of HO-1 and NQO-1 by 27-OH was prevented by pretreating cells with selective inhibitors of ERK and PI3K/Akt pathways (Fig. 4.21.). Similar to our findings, Iles et al. reported that in epithelial cells HNE induced HO-1 via ERK signaling pathway (Iles et al., 2005). In agreement with this, in human vascular smooth muscle cells challenged with moderately oxidized LDL, HO-1 induction through activation of MAPK pathway was observed (Anwar et al., 2005). While HO-1 upregulation by defined metabolites of the prostaglandin J(2) series was observed in HepG2 hepatoma cells, this induction was not inhibited by the ERK1/2 inhibitor PD98059, suggesting that enhancement of this antioxidant enzyme might be modulated through more than a single upstream pathway (Liu et al., 2004).

Finally, as shown in Fig. 4.22, in U937 cells pretreated with NAC or DPI, the induction of Nrf2 antioxidant signaling by low concentrations of 27-OH was prevented. In consistent with our findings, several studies demonstrated that inhibiting NADPH oxidase activity with DPI reduced nuclear transposition of Nrf2 in response to hypoxia in epithelial cells (Papaiahgari et al., 2006, 2004).

5.3. Conclusions

According to data obtained, it appears that 27-OH triggers survival signals involving ERK/Akt axis, at least in monocytic cells and its pro-oxidant effect is the key mechanism in this survival response. The study also provides evidence for a "Trojan horse" action of this oxysterol (Biasi et al., 2004). Notably, when cells challenged with relatively low, and not directly toxic amounts of 27-OH that reflects a more realistic approach, the oxysterol's pro-survival effect was observed. Instead of killing the cell directly, 27-OH and also by related compounds might delay the irreversible damage they can cause, at the same time initiating pro-inflammatory and pro-fibrogenic pathways. Therefore, it can be suggested that delayed macrophage apoptosis would favor growth and consequent destabilization of advanced atherosclerotic plaques (Martinet et al., 2012).

In summary, as schematically shown in Fig. 5.1 (graphical abstract) the ROS-mediated up-regulation of ERK/Akt phosphorylation, induced by low micromolar concentration of 27-OH is primary responsible for a significant induction Nrf2 and stimulated expression and synthesis of Nrf2 target enzymes, HO-1 and NQO-1. Moreover, molecular events modulate the pro-oxidant effect of 27-OH by a transient inhibition and, interestingly, able to prolonge the survival of cells challenged by the oxysterol. On the other hand, relatively high micromolar amount of 27-OH did not exert any survival effect in promonocytic cells, but a constant pro-oxidant effect was observed.

Based on the literature, survival signaling elicited by 27-OH should appear beneficial and might involved in processes related to inflammation. On the other hand, oxysterol modulated cell survival could have detrimental effects like favoring the tumorigenesis in the case of chronic inflammation. Therefore, the potential beneficial or detrimental effect of these compounds appears to depend on different physiological processes and their grade of disease progression. It is worth to consider whether oxysterols are friends or foes.

In conclusion, the findings obtained from this thesis highlight that cholesterol oxidation by-products involved in the pathogenesis of oxidative stress related chronic diseases like atherosclerosis and cancer.. Therefore, to understand deeply the complex mechanism and to generate synthetic or natural molecules targeting this survival mechanism might be a very promising tool in the prevention such diseases.

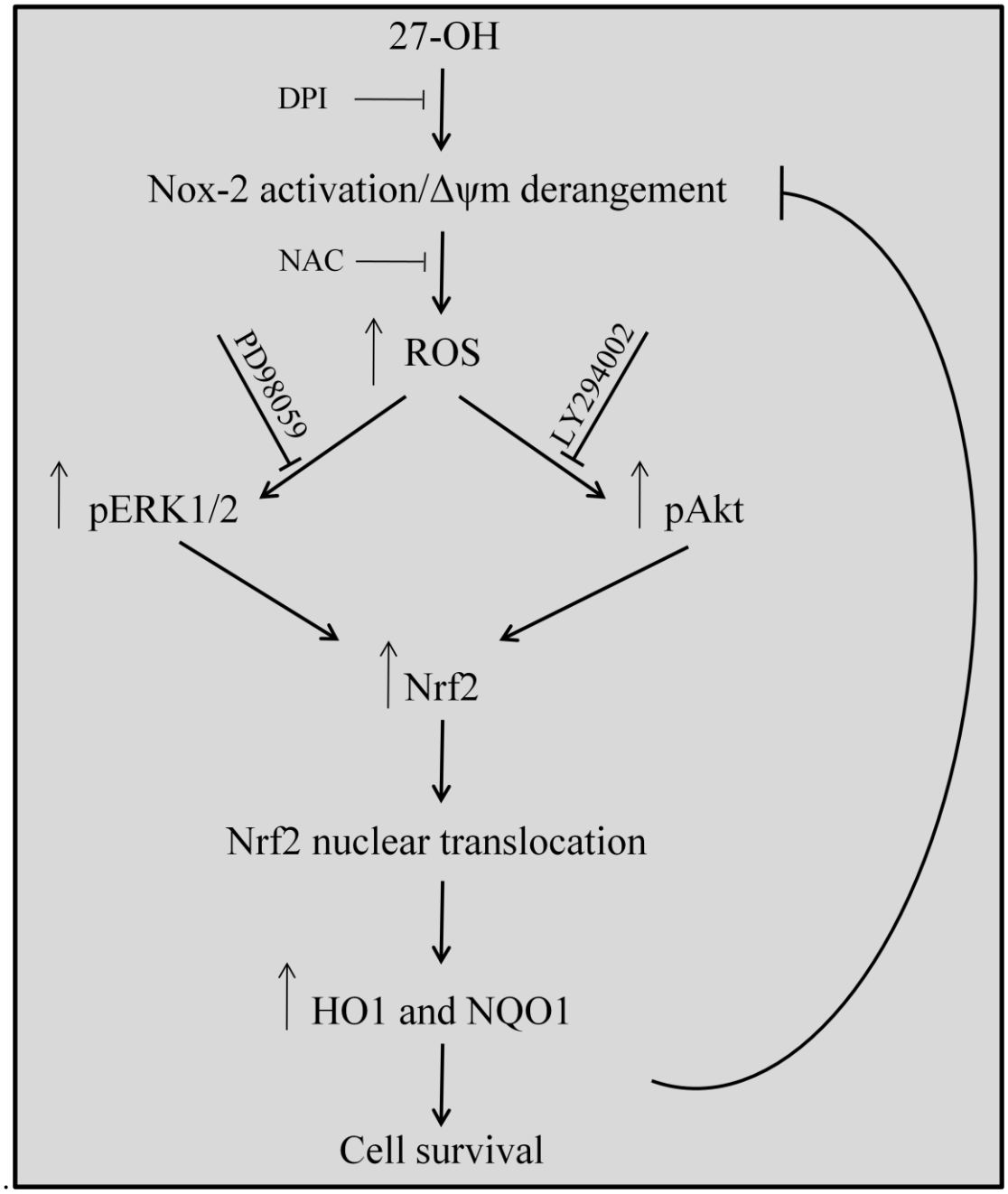

Figure 5.1. Schematic flow sheet of 27-hydroxycholesterol-induced redox and Nrf2 modulated survival signaling

5.4. Future Studies

The combined modulation of ERK/Akt phosphorylation and cellular redox state, as well as the induction of Nrf2 antioxidant response, here demonstrated for a low concentration of 27-OH, might be investigated also for other lipid oxidation products implicated in the pathogenesis of atherosclerosis and other inflammation-driven chronic

diseases. Furthermore, to understand whether a cell specificity is the fact that the survival mechanism evoked by 27-OH in U937 monocytic cells, our approach could be tested in other vascular cell lines such as endothelial and smooth muscle cells.

In recent years, research on autophagy is a growing field with the aim of elucidation of its complex mechanism. It has become accepted that although autophagy has been regarded as cell survival mechanism, under certain conditions, excessive autophagy may leads to a caspase-independent, non-apoptotic type of cell death (Type II cell death) (Galluzzi et al., 2009). Despite the increasing interest in understanding the mechanism of autophagy, there is limited information on how cellular signaling pathways, regulate this complex process. It is now well established that autophagy is stimulated in advanced atherosclerotic plaques by inflammation and oxidized lipids where progression of atherosclerosis is characterized by formation of these plaques (Martinet and De Meyer, 2009). The protective role of autophagy in atherosclerosis involves the removal of damaged organelles by autophagy in response to mild oxidative stress which contributes to cellular recovery (Kiffin et al., 2006). Further studies are required to distinguish the relation between oxysterols and autophagy. Of note, relating this crosstalk to atherosclerosis progression and developing therapeutic interventions to target such diseases are exciting prospects for future studies.

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APPENDIX

APPENDIX A

Chemicals (in alphabetical order)

Name of Chemical	**Supplier Company**
2-Mercaptoethanol	Fluka, Switzerland
27-Hydroxycholesterol	Steraloids Inc., USA
27-OH-d7	Avanti PolarLipids, USA
4-Hydroxyphenylacetic acid	Sigma, Germany
Acetic Acid	Merck, Germany
Acrylamide/Bis-acrylamide	Sigma, Germany
Acrylamide/Bis-acrylamide	Biorad, USA
Ammonium Persulfate	Sigma, Germany
Bradford solution	Biorad, USA
BSA	Promega, USA
BSA	Amresco, USA
Bromophenol Blue	Sigma, Germany
Coomassie Brilliant Blue	Merck, Germany
DAPI	Roche, Germany
DC protein assay	Biorad, Germany
Developer/Replenisher	Agfa, Belgium
DHE	Sigma, Germany
Distilled water	Milipore, France
DMSO	Sigma, Germany
DPI	Sigma, USA

DTT	Sigma, Germany
EDTA	Riedel-de Haen, Germany
EGTA	Riedel-de Haen, Germany
Ethanol	Riedel-de Haen, Germany
Ferrocytochrome c	Sigma, Germany
Fixer E.O.S.	Agfa, Belgium
Foetal Bovine Serum (FBS)	Pan, Fermany
Glycerol	Riedel-de Haen, Germany
Glycine	Molekula, UK
HCl	Merck, Germany
HEPES	Molekula, UK
Hyperfilm ECL	Amersham, UK
Isopropanol	Riedel-de Haen, Germany
JC-1	Life Technologies, Italy
KCl	Amresco, USA
KH_2PO_4	Riedel-de Haen, Germany
KOH	Riedel-de Haen, Germany
L-Glutamine	Sigma, Germany
Liquid nitrogen	Karbogaz, Turkey
LY294002	Calbiochem, USA
Methanol	Riedel-de Haen, Germany
$MgCl_2$	Sigma, Germany
Milk Diluent concentrate	KPL, USA
Na_2HPO_4	Merck, Germany
NAC	Sigma, Germany
NaCl	Riedel-de Haen, Germany
NP-40	Sigma, Germany
PD98059	Calbiochem, USA
Penicillin-Streptomycin	PAN, Germany
Phenol	Amersco, USA
phoSTOP phosphatase inhibitor	Roche, Switzerland
PMSF	Sigma, Germany
Protease inhibitor coctail tablet	Roche, Switzerland
PVDF membrane	Amersham, UK
RPMI 1640	PAN, Germany
Sodium Dodecyl Sulphate	Sigma, Germany
TEMED	Sigma, Germany
Tris	Molekula, UK
Tween20	Molekula, UK
X-ray Film Biomax MS-1	Sigma, Germany
X-ray film	Sigma, Germany

APPENDIX B

Antibodies and enzymes (in alphabetical order)

Name	**Supplier Company**
Anti-β-Actin	Cell Signaling, USA
Anti-Akt	Cell Signaling, USA
Anti-p-Akt	Cell Signaling, USA
Anti-p-Bad	Cell Signaling, USA
Anti-Bcl-xl	Cell Signaling, USA
Anti-Bim	Cell Signaling, USA
Anti-cleaved caspase 3	Cell Signaling, USA
Anti- ERK1/2	Cell Signaling, USA
Anti-p-ERK1/2	Cell Signaling, USA
Anti-HO-1	Cell Signaling, USA
Anti-Lamin A/C	Cell Signaling, USA
Anti-mouse HRP	Cell Signaling, USA
Anti-Nrf2	Cell Signaling, USA
Anti-NQO-1	Cell Signaling, USA
Anti-rabbit HRP	Cell Signaling, USA
Anti-rabbit FITC	Abcam, UK
RNase -Superase In	Life technologies, USA

APPENDIX C

Molecular biology kits and reagents (in alphabetical order)

Name	**Supplier Company**
AnnexinV-FITC	Alexis, USA
ECL Advance	Amersham, UK
Hs_NFE2L2 s9493 siRNA	Applied Biosystems, USA
High-Capacity cDNA synthesis Kit	Life Technologies, USA
Negative Control siRNA	Applied Biosystems, USA
NeoFX transfection reagent	Applied Biosystems, USA
Quantitech RT Kit	Qiagen, Netherlands
Quantitech SYBRgreen KİT	Qiagen, Netherlands
Taq PCR Master Mix Kit	Qiagen, Netherlands
Trizol	Life Technologies, USA
WST-1 (Cell Proliferation Kit)	Roche, Germany

APPENDIX D

Buffers and solutions (in alphabetical order)

Standard buffers and solutions used in this study were prepared according to the protocols in *Molecular Cloning: A Laboratory Manual*, Sambrook et al., 2001. (Sambrook and MacCallum, 2013).

Annexin-V staining buffer

100 μl cell suspension in FACS incubation buffer
2 μl Annexin-V (Alexis)

Blocking solution

5 g nonfat dried milk
100 ml washing solution

Complete lysis buffer

20 mM Tris-HCl (pH 7.5)
150 mM NaCl
NP-40 0.5% (v/v)
1 mM EDTA
0.5 mM PMSF
1 mM DTT
Protease and phosphatase inhibitor mix

Cytosolic protein extraction buffer

20 mM Hepes pH 7.9
0.35 M NaCl
20% glycerol
1% Igepal CA-630
1 mM $MgCl_2$
0.5 mM EDTA
0.1 mM EGTA
Protease inhibitors

DAPI stock solution (1000X)

10 mg DAPI

10 ml ddH_2O
Store at -20°C

DAPI working solution (1X)

1 µl DAPI stock solution (1000X)
1 ml Methanol (Store at +4°C up to 6 months)

FACS incubation buffer

10 mM HEPES
140 mM NaCl
2,5 mM $CaCl_2$
pH: 7,4

Immunoblotting washing solution

100 ml PBS (10X)
900 ml ddH_2O
2 ml Tween20 (final: 0,2%)

Phosphate-buffered saline (PBS) (10X)

80 g NaCl
2.25 g KCl
23.27 g Na_2HPO_4
2.05 g KH_2PO_4
pH: 7,4
Adjust to 1000ml with ddH_2O

PBS-Tween20 (PBST) solution (1X)

0.5 ml Tween20
Adjust to 1000 ml with 1X PBS

Primary antibody incubation solution

2 ml 5% (w/v) milk blocking solution
1µg primary antibody

Running buffer (10X)

144.1g glycine
30.3g Tris
10g SDS
Adjust to 1000ml with ddH_2O

SDS Seperation Gel

3.75 ml Tris/SDS (1.5M pH 8.8)
5.25 ml H_2O
6 ml Acryl: Bisacryl (30%)
50 µl 10% APS
10 µl TEMED

SDS Stacking Gel

1.25 ml Tris/SDS (0.5M pH 6.8)
3.05 ml H_2O
0.65 ml Acryl: Bisacryl (30%)
25 µl 10% APS
10 µl TEMED

Secondary antibody incubation solution

5 ml 5% (w/v) milk blocking solution
1 µg secondary antibody

T1 Buffer

10 mM HEPES-KOH (pH: 7,9)
2 mM MgCl2.6H2O
0,1 mM EDTA
10 mM KCl
1% NP-40
1 mM DTT (freshly added)
0,5 mM PMSF (freshly added)
Complete protease inhibitors (freshly added)

T2 Buffer

50 mM HEPES-KOH (pH: 7,9)
2 mM $MgCl_2.6H_2O$
0,1 mM EDTA
50 mM KCl
400mM NaCl
10% (v/v) Glycerol
1 mM DTT (freshly added)
0,5 mM PMSF (freshly added)
Complete protease inhibitors (freshly added)

Transfer buffer (stock: 10X)

144 g glycine

30.3 g Tris
Adjust to 1000ml with ddH_2O

Transfer buffer (working: 1X)

100 ml Transfer buffer (10X)
700 ml ddH_2O
200 ml Methanol

APPENDIX E

Protein Molecular Weight Marker

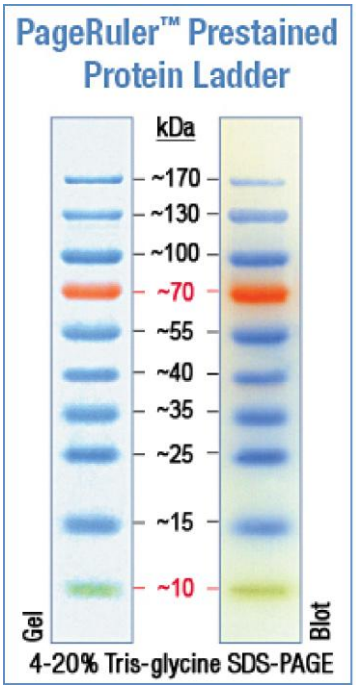

Figure E.1: Protein Molecular Weight Marker (Fermentas, Germany)

APPENDIX F

Equipments (in alphabetical order)

Name	**Supplier Company**
Autoclave	Hirayama,Hiclave HV-110,Japan
Balance	Sartorius, BP221S, Germany
	Schimadzu, Libror EB-3200 HU, Japan
Cell Counter	Cole Parmer, USA
Centrifuge	Eppendorf, 5415D, Germany
	Eppendorf, 5415D, Germany
	Eppendorf, 5415R, Germany
CO_2 Incubator	Binder,Germany
Computer Software	FlowJo V10
	Image J 1.42q
	Photoshop CS5
	Irfanview 4.20
	LSM 510 Image Examiner
	MS Office 2007
Deepfreeze	-80^{o}C, Kendro Lab Prod., Heraeus Hfu486 Basic, Germany
	-20^{o}C,Bosch,Turkey
Distilled Water	Millipore, Elix-S, France
Flow Cytometer	BD FACS Canto,USA
Gel Documentation	Biorad, UV-Transilluminator 2000, USA
Heater	Thermomixer Comfort, Eppendorf, Germany
Hematocytometer	Hausser Scientific,Blue Bell Pa.,USA
Ice Machine	Scotsman Inc., AF20, USA
Incubator	Memmert, Modell 300, Germany
	Memmert, Modell 600, Germany
Laminar Flow	Kendro Lab. Prod., Heraeus, HeraSafe HS12, Germany
Liquid Nitrogen Tank	Taylor-Wharton,3000RS,USA
Magnetic Stirrer	VELP Scientifica, ARE Heating Magnetic Stirrer, Italy
Mass Spectrometer	HP 5970 MSD, Agilent, USA
Microliter Pipettes	Gilson, Pipetman, France
	Eppendorf, Germany
	Mettler Toledo Volumate, USA
Microscope	Zeiss Axiovert 200 M, Milan Italy
	Zeiss LSM 510; Carl Zeiss S.p.A., Arese, Milan, Italy
Microtiter plate reader	Biorad, USA
pH meter	WTW, pH540 GLP MultiCal, Germany

Power Supply	Biorad, PowerPac 300, USA
	Wealtec, Elite 300, USA
Refrigerator	Bosch,Turkey
Spectrophotometer	ND-1000, Nanodrop, USA
	Schimadzu UV-1208, Japan
	Scimadzu UV-3510, Japan
Thermocycler	Eppendorf, Mastercycler Gradient, Germany
Vortex	Velp Scientifica,Italy
Water bath	Huber, Polystat cc1, Germany

Peer-reviewed publications arising from this thesis:

1. B Vurusaner, P Gamba, G Testa, S Gargiulo, F Biasi, G Leonarduzzi, H Basaga, G Poli.Survival signaling elicited by 27-hydroxycholesterol through the combined modulation of cellular redox state and ERK/Akt phosphorylation. Free Rad. Biol. Med. 77:376-385 (2014).

2. B Vurusaner, P Gamba, S Gargiulo, G Testa, E Staurenghi, G Leonarduzzi, G Poli, H Basaga. Nrf2 antioxidant defense is involved in survival signaling elicited by 27-hydroxycholesterol in human promonocytic cells. Free Rad. Biol. Med. 91, 93–104. (2016).

Text from the following review paper has been used for the writing of the Introduction section of this thesis:

1. B Vurusaner, G Leonarduzzi, P Gamba, G Poli, H Basaga. Oxysterols and mechanism of survival signaling. (Submitted)

Related publications outside the thesis:

1. Vurusaner, B., Poli G., Basaga, H.: Tumor suppressor genes and ROS: Complex networks of interactions. Free Rad. Biol. Med. 52: 7-18 (2012).