Oxysterols and mechanisms of survival signaling.

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Abstract	Oxysterols, a family of oxidation products of cholesterol, are increasingly drawing attention of scientists to their multifaceted biochemical properties, several of them of clear relevance to human pathophysiology. Taken up by cells through both vesicular and non vesicular ways or often generated intracellularly, oxysterols contribute to modulate not only the inflammatory and immunological response but also cell viability, metabolism and function. Indeed, the signaling pathways and the transcription factors whose activation they can influence are quite a number. Moreover, oxysterols have been recognized as elective ligands for the most important nuclear receptors. The outcome of such a complex network of intracellular reactions promoted by these cholesterol oxidation products appears to be largely dependent upon the type of cells, the dynamic conditions of the cellular and tissue environment but also certainly on the actual concentration of the oxysterol. Here focus has been given to the cascade of molecular events exerted by relatively low concentrations of certain oxysterols that elicit survival and functional signals

	in the cells, with the aim to contribute to further expand the actual knowledge about the biological and possibly physiological potential of the biochemical reactions triggered and modulated by oxysterols.
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Dear Editor and Associate Editors,

Please find our revised manuscript titled as **'Oxysterols and mechanisms of survival signaling.'** for a possible publication as an original review article in Molecular Aspects of Medicine for special issue 'Hormetic and regulatory effects of lipid oxidation products'by invitation of Guest Editor Giuseppe Poli. This is the resubmission of our work and prepared according to the regulations in the "Manuscript Guidelines" and contains a total of 13.762 words together with 4 figures that were added in actual print sizes. Please find the amendments to the initial manuscript as uploaded to "MAM_2016_4_Revised with highlighted amendments" document. All the amendments and answers are organized point-by-point to carefully address the reviewers' points as uploaded to "Response to reviewers' requests and comments_MAM" section. All co-authors have seen and agreed with the contents of the above mentioned manuscript and there is no financial interest to report. We certify that the submitted original manuscript is not under review by any other journal.

We thank you in advance for your consideration.

Best regards,

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Response to Reviewers

Reviewer #1:

1) "It could be better to replace parts 2 and 3 by only one part called "Oxysterols, signal transduction and transcriptional activity". Each transcription factor targeted by oxysterols could be presented separately."

Following the Reviewer's suggestion, we have re-organized the manuscript in a more logical and better readable way:

1. Introduction
2. Oxysterols, signal transduction and transcriptional activity
    - 2.1 Oxysterols and signal transduction
    - 2.2 Oxysterol-mediated activation of transcription factors
3. Survival signaling pathways and oxysterols
    - 3.1 Principal mechanisms of survival signaling
    - 3.2 Oxysterols and cell survival
4. Conclusions

We hope that this new version is satisfactory for the Reviewer.

2) "Part 4 Survival Signaling: The presentation of MAPKs, PI3K/Akt…is OK, but some survival systems have been omitted in this part (ER stress, autophagy, sphingosine 1-phosphate etc…). One suggestion is to have a more general task for instance "Oxysterols and survival signalling pathways" and discuss each survival signalling together with its interactions with oxysterols and the cellular consequences."

In paragraph 3.1 we have included the general description regarding Nrf2 and autophagy (that were already present in the manuscript but in different paragraphs), ER stress and sphingosine 1-phosphate (new parts).

In paragraph 3.2, the interactions between the above cited survival signaling and the principal oxysterols has been reported.

3) "Some sentences are too long and too convoluted (ex page 9"There is a strong experimental…. (VCAM1), etc…"

Long and convoluted sentences have been simplified and rewritten.

Reviewer #2:

1) "…, paragraph 4 "Survival signals" and the related 4.1, 4.2 and 4.3, sound a bit too long. They refer to general knowledge on pro-survival pathways, with no reference to the oxysterols. I suggest including the most important statements of this part in the previous paragraphs 2 and 3. …..…"

Following the reviewer's suggestion, we have re-organize the manuscript in a more logical and better readable way:

1. Introduction
2. Oxysterols, signal transduction and transcriptional activity
    - 2.1 Oxysterols and signal transduction
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    - 3.3 Principal mechanisms of survival signaling
    - 3.4 Oxysterols and cell survival
4. Conclusions

We hope that this new version is satisfactory for the Reviewer.

2) "In paragraph 5.2.1 "…..… I suggest revising carefully the literature cited. For instance, the Authors talk about "recent studies" referring to Anwar 2005, Yu 2000, Papaiahgari 2004-2006, Martin 2004, Iles 2005 or Li 1996. Are these the most recent papers on the regulation of Nrf2 by ERK, Akt, p38 or PKC?"

The statement "recent studies" has been removed when it was not suitable. A few more recent articles on Nrf2 are now included in paragraph 3.1.

3) "In the Introduction, the Authors refer to some unpublished data from Domenicotti et al. If they refer to "Marengo B, …..…", then it should be cited properly."

The reference "Marengo et al, 2015" is now cited properly

Oxysterols and mechanisms of survival signaling

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Abstract

Oxysterols, a family of oxidation products of cholesterol, are increasingly drawing attention of scientists to their multifaceted biochemical properties, several of them of clear relevance to human pathophysiology. Taken up by cells through both vesicular and non vesicular ways or often generated intracellularly, oxysterols contribute to modulate not only the inflammatory and immunological response but also cell viability, metabolism and function by modulating several signaling pathways. Moreover, they have been recognized as elective ligands for the most important nuclear receptors. The outcome of such a complex network of intracellular reactions promoted by these cholesterol oxidation products appears to be largely dependent on the type of cells, the dynamic conditions of the cellular and tissue environment but also on the concentration of the oxysterols. Here focus has been given to the cascade of molecular events exerted by relatively low concentrations of certain oxysterols that elicit survival and functional signals in the cells, with the aim to contribute to further expand the knowledge about the biological and physiological potential of the biochemical reactions triggered and modulated by oxysterols.

Key words

Oxysterols, Survival, Nrf2, Autophagy

Abbreviations

5,6-S, 5,6-secosterol; 24OH, 24-hydroxycholesterol; 25OH, 25-hydroxycholesterol; 27OH, 27-hydroxycholesterol; 7K, 7-ketocholesterol; 7βOH, 7β-hydroxycholesterol; AGE, advanced glycation end products; AMPK, AMP-activated protein kinase; AP-1, activator protein-1; ARE, antioxidant response element; ASK, apoptosis signal-regulating kinase; ATF, activating transcription factor; Aβ, amyloid-β; CHOP, CEBP-homologous protein; CREB, cyclic AMP response element binding protein; DAG, diacylglycerol; DAPK, death associated protein kinase; EGF, epidermal growth factor; EIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GRP78, glucose-regulated protein 78; GSK3, glycogen synthase kinase 3; HNE, 4-hydroxynonenal; HO-1, heme oxygenase 1; HUVEC, human umbilical-vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; IGFBP1, insulin-like growth factor-binding protein 1; IKK, inhibitory κB kinase; IL, interleukin; IP3, inositol 1,4,5-triphosphate; IRE1, inositol requiring protein-1; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; LOX-1, lectin-like oxLDL scavenger receptor 1; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; MCP-1, monocyte chemotactic protein 1; MEK, mitogen-activated protein kinase ERK kinase; Mnk, MAPK interacting kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; NOX, NADPH oxidase; NQO-1, NADPH:quinone oxidoreductase 1; Nrf2, nuclear erythroid 2-related factor 2; OSBP, oxysterol-binding protein; oxLDL, oxidized low density lipoprotein; oxPAPC, oxidized 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine; PDGF, platelet-derived growth factor; PDK, 3-phosphoinositide-dependent protein kinase; PERK, protein kinase RNA-like ER kinase; PI3K, phosphatidylinositol 3-kinase; PIP2, PIP3, phosphatidyl inositol di/triphosphate; PK, protein kinase; PLC, phospholipase C; PM, plasmamembrane;−PP2A, protein phosphatase 2A; PPAR, peroxisome proliferator-activated receptor; RNS, reactive nitrogen species; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; RXR, retinoid X receptor; SCAP, SREBP cleavage activating protein; SMC, smooth muscle cell; Smo, Smoothened transducer; S1P, sphingosine 1-phosphate; SREBP, sterol regulatory element binding protein; TLR, Toll like receptor; Triol, cholestan-3β,5α,6β-triol; UPR, unfolded protein response; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; XBP1, X-box-binding protein 1.

1.Introduction

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 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 exerts a number of effects not simply *per se* but mainly through the biochemical properties exerted by its metabolites. Among cholesterol metabolites, an increasing attention is drawn by the family of cholesterol oxidation products termed oxysterols. Oxysterols are 27-carbon molecules that, unlike cholesterol, have 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 cholesterol, thus suggesting their involvement in many of the biochemical and biological effects ascribed to cholesterol (Leonarduzzi et al., 2002; Schroepfer, 2000). In **Figure 1**, the oxidation sites in the cholesterol molecule are depicted, and **Table 1** reports on the most representative oxysterols of non enzymatic and enzymatic origin.

In the last years 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, and in gene regulation. Evaluating the 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). In particular, the molecular aspects of their pro-inflammatory effects have been well deepened, and a growing bulk of experimental findings points to a significant contribution paid by these cholesterol derivatives to the progression of

inflammatory-based chronic pathologies, such as vascular aging, atherosclerosis, Alzheimer's disease, multiple sclerosis, inflammatory bowel disease and colorectal cancer, non alcoholic liver disease, retinopathies, diabetes mellitus (Biasi et al., 2013; Gamba et al., 2012, 2015; Gargiulo et al., 2015; Poli et al., 2013).

Nowadays, new emphasis to the beneficial effects exerted by at least certain oxysterols, has been given by the largely proven evidence that side-chain cholesterol oxides like 24-, 25- and 27-hydroxycholesterol (24OH, 25OH and 27OH) are among the best ligands of a variety of physiologically important nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs) and liver X receptors (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.,1996,1999).

It has been shown that 27OH, a good LXR ligand, rather polarized human macrophages towards an anti-inflammatory state (Marengo et al., 2015), while the sterol ring-derived oxysterol, namely 7keto-cholesterol (7K), definitely not binding to LXRs, induced and sustained mainly pro-inflammatory reactions in human monocyte-derived macrophages (Buttari et al., 2013). 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.

So far, the focus was only on the negative effects of oxysterols, including their potential pro-apoptotic effect on various cell types. In the last years, an increasing bulk of studies is giving evidence of the involvement of certain oxysterols, in particular 27OH, in the modulation of cell survival pathways (Berthier et al., 2005; Riendeau and Garenc, 2009; Vurusaner 2014, 2015).

Before trying to straighten out the knowledge about oxysterols and their modulation of survival signaling, it is noteworthy to mention that 27OH, together with 25OH, has been

shown to exert a broad of antiviral effects 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 (see Lembo et al., in this issue).

2. Oxysterols, signal transduction and transcriptional activity

2.1 Oxysterols and signal transduction

There is not an unique way by which oxysterols can trigger the various cell signaling pathways within cells. Furthermore, uptake and cellular trafficking 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 plasmamembrane (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 diphosphate (PIP2) and phosphatidyl inositol 3,4,5 triphosphate (PIP3) (Wang and Richards, 2012), namely two key regulators of several signaling pathways, including the PI3K/Akt survival signaling cascade (Di Paolo and De Camilli, 2006). The effect of the various oxysterols on lipid rafts formation and stability is not homogeneous. While 27OH and 25OH seem to favour raft physiological functions, 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 (PLC)/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 protein kinase C (PKC)/mitogen-activated protein kinase ERK kinase (MEK)/extracellular signal-regulated kinases (ERK) pathway (Leonarduzzi et al., 2010).

Another primary trigger of the PKC/MEK/ERK pathway, is represented by NADPH oxidase (NOX), located in caveolae and lipid rafts (Jin et al., 2011), whose assembly and activation within PM 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 of reactive oxygen species (ROS) under physiological conditions and oxysterols were shown able to up-regulate 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, 2015).

Still on PM, at least certain oxysterols of pathophysiological relevance, like 25OH and 27OH, 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 Patched receptor and the Smoothened transducer (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 to be strictly dependent on lipid raft integrity and function (Shi et al., 2013).

Moreover, the internalization of oxidized low density lipoproteins (oxLDL) 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 up-regulate expression and synthesis of CD36 (Leonarduzzi et al., 2008, 2010).

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's transport within different cell compartments (Maxfield and Wustner, 2002), certainly even if not only involving oxysterol-binding proteins (OSBPs). 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 were displaying a key role in the modulation of ERK1/2 phosphorylation level, by forming an active oligomer with the serine/threonine protein phosphatase 2A (PP2A) (Wang et al., 2005). Anyway, 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 PM 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.

2.2. 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 appear to be redox modulated. Among them there are sterol regulatory element binding proteins (SREBPs), nuclear factor κ B (NF-κB), Toll like receptors (TLRs), nuclear factor erythroid 2-related factor 2 (Nrf2), LXRs, retinoid X receptor (RXR), PPARs, retinoic acid receptor-related orphan receptors (RORs), estrogen receptors (**Figure 2**). The mentioned transcription factors are described below.

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-κB, through the ERK/c-Jun N-terminal kinase (JNK) pathway (Leonarduzzi et al., 2005; Umetani et al., 2014), with or without the involvement of Erα (Umetani et al., 2014). There is strong experimental evidence that a variety of cholesterol oxidation products may up-regulate a large number of inflammation-related genes. Namely, expression of these genes is NF-κB-dependent, like those coding for interleukin (IL)-1β, -6, -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-κB nuclear translocation and consequent gene transcription stem from the activation of TLRs, a family of receptors primarily involved in the innate immunity

and localized on the PM and/or in endosomes, that may also be induced by oxysterols such as 27OH and 25OH (Gargiulo et al., 2015; Poli et al., unpublished data).

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

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., 1996, 1999). The two oxysterols mainly investigated for their LXR binding property are 24OH and 27OH and their involvement in the physiological regulation of cholesterol and lipid metabolism strongly proposed (Björkhem, 2009, 2013). 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 (Buttari et al., 2013).

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 7αOH, 7βOH, 7K (Wang et al., 2010a) and, with regard to side chain cholesterol oxides, by 24OH (Wang et al., 2010b).

Finally and importantly, 27OH was definitely demonstrated to act as competitive ligand for estrogen receptor-α and -β, by this way triggering intracellular signals potentially able to modulate cancer cell growth and atherosclerosis progression (Lee et al., 2014; Umetani et al., 2007, 2014). 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 25OH, which was shown to signal trough estrogen receptor-α (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 favour 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 apoptosis (de Medina et al., 2011).

3. Survival signaling pathways and oxysterols

Over the last decade, numerous *in vitro* studies have characterized the potential pro-apoptotic effect of oxysterols in a 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 pathologies including atherosclerosis and neurodegenerative diseases.

Based on the presently available data, oxysterols show differences in the degree of cytotoxicity and ability to induce cell death, but these cellular effects have been mostly studied for single compounds. However, oxysterols in oxLDL, foods and the core region of atherosclerotic plaques are always present as a mixture and literature about the way oxysterols act collectively is limited. In this connection, Biasi et al. reported that the cytotoxicity of single 7K and 7βOH is quenched when cells are challenged with a mixture composed of both oxysterols (Biasi et al., 2004). In particular, murine macrophages treated with 7K underwent apoptosis through the mitochondrial pathway, whereas when the same cells were treated with 7K and an equimolar concentration of 7βOH, the pro-apoptotic effect of the first oxysterol was markedly attenuated. Notably, the 7K-induced intracellular ROS rise, dependent upon NOX activation, was inhibited in the presence of the oxysterol mixture, suggesting the occurrence of a substrate-based competition among oxysterols at the level of NOX (Biasi et al., 2004; Leonarduzzi et al. 2004, 2006). In agreement with this, Aupeix et al. showed that the challenge of U937 human promonocytic cell line with 7βOH (30-40 μM) alone exerted pro-apoptotic effects, that significantly diminished when an identical amount of 25OH was simultaneously added (Aupeix et al., 1995).

Interestingly, it has recently been demonstrated that oxysterols of pathophysiological interest could show both beneficial and detrimental properties at the same time, i.e. that they could

trigger both survival and death signals within cells. In **Figure 3** the main survival signals modulated by oxysterols are summarized.

3.1 Principal mechanisms of survival signaling

Activation of receptor signaling initiates multiple signal transduction pathways involved in cell survival. The three best characterized signaling pathways activated in response to receptor tyrosine kinases (RTKs) are the mitogen-activated protein kinases (MAPKs) cascade, the phosphatidylinositol 3 kinase (PI3K)/Akt cascade and the PLC cascade (Katz et al., 2007). Besides these pathways, Nrf2 transcription factor also plays a key role in cell survival by protecting cells against oxidative damage (Vurusaner et al., 2015). Moreover, there is now growing evidence suggesting that endoplasmic reticulum (ER) stress (Schleicher et al., 2010) and autophagy (Scherz-Shouval and Elazar, 2011) modulate the balance between cell survival and death.

MAPKs. Cells can sense and respond to stress in various ways, including initiation of cell death and promotion of cell survival. There are many different types of response to stress that depend on the type, strength and duration of the stimuli involving a complex network of signaling pathways. Among these pathways MAPKs, a group of proline-directed serine/threonine kinases, are the best characterized signaling molecules (Arciuch et al., 2009). MAPKs regulate stress signals in a three layer cascade fashion with a MAPK kinase kinase (MAPKKK) phosphorylating and activating its substrate MAPK kinase (MAPKK) which are dual-specificity kinases that in turn phosphorylate serine and threonine residues in their MAPK substrate (Trachootham et al., 2008).

In mammals, three distinct cascades of MAPKs have been elucidated: ERK 1/2, JNK, 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 a 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 (Ramos, 2008).

JNK is encoded by three genes, termed JNK1, JNK2 and JNK3, and these genes alternatively splice resulting in 10 or more JNK isoforms (Arciuch et al., 2009). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is present in the brain, heart and testis. JNK signaling cascade regulates cell death and the development of multiple cell types in the immune system, whereas JNK1 and JNK2 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 by inflammatory cytokines. JNK phosphorylation is catalyzed by two protein kinases MAPKK4 and MAPKK7 which are dual specifity kinases that selectively phosphorylate JNK on Tyr and Thr, respectively (Davis, 2000). JNK translocates to the nucleus where it phosphorylates and up-regulates several transcription factors, including c-Jun, JunA, JunB, activating transcription factor 2 and Elk-1 (Katz et al., 2007).

The p38 kinase family consists of four members namely α, β, γ and δ and these enzymes are activated by hormones, cytokines, G protein-coupled receptor ligands and cellular stress

(Arciuch et al., 2009). Activation of p38 kinases is mediated by the MAPKK3 and MAPKK6, and following the activation, p38 phopshorylates its substrates including MAPK interacting kinases 1 and 2 (Mnk1 and Mnk2), and eukaryotic initiation factor 4e (Roux and Blenis, 2004). Many studies have shown that p38 MAPKs have critical role in molecular 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 may result in many diseases and cancer. Thus, MAPKs are molecular targets for drug development, and pharmalogical manipulation of these kinases will likely help the treatment of human diseases related to disproportionate apoptosis.

PI3K /Akt. The PI3K/Akt pathway has been established as one of the most critical signaling pathways 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 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 PM. Following activation, PI3K converts the PIP2 lipids to PIP3, a second messenger that recruits 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt (also knows as PKB) to the PM (Arciuch et al., 2009; Cantley, 2002). Subsequently, PDK1 phosphorylates Akt at two regulatory sites, Thr^{308} in the activation loop and Ser^{473} in the C-terminal tail: phosphorylation of both sites is required for full activation of Akt (Arciuch et al., 2009). Akt activation leads to phosphorylation of various substrate proteins, including caspase-9, Mdm2, glycogen synthase kinase 3 (GSK3) and forkhead transcription factor (FKHRL1), which targets FasL, Bim, insulin-like growth

factor-binding protein 1 (IGFBP1), and Puma. A large amount of evidence has suggested that Bad is one of the direct targets of Akt in promoting cell survival and that phopshorylation of Bad on Ser^{136} by Akt inactivate this pro-apoptotic factor (Song et al., 2005; Trachootham et al., 2008).

Akt can also exert its anti-apoptotic functions by phosphorylating inhibitory κB kinase (IKK) and the cyclic AMP response element-binding protein (CREB), with consequent elevated transcription of genes encoding the anti-apoptotic proteins Bcl-2, Bcl-XL, and Mcl-1. Another target of Akt is apoptosis signal-regulating kinase 1 (ASK1) and its Akt-mediated phosphorylation inhibits its ability to activate JNK/p38 and prevents 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).

PKCs. PLCγ activation is stimulated by G protein coupled receptors that interact with G proteins of the G_q family. Active PLCγ enzyme catalyzes the hydrolysis of PIP2 to produce inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Binding of IP3 receptors on the membrane of the endoplasmic reticulum causes the release of intracellular Ca^{2+} which is followed by the activation of PKC family members (Katz et al., 2007; O'Gorman and Cotter, 2001).

The PKC is an ubiquitous family of serine/threonine kinases and has at least 10 members containing a highly conserved kinase core at the C-terminal and an N-terminal autoinhibitory 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, □ and η) contain DAG-sensitive C1 domains but Ca^{2+}-insensitive C2 domain while the atypical PKCs (ζ and ι) are both Ca^{2+} and DAG-independent. PKC isoforms play diverse role in signal

transduction, cell proliferation, differentiation, death, mitogenesis and stress responses (Arciuch et al., 2009; Guo et al., 2004). Most of the PKC family members have been shown to contribute to cell survival whereas members such as PKCα and PKCδ have been associated with apoptosis induction through inhibition of the Akt survival pathway and activation of p38 MAPK (Matsuzawa and Ichijo, 2005; Yang et al., 2008).

Nrf2. Nrf2, 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 by the interaction with Kelch-like ECH-associated protein 1 (Keap1), an actin-binding cytoskeletal protein (Motohashi and Yamamoto, 2004). Under oxidative stress, Nrf2 dissociates from Keap1, thus also from cytoskeleton, and migrates into the nucleus where it heteromerizes with Maf (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog) and binds to ARE sequences; Nrf2 then codifies for various antioxidant enzymes including aldoketoreductase, glutathione peroxidase, glutathione reductase, heme oxygenase 1 (HO-1), and NADPH:quinone oxidoreductase 1 (NQO-1) (Dinkova-Kostova, 2002; Dinkova-Kostova et al., 2001; Iqbal et al., 2003; Nguyen et al., 2009). While a growing bulk of evidence showed that the protective adaptive response to ROS/reactive nitrogen species (RNS) as induced by Nrf2 is mediated by enhanced expression of these cytoprotective enzymes, the specific upstream signal transduction pathways recruited to stimulate transcription of these phase II genes are yet poorly defined (Lee and Choi, 2003; Mann et al., 2007). In this relation, some studies demonstrated the clear involvement of several protein kinase pathways including ERK1/2, JNK, p38 and PKCs 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). More recently, Wang et al. demonstrated that palmitic acid stimulated hepatocyte

proliferation through the activation of Nrf2, which depends on ROS-induced activation of p38/ERK-Akt cascade (Wang et al., 2011). In bovine aortic endothelial cells, He at al. showed that JNK signaling pathway is involved in advanced glycation end products (AGE)-induced activation of Nrf2 with consequent expression of its target antioxidant genes (He et al., 2011). A cancer chemopreventive agent induced activation of Nrf2 through ERK1/2, p38, JNK, and PI3K kinases in kidney derived cells (Rojo et al., 2012).

ER stress. Perturbations in ER functions including protein folding, storing intracellular Ca^{2+} and lipid biosynthesis results in ER stress (Schröder, 2008). In eukaryotic cells, ER stress is sensed by 3 ER resident proteins: inositol requiring protein-1 (IRE1), activating transcription factor (ATF) 6 and protein kinase RNA-like ER kinase (PERK). Activation of these transmembrane sensors triggers unfolded protein response (UPR) that relieves ER stress and reestablishes homeostasis by several mechanisms, including inhibition of protein translation and induction of ER-resident chaperons (Kim et al., 2008a). IRE1 and ATF6 are crucial for chaperone production, which maintain the proper protein folding and promote cell survival. IRE1 also promotes the expression of X-box-binding protein 1 (XBP1), a transcription factor that facilitates degradation of misfolded proteins. In addition to XBP1, IRE1 pathways can also lead to apoptosis. Among the resident chaperons, the chaperone glucose-regulated protein 78 (GRP78) plays a key role in regulating ER stress by binding to transmembrane ER stress sensors inhibiting their activation. Altered protein folding in the ER stimulates GRP78 to release transmembrane signaling proteins including IRE1, PERK and ATF6 that allow oligomerization of IRE1 and PERK thus leading to UPR. As a result of oligomerization, PERK is activated. Activation of PERK results in the phosphorylation of eukaryotic translation initiation factor 2α (EIF2α) to slow down protein translation. However, in contrast, when EIF2α is phosphorylated and inactivated, certain mRNAs, including ATF4, are translated. ATF4 regulates the expression of several genes involved in restoring cellular redox

and ER homeostasis as well as autophagy genes. Moreover, ATF4, ATF6 and XBP1 assemble on the promoter of the gene encoding CEBP-homologous protein (CHOP), which participates as a down-stream component of ER stress pathways. Overexpression of CHOP is a potent inducer of apoptosis through a mechanism controlling the expression of Bim and Bcl-2 genes (Kim et al., 2008a; Scull and Tabas, 2010; Tabas, 2011).

The UPR-induced signal-transduction events ameliorate the accumulation of unfolded protein aggregates that are commonly accompanied by the activation of signaling pathways including ERK, JNK, p38, Akt and also NF-κB (Kaneko et al., 2003). In addition, ER stress associated with autophagy could activate alternative mechanisms to remove unfolded proteins independently from the proteasome system (Ogata et al., 2006).

Despite the beneficial functions of a transient UPR, prolonged ER stress leads to a wide range of diseases, including cardiovascular, stroke, diabetes, neurodegeneration and cancer. Prolonged activation of UPR occurs in various pathological conditions, including hypoxia, ischemia, heat shock, oxidative stress, proteasome inhibition and Ca^{2+} depletion of ER stores (Kim et al., 2008a; Oyadomari and Mori, 2004).

Autophagy. Selective autophagy of specific organelles and proteins can be induced in response to diverse stimuli and is involved in housekeeping degradation of aggregated proteins (aggrephagy), damaged mitochondria (mitophagy), and invading bacteria (xenophagy) via ubiquitin signaling (Deretic and Levine, 2009; Kirkin et al., 2009; Youle and Narendra, 2011). Macroautophagy (generally referred to as autophagy) breaks down macromolecules and allows cellular recycling to generate energy in order to survive to cellular stress. In this multi-step process, the cell material to be eliminated is sequestered within double-membrane vesicles known as autophagosomes, then the outer-membrane of the autophagosome fuses with lysosome for subsequent degradation (He and Klionsky, 2009). In the last years, it was made clear that even if autophagy can be considered a cell survival

mechanism, under certain conditions its excessive enhancement may lead to a caspase-independent non-apoptotic type of cell death (type II cell death) (Galluzzi et al., 2009). Several lines of evidence suggests that a cross-talk exists between autophagy and classical apoptosis. Important molecular players of such a connession appeared to be the anti-apoptotic genes Bcl-2, Bcl-xl and autophagy genes such as autophagy related 5 (Atg5), autophagy related 4D (Atg4D) and beclin1 (Dewaele et al., 2010).

Despite the increasing interest in understanding the mechanism of autophagy, there is limited information about the way cellular signaling molecules like kinases regulate this complex process. There are several reports that suggest a regulatory role for serine/threonine kinases involving mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), Akt, MAPKs (ERK, p38 and JNK) and PKCs in various steps of autophagy such as vesicle maturation, autophagosome inception and autophagy-related gene expression (Suffixidharan et al., 2011). It is generally accepted that PI3K/mTOR pathway primarily inhibits autophagy whereas the MAPK pathway and PKCs either positively or negatively regulate autophagy depending on the cellular context and inducers involved.

A role for autophagy in the cellular response to oxidative stress has been described. There are findings showing that increased ROS levels stimulate autophagic processes which, in turn, reduce ROS levels (Scherz-Shouval and Elazar, 2011). It has been described the important role of lipids, including sphingolipids, in regulating autophagy. In particular, ceramides and sphingosine 1-phosphate (S1P) is considered a physiological regulator of autophagy in relation to cellular growth, survival, and aging. These two sphingolipids act as second messengers to regulate the balance between cell death and survival, however, their effect on cell fate are opposite: S1P promotes cell survival and proliferation, whereas ceramide induces growth arrest and cell death (Harvald et al., 2015; Ogretmen and Hannun, 2004; Spiegel et al., 2003). Interestingly, it has been observed that S1P is implicated in the mitogenic response

of mesenchymal cells to a low oxidative stress; conversely, high oxidative stress induces growth arrest through S1P depletion (Cinq-Frais et al., 2013).

3.2. Oxysterols and cell survival

The first evidence of the involvement of oxysterols in cell survival was provided by Berthier and colleagues, in an *in vitro* study where THP-1 human monocytic cells were challenged with a high concentration of 7K (100 μM), namely one of the most abundant oxysterols in human tissues, provided with a strong pro-apoptotic effect. The treatment of THP-1 cells with 7K induces an early and transient up-regulation of MEK/ERK signaling pathway which transiently inactivated the pro-apoptotic protein Bad, thus significantly delaying the apoptotic process initiated by 7K itself (Berthier et al., 2005).

More recently, another group performed experiments on another human promonocytic cell line (U937) with low micromolar concentration of 27OH, showing a significant stimulation of cell viability due to the phosphorylation of Akt at residue Thr^{308}, postponing apoptotic death; conversely, higher concentrations of 27OH rapidly triggered lysosomal-independent apoptosis (Riendeau and Garenc, 2009). Moreover, using low micromolar doses of another oxysterol, cholestan-3β,5α,6β-triol (Triol), Jusakul et al. demonstrated that activation of pro-survival signaling in human MMNK-1 cholangiocytes involved ERK1/2 and p38α phosphorylation (Jusakul et al., 2013).

In a very recent study, our group provided clear evidence of survival signaling by 27OH. Low micromolar concentration (10 μM) of 27OH triggered survival signals in U937 human promonocytic cell line through the activation of ERK and PI3K/Akt, with consequent phosphorylation of the pro-apoptotic protein Bad; this reaction prevents the loss of transmembrane mitochondrial potential, thereby delaying cell apoptotic death. In addition, activation of both survival pathways appeared dependent upon a transient ROS increase,

mediated by the up-regulation of NOX2; thus, the prooxidant effect of 27OH resulted to be a key early mechanism in the survival signaling operated by the oxysterol, at least on U937 cells and at low concentration (Vurusaner et al., 2014) (**Figure 4**).

In agreement with our results, another oxysterol, 7βOH, was shown to be anti-apoptotic and to induce cell proliferation when added at low concentrations (below 20 μg/ml) to human umbilical-vein endothelial cells (HUVEC); this effect was dependent on the activation of MEK/ERK cascade, but apparently independent from ROS production (Trevisi et al., 2009). However, at higher concentrations, 7βOH led HUVEC to apoptosis, showing then a dual effect on endothelial cell viability, depending on the concentration.

Also oxLDL displayed a dual effect on cell viability and proliferation of endothelial cells (Galle et al., 2001). At low oxLDL concentrations (5-10 μg/mL) a proliferative effect was observed, while at high concentrations (50-300 μg/mL) cells underwent apoptotic death. Interestingly, a NOX-dependent increase of ROS appeared involved in both conditions (Galle et al., 2001; Heinloth et al., 2000).

With regard to 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 activation of both ERK1/2 and Akt as well as subsequent phosphorylation of Bad and IkBα (Hundal et al., 2001). In another study, THP-1 monocytic cells were challenged with 50 μg/ml oxLDL, and such a treatment attenuated staurosporine-induced apoptosis through the up-regulation of ERK but not PI3K/Akt signaling (Namgaladze et al., 2008). Of note, similarly to oxLDL, both 27OH and 24OH were shown to antagonize staurosporin mediated apoptosis in human neuroblastoma SH-SY5Y cells (Emanuelsson and Norlin, 2012).

While it is well established that oxLDL activate pro-survival signaling pathways promoting macrophage proliferation and survival, little is known regarding the upstream signaling events, and the pattern recognition receptors involved. Riazy et al. excludes a role for

endocytic pattern recognition receptors, scavenger receptor A and CD36 in the oxLDL-mediated survival of bone marrow derived macrophages (Riazy et al., 2011). Moreover, oxLDL-induced proliferation of SMCs appeared to be partly dependent on the bioactive sphingolipid S1P, via activation of sphingomyelinase, ceramidase, and sphingosine kinase (Augè et al., 1999; Levade et al., 2001). Furthermore, by using specific inhibitors, it has been also demonstrated that sphingomyelin/ceramide pathway was involved in 7k and α-epoxycholesterol-induced SMC proliferation (Liao et al., 2010). Nevertheless, there are few results about the link between oxysterols and the pro-survival pathway involving S1P.

It appears that both MEK/ERK and PI3K/Akt signaling pathways would play a critical role in the pro-survival effect inducible by modified lipoproteins, whereby the balance between anti-apoptotic pathways (ERK, Akt) and stress-activated pro-apoptotic pathways (JNK, p38) would determine the final effect: either cell survival or apoptosis.

Another important aspect regarding pro- and anti-apoptotic signaling by oxysterols is the likely modulation of cytosolic free Ca^{2+}. Lizard and colleagues demonstrated that 7K-induced apoptosis in THP-1 cells was triggered by a sustained influx of extracellular Ca^{2+} followed by activation of calcineurin and dephosphorylation of Bad (Berthier et al., 2004). Moreover, activation of calcineurin was proved to be induced by the translocation of transient receptor potential calcium channel 1, a component of the store-operated Ca^{2+} entry channel, into lipid raft domains of PM. Notably, the same group showed that 7K, in parallel to the pro-apoptotic signaling, exerted a Ca^{2+}-dependent activation of MEK/ERK survival pathway via Ca^{2+}-sensitive proline rich tyrosine kinase 2 (Berthier et al., 2005). A net increase of intracellular Ca^{2+} was shown to be required also for the pro-survival effect inducible by oxLDL (Matsumura et al., 1997). More recently, to clarify the down-stream pathways that are activated by intracellular Ca^{2+} increase, bone marrow derived macrophages were challenged with oxLDL; these oxidized micelles prevented macrophage apoptosis through mobilization

of intracellular Ca^{2+}, that in turn activated the Ca^{2+}-sensitive kinase eukaryotic elongation factor 2 (Chen et al., 2009).

An increasing bulk of evidence points to the redox regulation of several transcription factors such as Nrf2, NF-κB, Jun/activator protein-1 (AP-1) and p53, whose modulation may lead to cell cycle changes and integration of pro- and anti-apoptotic signals (Trachootham et al., 2008).

While AP-1 and p53 are mostly involved in the induction of cell death, up-regulation of Nrf2 and NF-κB rather promotes cell proliferation and survival.

At present, little is known about oxysterol-mediated survival effects operated at the transcriptional level. Here we will briefly review this matter focusing on the redox sensitive Nrf2 pathway which plays a critical role in protecting cells against oxidative/electrophilic stress following the exposure to a variety of exogenous and endogenous stimuli and promotes cell survival.

The activation of Nrf2 by components of oxLDLs in vascular cells suggests the involvement of this transcription factor and its related antioxidant response in the signaling pathway sustaining atherosclerosis progression (Ishii et al., 2004). Moreover, oxLDLs were shown to activate Nrf2 much strongly in murine macrophages than in smooth muscle cells (SMCs), while 4-hydroxynonenal (HNE), a major product of lipid peroxidation, stimulated Nrf2 nuclear translocation in both cell types. These findings suggest that Nrf2 modulation could depend on the type of the cells and of the chemical inducers.

In a very recent study, C6 glioma cells were challenged with either 10 or 20 μM 27OH to study its role in Nrf2 modulation in neurodegenerative diseases, including Alzheimer's disease. At the concentration employed, 27OH resulted to be pro-oxidant and neurotoxic, being able to significantly down-regulate the expression of Nrf2 and of its down-stream

antioxidant genes HO-1, NQO-1 and γ-glutamyl-cysteine synthetase, both at gene and protein level (Ma et al., 2015).

Nrf2-target genes, such as HO-1 and NQO-1, are critical in the cellular response against pro-oxidative stimuli such as oxLDLs and oxidized phospholipids, in macrophages, endothelial cells, and SMCs (Araujo et al., 2012). In this relation, the oxidized 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine (oxPAPC) was shown to induce HO-1, NQO-1 and glutamate-cysteine ligase modifier subunit expression in endothelial cells in a Nrf2-dependent manner (Jyrkkänen et al., 2008). Similarly, HO-1 induction via Nrf2 signaling was observed in vascular SMCs in response to moderately oxLDLs (Anwar et al., 2005).

Moreover, Nrf2 nuclear translocation is modulated by ERK1/2, p38, PKCs and PI3K/Akt pathways, but the activation of these upstream signals depends on the type of the inducer and of the targeted cells. As far as it regards, Papaiahgari and colleagues showed that, in pulmonary epithelial cells, in response to hyperoxia, a ROS-dependent ERK and Akt activation up-regulated Nrf2-mediated gene expression (Papaiahgari et al., 2004, 2006). Further, the survival signaling pathway PI3K/Akt-dependent was demonstrated to be responsible for Nrf2 protein increase and HO-1 expression in PC12 pheochromocytoma cells (Martin et al., 2004). Also HNE-mediated induction of HO-1 expression and synthesis in macrophages and epithelial cells was suggested to be dependent on the activation of ERK survival pathway (Iles et al., 2005; Li et al., 1996).

Inconsistent results were obtained in hepatoma cells challenged with 15-deoxy-delta 12,14-prostaglandin J2, where induction of HO-1 synthesis was not quenched by using the ERK1/2 inhibitor PD98059. This evidence suggests that the up-regulation of this heme catabolism-related enzyme might depend on the activation of more than a single signaling pathway (Liu et al., 2004). Conversely, the anti-apoptotic effect of 15-deoxy-delta 12,14-prostaglandin J2

was confirmed in rat pheochromocytoma cells through the involvement of ERK/Akt-dependent Nrf2 activation (Kim et al., 2008b).

Therefore, based on the presently available data, the activation of Nrf2 by different types of inducers, including lipid oxidation products, leads to cell survival and protection against oxidative stress, a condition that does not necessarily imply positive outcome, since Nrf2-dependent delay or quenching of apoptotic cell death might also result in tumorigenesis and drug resistance (Niture and Jaiswal, 2012).

There is now growing evidence indicating that ER stress is activated by oxidized lipids and modulates the balance between survival and apoptotic effects induced by lipid oxidation products. Sanson et al. demonstrated that oxLDLs trigger ER stress induction that can be prevented by the ER-associated chaperone oxygen-regulated protein 150 in human endothelial cells (Sanson et al., 2009). Moreover, 7K and HNE induced ER stress in vascular cells, characterized by the detection of ER stress markers (phospho Ire1 and PERK) and activation of their down-stream pathways. Interestingly, ER stress is involved in the apoptotic effect of oxLDLs through the Ire1/JNK pathway. Moreover, the antioxidant N-acetylcysteine prevented the ER stress induced by oxLDLs, 7K and HNE, suggesting that oxidative stress is involved in the activation of ER stress. In agreement with these findings, Pedruzzi et al. showed that 7K induced ER stress and apoptosis in aortic SMCs via the activation of Nox-4 (Pedruzzi et al., 2004). Increased ER stress also occurs in unstable plaques; in coronary artery SMCs and THP-1 monocytes, 7K induced ER stress and apoptosis (Myoishi et al., 2007).

In human aortic endothelial cells, oxidized phospholipids induced UPR activation and up-regulated the expression of inflammatory genes (Gargalovic et al., 2006). The ER stress induced by oxLDLs in endothelial cells was prevented by AMP-activated protein kinase (AMPK) (Dong et al., 2010). Low doses of 7K induced a more subtle ER stress and activated pattern recognition receptors (PRRs) which alter the balance between survival and death in

macrophages (Devries-Seimon et al., 2005; Seimon et al., 2006). Another study showed that lipid oxidation products, including oxysterols, present in advanced atherosclerotic plaques, contribute to trigger ER stress (Tabas, 2010). Altogether, these studies indicate that ER stress is induced by oxysterols and oxLDLs, and that it could play a key role in the progression of atherosclerosis.

A prolonged ER stress has been also identified as a pathogenic mechanism in diseases associated with neurodegeneration, such as Alzheimer's disease. In retinal pigment cells, 27OH increased the level of ER stress, together with amyloid-β (Aβ) production and oxidative stress (Dasari et al., 2010). Furthermore, it has been shown that 27OH activated CHOP thus down-regulating leptin, a hormone which reduces Aβ production and tau phosphorylation in neuroblastoma cells (Marwarha et al., 2012). In the given mechanism, CHOP was shown as a negative regulator of C/EPα, a transcription factor which regulates leptin expression. Another oxysterol, namely 24OH, has been shown to suppress Aβ production via amyloid precursor protein down-regulation, which occurred through the expression of GRP78 ER chaperone (Urano et al., 2013). According to these results, it can be suggested that the up-regulation of the nontoxic ER chaperon inducers, such as 24OH, may represent a therapeutic target for AD. Additional studies are needed to fully understand the relation between oxysterols and ER stress that may suggest new therapeutic implications in ER stress-driven pathologies.

It is now well established that autophagy appears stimulated in advanced atherosclerotic plaques by inflammation and oxidized lipids (Martinet and De Meyer, 2009). The protective role of autophagy in atherosclerosis involves the removal of damaged organelles in response to mild oxidative stress (Kiffin et al., 2006). In an *in vitro* pharmacological study, 7K-stimulated autophagy was shown to attenuate SMCs apoptosis induced by low concentrations of lipophilic statins (Martinet et al., 2008). Most likely, autophagy up-regulation in statin-

treated cells allowed to quench the release of cytohrome c into the cytosol and caspase activation, by this way limiting the extent of apoptotic cell death (Gozuacik and Kimchi, 2004; Martinet et al., 2008).

Further, oxidized lipids, such as oxysterols and HNE, are able to stimulate autophagy in atherosclerosis. Signs of autophagy, such as autophagolysosomes and increase of LC3-II, were detectable when epithelial cells were treated with oxLDLs but not with native LDLs (Nowicki et al., 2007). Moreover, 7K-induced autophagy was demonstrated in SMCs, in terms of myelin figure formation, intense protein ubiquitination, and LC3-II increase (Martinet et al., 2004). Interestingly, cultured SMCs treated with aggregated LDLs showed up-regulation of death-associated protein kinase (DAPK), a Ser/Thr death kinase that regulate membrane blebbing and autophagic (type II) programmed cell death (Inbal et al., 2002; Martinet et al., 2002). Indeed, DAPK was confirmed to induce survival in SMCs, since the antisense depletion of this kinase promoted caspase-dependent apoptosis (Jin and Gallagher, 2003).

Several studies focused on lectin-like oxLDL scavenger receptor 1 (LOX-1), a major receptor responsible for binding, internalization and degradation of oxLDLs, with a primary role in atherosclerosis development (Sawamura et al., 1997). In an *in vitro* study, autophagy activation in HUVECs challenged with oxLDLs was proven to be mediated by a ROS/LOX-1 pathway (Ding et al., 2013a). Such an autophagic response would contribute to the degradation of oxLDLs, thus favoring cell survival. In a similar way, treatment of vascular SMCs with a relatively low concentration of oxLDLs was shown to trigger autophagy with increased beclin-1and Atg5 expression, as well as LC3-II/LC3-1; on the contrary, high levels of oxLDLs induced SMC apoptosis and, under this experimental condition, autophagy was suppressed (Ding et al., 2013b).

Thus, autophagy may protect vascular cells against apoptosis depending on cell types, oxidant concentration and time of exposure (Perotta and Aquila, 2015) and 7K could be one of the triggering factors of autophagy itself. The same oxysterol was suggested to be primarily involved in another pro-survival mechanism involving autophagy, namely the up-regulation of a mitochondrial enzyme, proline oxidase, in oxLDLs challenged cancer cells,. Activation of this enzyme would increase intracellular ROS and, subsequently, cell autophagic response, by this way counteracting cancer cell apoptosis (Zabirnyk et al., 2010).

No published papers concerning the potential stimulation of pro-survival autophagy by oxysterols other than 7K are presently available, even if the involvement of 27OH at low micromolar concentrations appears very likely (Vurusaner et al., unpublished data). On the other hand, a relatively high concentration (50 uM) of 7K, but also of 7βOH, and 24(S)OH, led 158N murine oligodendrocytes to a type of death termed oxyapoptophagy (Nury et al., 2013). Once again, it seems that at least certain oxysterols may modulate the actual outcome of cell autophagic response mainly depending upon their concentration, being able to trigger pro-survival autophagy only when present in low amount.

By reviewing the available literature on oxysterol-induced pro-survival signals we are developing the opinion that, in general terms, this is a complex phenomenon depending on cell types, environmental factors, cell senescence, oxysterol concentration and exposure time. Notably, when relatively low and not directly toxic concentrations of oxysterols are administered in a biologically representative mixture, they tend to have a “Trojan-horse”-like behaviour (Biasi et al., 2004; Leonarduzzi et al., 2004). Namely, instead of killing cells directly, oxysterols might delay its irreversible damage, in the meantime initiating pro-inflammatory and pro-fibrogenic pathways. On the contrary, relatively higher amounts of the same compounds induce earlier and direct cell death (Leonarduzzi et al., 2002). Moreover, delayed macrophage apoptosis would favour growth and destabilization of advanced

atherosclerotic plaques (Martinet et al., 2012). Thus, the elucidation of the molecular mechanisms underlying oxysterol-induced pro- and anti-apoptotic signaling might contribute to a better understanding of the pathogenesis of several oxysterol-associated disease processes.

4. Conclusions

Oxysterols have been shown to contribute to the modulation of a wide variety of signaling pathways, thus influencing a number of transcription factors. Further, especially oxysterols of enzymatic origin have been recognized to be primary ligands of key nuclear receptors. As repeatedly stated in this review, the type of cells, the cellular and tissue environment and, above all, the actual concentration of the cholesterol oxides, resulted to be the main conditioning factors as far as their final effects are concerned.

We deemed of particular interest trying to comprehensively analyse the potential role of various oxysterols at least, with regard to the modulation of survival signaling. Oxysterols appear as primarily involved in the pathogenesis of inflammation-driven chronic pathologies like cancer, atherosclerosis, neurodegenerative diseases, thus possibly useful is to draw the attention to the high potentiality of oxysterols in triggering and sustaining survival response in cells challenged with the various stressors.

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Table 1 - Origin of plasma and tissue oxysterols

Oxysterols of not enzymatic origin

Deriving from the diet

Mainly formed by autoxidation of foodstuff containing cholesterol (meat, cheese, milk, dairy products, etc.) as induced by heat, light exposure, refrigeration, freeze-drying.

Generated in the body by:

Oxidation driven by reactive oxygen species and the leukocyte/H2O2/HOCl system, the most frequent source being the inflammatory processes; attack by peroxyl and alkoxyl radicals.

The quantitatively most represented compounds of this subgroup are:

7ketocholesterol, 7β-hydroxycholesterol, 5α,6α-epoxycholesterol,

5β,6β-epoxycholesterol, 3β,5α,6β- trihydroxycholestane

Oxysterols of enzymatic origin

Generated in the body by:

cholesterol 27-hydroxylase (CYP27A1) (various tissues)

cholesterol 25-hydroxylase (various tissues)

cholesterol 24-hydroxylase (CYP46A1) (mainly brain)

cholesterol 7α-hydroxylase (CYP7A1) (liver, prostate)

27-hydroxycholesterol, 25-hydroxycholesterol, 24-hydroxycholesterol,

7α-hydroxycholesterol

Legends to figures

Fig. 1 Cholesterol structure and its main oxidation sites (in red).

Fig. 2 Transcription factors activated by oxysterols.

ER: estrogen receptor; IκB: inhibitory kappa B; INSIG: insulin induced gene 1; LXR: liver X receptor; MAF: v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog; MYD88: myeloid differentiation primary response gene 88; NF-κB: nuclear factor κB; NOX: NADPH oxidase; Nrf2: nuclear erythroid 2-related factor 2; PPAR: peroxisome proliferator-activated receptor; ROR: retinoic acid receptor-related orphan receptor; ROS: reactive oxygen species; RXR: retinoid X receptor; SCAP: SREBP cleavage activating protein; SREBP: sterol regulatory element binding protein; TLR4: Toll like receptor 4.

Fig. 3 Main signal transduction pathways involved in cell response to various stimuli.

Ca: Calcium; DAG: diacylglycerol; ERK1/2: extracellular signal-regulated kinase; IP3: inositol 1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; MEK1/2: mitogen-activated protein kinase ERK kinase 1/2; MEKK1/4: mitogen-activated protein kinase kinase 1/4; MKK: MAP kinase kinase; MLKs: mixed-lineage kinases; PDK1: 3-phosphoinositide-dependent protein kinase 1; PI3K: phosphatidylinositol 3-kinase; PIP3: phosphatidyl inositol triphosphate; PKB, PKC: protein kinase B and C; PLC γ: phospholipase C-γ

Fig. 4 Anti-apoptotic signaling cascade operated by low micromolar concentration of 27-hydroxycholesterol in U937 promonocytic cells.

27-OH: 27-Hydroxycholesterol; DPI: diphenyleneiodonium chloride, inhibitor of NADPH oxidases; LY294002: inhibitor of PI3K; PD98059: inhibitor of MEK; pAkt: phosphorylated Akt; pBad: phosphorylated anti-apoptotic Bad protein; pERK: phosphorylated ERK kinase; NAC: N-acetylcysteine; Nrf2: nuclear erythroid 2-related factor 2.

Oxysterols and mechanisms of survival signaling

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Abstract

Oxysterols, a family of oxidation products of cholesterol, are increasingly drawing attention of scientists to their multifaceted biochemical properties, several of them of clear relevance to human pathophysiology. Taken up by cells through both vesicular and non vesicular ways or often generated intracellularly, oxysterols contribute to modulate not only the inflammatory and immunological response but also cell viability, metabolism and function by modulating several signaling pathways. Moreover, they have been recognized as elective ligands for the most important nuclear receptors. The outcome of such a complex network of intracellular reactions promoted by these cholesterol oxidation products appears to be largely dependent on the type of cells, the dynamic conditions of the cellular and tissue environment but also on the concentration of the oxysterols. Here focus has been given to the cascade of molecular events exerted by relatively low concentrations of certain oxysterols that elicit survival and functional signals in the cells, with the aim to contribute to further expand the knowledge about the biological and physiological potential of the biochemical reactions triggered and modulated by oxysterols.

Key words

Oxysterols, Survival, Nrf2, Autophagy

Abbreviations

5,6-S, 5,6-secosterol; 24OH, 24-hydroxycholesterol; 25OH, 25-hydroxycholesterol; 27OH, 27-hydroxycholesterol; 7K, 7-ketocholesterol; 7βOH, 7β-hydroxycholesterol; AGE, advanced glycation end products; AMPK, AMP-activated protein kinase; AP-1, activator protein-1; ARE, antioxidant response element; ASK, apoptosis signal-regulating kinase; ATF, activating transcription factor; Aβ, amyloid-β; CHOP, CEBP-homologous protein; CREB, cyclic AMP response element binding protein; DAG, diacylglycerol; DAPK, death associated protein kinase; EGF, epidermal growth factor; EIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GRP78, glucose-regulated protein 78; GSK3, glycogen synthase kinase 3; HNE, 4-hydroxynonenal; HO-1, heme oxygenase 1; HUVEC, human umbilical-vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; IGFBP1, insulin-like growth factor-binding protein 1; IKK, inhibitory κB kinase; IL, interleukin; IP3, inositol 1,4,5-triphosphate; IRE1, inositol requiring protein-1; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; LOX-1, lectin-like oxLDL scavenger receptor 1; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; MCP-1, monocyte chemotactic protein 1; MEK, mitogen-activated protein kinase ERK kinase; Mnk, MAPK interacting kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; NOX, NADPH oxidase; NQO-1, NADPH:quinone oxidoreductase 1; Nrf2, nuclear erythroid 2-related factor 2; OSBP, oxysterol-binding protein; oxLDL, oxidized low density lipoprotein; oxPAPC, oxidized 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine; PDGF, platelet-derived growth factor; PDK, 3-phosphoinositide-dependent protein kinase; PERK, protein kinase RNA-like ER kinase; PI3K, phosphatidylinositol 3-kinase; PIP2, PIP3, phosphatidyl inositol di/triphosphate; PK, protein kinase; PLC, phospholipase C; PM, plasmamembrane;−PP2A, protein phosphatase 2A; PPAR, peroxisome proliferator-activated receptor; RNS, reactive nitrogen species; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; RXR, retinoid X receptor; SCAP, SREBP cleavage activating protein; SMC, smooth muscle cell; Smo, Smoothened transducer; S1P, sphingosine 1-phosphate; SREBP, sterol regulatory element binding protein; TLR, Toll like receptor; Triol, cholestan-3β,5α,6β-triol; UPR, unfolded protein response; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; XBP1, X-box-binding protein 1.

1.Introduction

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 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 exerts a number of effects not simply *per se* but mainly through the biochemical properties exerted by its metabolites. Among cholesterol metabolites, an increasing attention is drawn by the family of cholesterol oxidation products termed oxysterols. Oxysterols are 27-carbon molecules that, unlike cholesterol, have 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 cholesterol, thus suggesting their involvement in many of the biochemical and biological effects ascribed to cholesterol (Leonarduzzi et al., 2002; Schroepfer, 2000). In **Figure 1**, the oxidation sites in the cholesterol molecule are depicted, and **Table 1** reports on the most representative oxysterols of non enzymatic and enzymatic origin.

In the last years 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, and in gene regulation. Evaluating the 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). In particular, the molecular aspects of their pro-inflammatory effects have been well deepened, and a growing bulk of experimental findings points to a significant contribution paid by these cholesterol derivatives to the progression of

inflammatory-based chronic pathologies, such as vascular aging, atherosclerosis, Alzheimer's disease, multiple sclerosis, inflammatory bowel disease and colorectal cancer, non alcoholic liver disease, retinopathies, diabetes mellitus (Biasi et al., 2013; Gamba et al., 2012, 2015; Gargiulo et al., 2015; Poli et al., 2013).

Nowadays, new emphasis to the beneficial effects exerted by at least certain oxysterols, has been given by the largely proven evidence that side-chain cholesterol oxides like 24-, 25- and 27-hydroxycholesterol (24OH, 25OH and 27OH) are among the best ligands of a variety of physiologically important nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs) and liver X receptors (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.,1996,1999).

It has been shown that 27OH, a good LXR ligand, rather polarized human macrophages towards an anti-inflammatory state (Marengo et al., 2015), while the sterol ring-derived oxysterol, namely 7keto-cholesterol (7K), definitely not binding to LXRs, induced and sustained mainly pro-inflammatory reactions in human monocyte-derived macrophages (Buttari et al., 2013). 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.

So far, the focus was only on the negative effects of oxysterols, including their potential pro-apoptotic effect on various cell types. In the last years, an increasing bulk of studies is giving evidence of the involvement of certain oxysterols, in particular 27OH, in the modulation of cell survival pathways (Berthier et al., 2005; Riendeau and Garenc, 2009; Vurusaner 2014, 2015).

Before trying to straighten out the knowledge about oxysterols and their modulation of survival signaling, it is noteworthy to mention that 27OH, together with 25OH, has been

shown to exert a broad of antiviral effects 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 (see Lembo et al., in this issue).

2. Oxysterols, signal transduction and transcriptional activity

2.1 Oxysterols and signal transduction

There is not an unique way by which oxysterols can trigger the various cell signaling pathways within cells. Furthermore, uptake and cellular trafficking 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 plasmamembrane (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 diphosphate (PIP2) and phosphatidyl inositol 3,4,5 triphosphate (PIP3) (Wang and Richards, 2012), namely two key regulators of several signaling pathways, including the PI3K/Akt survival signaling cascade (Di Paolo and De Camilli, 2006). The effect of the various oxysterols on lipid rafts formation and stability is not homogeneous. While 27OH and 25OH seem to favour raft physiological functions, 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 (PLC)/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 protein kinase C (PKC)/mitogen-activated protein kinase ERK kinase (MEK)/extracellular signal-regulated kinases (ERK) pathway (Leonarduzzi et al., 2010).

Another primary trigger of the PKC/MEK/ERK pathway, is represented by NADPH oxidase (NOX), located in caveolae and lipid rafts (Jin et al., 2011), whose assembly and activation within PM 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 of reactive oxygen species (ROS) under physiological conditions and oxysterols were shown able to up-regulate 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, 2015).

Still on PM, at least certain oxysterols of pathophysiological relevance, like 25OH and 27OH, 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 Patched receptor and the Smoothened transducer (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 to be strictly dependent on lipid raft integrity and function (Shi et al., 2013).

Moreover, the internalization of oxidized low density lipoproteins (oxLDL) 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 up-regulate expression and synthesis of CD36 (Leonarduzzi et al., 2008, 2010).

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's transport within different cell compartments (Maxfield and Wustner, 2002), certainly even if not only involving oxysterol-binding proteins (OSBPs). 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 were displaying a key role in the modulation of ERK1/2 phosphorylation level, by forming an active oligomer with the serine/threonine protein phosphatase 2A (PP2A) (Wang et al., 2005). Anyway, 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 PM 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.

2.2. 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 appear to be redox modulated. Among them there are sterol regulatory element binding proteins (SREBPs), nuclear factor κ B (NF-κB), Toll like receptors (TLRs), nuclear factor erythroid 2-related factor 2 (Nrf2), LXRs, retinoid X receptor (RXR), PPARs, retinoic acid receptor-related orphan receptors (RORs), estrogen receptors (**Figure 2**). The mentioned transcription factors are described below.

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-κB, through the ERK/c-Jun N-terminal kinase (JNK) pathway (Leonarduzzi et al., 2005; Umetani et al., 2014), with or without the involvement of Erα (Umetani et al., 2014). There is strong experimental evidence that a variety of cholesterol oxidation products may up-regulate a large number of inflammation-related genes. Namely, expression of these genes is NF-κB-dependent, like those coding for interleukin (IL)-1β, -6, -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-κB nuclear translocation and consequent gene transcription stem from the activation of TLRs, a family of receptors primarily involved in the innate immunity

and localized on the PM and/or in endosomes, that may also be induced by oxysterols such as 27OH and 25OH (Gargiulo et al., 2015; Poli et al., unpublished data).

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

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., 1996, 1999). The two oxysterols mainly investigated for their LXR binding property are 24OH and 27OH and their involvement in the physiological regulation of cholesterol and lipid metabolism strongly proposed (Björkhem, 2009, 2013). 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 (Buttari et al., 2013).

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 7αOH, 7βOH, 7K (Wang et al., 2010a) and, with regard to side chain cholesterol oxides, by 24OH (Wang et al., 2010b).

Finally and importantly, 27OH was definitely demonstrated to act as competitive ligand for estrogen receptor-α and -β, by this way triggering intracellular signals potentially able to modulate cancer cell growth and atherosclerosis progression (Lee et al., 2014; Umetani et al., 2007, 2014). 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 25OH, which was shown to signal trough estrogen receptor-α (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 favour 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 apoptosis (de Medina et al., 2011).

3. Survival signaling pathways and oxysterols

Over the last decade, numerous *in vitro* studies have characterized the potential pro-apoptotic effect of oxysterols in a 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 pathologies including atherosclerosis and neurodegenerative diseases.

Based on the presently available data, oxysterols show differences in the degree of cytotoxicity and ability to induce cell death, but these cellular effects have been mostly studied for single compounds. However, oxysterols in oxLDL, foods and the core region of atherosclerotic plaques are always present as a mixture and literature about the way oxysterols act collectively is limited. In this connection, Biasi et al. reported that the cytotoxicity of single 7K and 7βOH is quenched when cells are challenged with a mixture composed of both oxysterols (Biasi et al., 2004). In particular, murine macrophages treated with 7K underwent apoptosis through the mitochondrial pathway, whereas when the same cells were treated with 7K and an equimolar concentration of 7βOH, the pro-apoptotic effect of the first oxysterol was markedly attenuated. Notably, the 7K-induced intracellular ROS rise, dependent upon NOX activation, was inhibited in the presence of the oxysterol mixture, suggesting the occurrence of a substrate-based competition among oxysterols at the level of NOX (Biasi et al., 2004; Leonarduzzi et al. 2004, 2006). In agreement with this, Aupeix et al. showed that the challenge of U937 human promonocytic cell line with 7βOH (30-40 μM) alone exerted pro-apoptotic effects, that significantly diminished when an identical amount of 25OH was simultaneously added (Aupeix et al., 1995).

Interestingly, it has recently been demonstrated that oxysterols of pathophysiological interest could show both beneficial and detrimental properties at the same time, i.e. that they could

trigger both survival and death signals within cells. In **Figure 3** the main survival signals modulated by oxysterols are summarized.

~~Intercellular communication is a crucial process in multicellular organisms. Growth factors, defined as polypeptides, 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 kinases (RTKs) contain an extracellular ligand binding domain, a transmembrane region and an intracellular portion 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). Many different RTK classes have been identified such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), 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 RTKs and also phosphorylation of relay proteins, each one able to trigger a separate cellular response.~~

3.1 Principal mechanisms of survival signaling

Activation of receptor signaling initiates multiple signal transduction pathways involved in cell survival. The three best characterized signaling pathways activated in response to receptor tyrosine kinases (RTKs) are the mitogen-activated protein kinases (MAPKs)

cascade, the phosphatidylinositol 3 kinase (PI3K)/Akt cascade and the PLC cascade (Katz et al., 2007). Besides these pathways, Nrf2 transcription factor also plays a key role in cell survival by protecting cells against oxidative damage (Vurusaner et al., 2015). Moreover, there is now growing evidence suggesting that endoplasmic reticulum (ER) stress (Schleicher et al., 2010) and autophagy (Scherz-Shouval and Elazar, 2011) modulate the balance between cell survival and death.

MAPKs. Cells can sense and respond to stress in various ways, including initiation of cell death and promotion of cell survival. There are many different types of response to stress that depend on the type, strength and duration of the stimuli involving a complex network of signaling pathways. Among these pathways MAPKs, a group of proline-directed serine/threonine kinases, are the best characterized signaling molecules (Arciuch et al., 2009). MAPKs regulate stress signals in a three layer cascade fashion with a MAPK kinase kinase (MAPKKK) phosphorylating and activating its substrate MAPK kinase (MAPKK) which are dual-specificity kinases that in turn phosphorylate serine and threonine residues in their MAPK substrate (Trachootham et al., 2008).

In mammals, three distinct cascades of MAPKs have been elucidated: ERK 1/2, JNK, 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 a 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 (Ramos, 2008).

JNK is encoded by three genes, termed JNK1, JNK2 and JNK3, and these genes alternatively splice resulting in 10 or more JNK isoforms (Arciuch et al., 2009). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is present in the brain, heart and testis. JNK signaling cascade regulates cell death and the development of multiple cell types in the immune system, whereas JNK1 and JNK2 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 by inflammatory cytokines. JNK phosphorylation is catalyzed by two protein kinases MAPKK4 and MAPKK7 which are dual specifity kinases that selectively phosphorylate JNK on Tyr and Thr, respectively (Davis, 2000). JNK translocates to the nucleus where it phosphorylates and up-regulates several transcription factors, including c-Jun, JunA, JunB, activating transcription factor 2 and Elk-1 (Katz et al., 2007).

The p38 kinase family consists of four members namely α, β, γ and δ and these enzymes are activated by hormones, cytokines, G protein-coupled receptor ligands and cellular stress (Arciuch et al., 2009). Activation of p38 kinases is mediated by the MAPKK3 and MAPKK6, and following the activation, p38 phopshorylates its substrates including MAPK interacting kinases 1 and 2 (Mnk1 and Mnk2), and eukaryotic initiation factor 4e (Roux and Blenis, 2004). Many studies have shown that p38 MAPKs have critical role in molecular 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 may result in many diseases and cancer. Thus, MAPKs are molecular targets for drug development, and pharmalogical manipulation of these kinases will likely help the treatment of human diseases related to disproportionate apoptosis.

PI3K /Akt. The PI3K/Akt pathway has been established as one of the most critical signaling pathways 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 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 PM. Following activation, PI3K converts the PIP2 lipids to PIP3, a second messenger that recruits 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt (also knows as PKB) to the PM (Arciuch et al., 2009; Cantley, 2002). Subsequently, PDK1 phosphorylates Akt at two regulatory sites, Thr^{308} in the activation loop and Ser^{473} in the C-terminal tail: phosphorylation of both sites is required for full activation of Akt (Arciuch et al., 2009). Akt activation leads to phosphorylation of various substrate proteins, including caspase-9, Mdm2, glycogen synthase kinase 3 (GSK3) and forkhead transcription factor (FKHRL1), which targets FasL, Bim, insulin-like growth factor-binding protein 1 (IGFBP1), and Puma. A large amount of evidence has suggested that Bad is one of the direct targets of Akt in promoting cell survival and that phopshorylation of Bad on Ser^{136} by Akt inactivate this pro-apoptotic factor (Song et al., 2005; Trachootham et al., 2008).

Akt can also exert its anti-apoptotic functions by phosphorylating inhibitory κB kinase (IKK) and the cyclic AMP response element-binding protein (CREB), with consequent elevated transcription of genes encoding the anti-apoptotic proteins Bcl-2, Bcl-XL, and Mcl-1.

Another target of Akt is apoptosis signal-regulating kinase 1 (ASK1) and its Akt-mediated phosphorylation inhibits its ability to activate JNK/p38 and prevents 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).

PKCs. PLCγ activation is stimulated by G protein coupled receptors that interact with G proteins of the G_q family. Active PLCγ enzyme catalyzes the hydrolysis of PIP2 to produce inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Binding of IP3 receptors on the membrane of the endoplasmic reticulum causes the release of intracellular Ca^{2+} which is followed by the activation of PKC family members (Katz et al., 2007; O'Gorman and Cotter, 2001).

The PKC is an ubiquitous family of serine/threonine kinases and has at least 10 members containing a highly conserved kinase core at the C-terminal and an N-terminal autoinhibitory 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, □ and η) contain DAG-sensitive C1 domains but Ca^{2+}-insensitive C2 domain while the atypical PKCs (ζ and ι) are both Ca^{2+} and DAG-independent. PKC isoforms play diverse role in signal transduction, cell proliferation, differentiation, death, mitogenesis and stress responses (Arciuch et al., 2009; Guo et al., 2004). Most of the PKC family members have been shown to contribute to cell survival whereas members such as PKCα and PKCδ have been associated with apoptosis induction through inhibition of the Akt survival pathway and activation of p38 MAPK (Matsuzawa and Ichijo, 2005; Yang et al., 2008).

Nrf2. Nrf2, 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 by the interaction with Kelch-like ECH-associated protein 1 (Keap1), an actin-binding cytoskeletal protein (Motohashi and Yamamoto, 2004). Under oxidative stress, Nrf2 dissociates from Keap1, thus also from cytoskeleton, and migrates into the nucleus where it heteromerizes with Maf (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog) and binds to ARE sequences; Nrf2 then codifies for various antioxidant enzymes including aldoketoreductase, glutathione peroxidase, glutathione reductase, heme oxygenase 1 (HO-1), and NADPH:quinone oxidoreductase 1 (NQO-1) (Dinkova-Kostova, 2002; Dinkova-Kostova et al., 2001; Iqbal et al., 2003; Nguyen et al., 2009). While a growing bulk of evidence showed that the protective adaptive response to ROS/reactive nitrogen species (RNS) as induced by Nrf2 is mediated by enhanced expression of these cytoprotective enzymes, the specific upstream signal transduction pathways recruited to stimulate transcription of these phase II genes are yet poorly defined (Lee and Choi, 2003; Mann et al., 2007). In this relation, some studies demonstrated the clear involvement of several protein kinase pathways including ERK1/2, JNK, p38 and PKCs 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). More recently, Wang et al. demonstrated that palmitic acid stimulated hepatocyte proliferation through the activation of Nrf2, which depends on ROS-induced activation of p38/ERK-Akt cascade (Wang et al., 2011). In bovine aortic endothelial cells, He at al. showed that JNK signaling pathway is involved in advanced glycation end products (AGE)-induced activation of Nrf2 with consequent expression of its target antioxidant genes (He et al., 2011). A cancer chemopreventive agent induced activation of Nrf2 through ERK1/2, p38, JNK, and PI3K kinases in kidney derived cells (Rojo et al., 2012).

ER stress. Perturbations in ER functions including protein folding, storing intracellular Ca^{2+} and lipid biosynthesis results in ER stress (Schröder, 2008). In eukaryotic cells, ER stress is sensed by 3 ER resident proteins: inositol requiring protein-1 (IRE1), activating transcription factor (ATF) 6 and protein kinase RNA-like ER kinase (PERK). Activation of these transmembrane sensors triggers unfolded protein response (UPR) that relieves ER stress and reestablishes homeostasis by several mechanisms, including inhibition of protein translation and induction of ER-resident chaperons (Kim et al., 2008a). IRE1 and ATF6 are crucial for chaperone production, which maintain the proper protein folding and promote cell survival. IRE1 also promotes the expression of X-box-binding protein 1 (XBP1), a transcription factor that facilitates degradation of misfolded proteins. In addition to XBP1, IRE1 pathways can also lead to apoptosis. Among the resident chaperons, the chaperone glucose-regulated protein 78 (GRP78) plays a key role in regulating ER stress by binding to transmembrane ER stress sensors inhibiting their activation. Altered protein folding in the ER stimulates GRP78 to release transmembrane signaling proteins including IRE1, PERK and ATF6 that allow oligomerization of IRE1 and PERK thus leading to UPR. As a result of oligomerization, PERK is activated. Activation of PERK results in the phosphorylation of eukaryotic translation initiation factor 2α (EIF2α) to slow down protein translation. However, in contrast, when EIF2α is phosphorylated and inactivated, certain mRNAs, including ATF4, are translated. ATF4 regulates the expression of several genes involved in restoring cellular redox and ER homeostasis as well as autophagy genes. Moreover, ATF4, ATF6 and XBP1 assemble on the promoter of the gene encoding CEBP-homologous protein (CHOP), which participates as a down-stream component of ER stress pathways. Overexpression of CHOP is a potent inducer of apoptosis through a mechanism controlling the expression of Bim and Bcl-2 genes (Kim et al., 2008a; Scull and Tabas, 2010; Tabas, 2011).

The UPR-induced signal-transduction events ameliorate the accumulation of unfolded protein aggregates that are commonly accompanied by the activation of signaling pathways including ERK, JNK, p38, Akt and also NF-κB (Kaneko et al., 2003). In addition, ER stress associated with autophagy could activate alternative mechanisms to remove unfolded proteins independently from the proteasome system (Ogata et al., 2006).

Despite the beneficial functions of a transient UPR, prolonged ER stress leads to a wide range of diseases, including cardiovascular, stroke, diabetes, neurodegeneration and cancer. Prolonged activation of UPR occurs in various pathological conditions, including hypoxia, ischemia, heat shock, oxidative stress, proteasome inhibition and Ca^{2+} depletion of ER stores (Kim et al., 2008a; Oyadomari and Mori, 2004).

Autophagy. ~~The term autophagy derives from the Greek words auto (self) and phagy (eating) and was first used by Christian de Duve to define a non selective pathway for the degradation of non-essential cellular constituents, that was activated under conditions of nutrient starvation (De Duve, 1963; De Duve and Wattiaux, 1966).~~ Selective autophagy of specific organelles and proteins can be induced in response to diverse stimuli and is involved in housekeeping degradation of aggregated proteins (aggrephagy), damaged mitochondria (mitophagy), and invading bacteria (xenophagy) via ubiquitin signaling (Deretic and Levine, 2009; Kirkin et al., 2009; Youle and Narendra, 2011). Macroautophagy (generally referred to as autophagy) breaks down macromolecules and allows cellular recycling to generate energy in order to survive to cellular stress. In this multi-step process, the cell material to be eliminated is sequestered within double-membrane vesicles known as autophagosomes, then the outer-membrane of the autophagosome fuses with lysosome for subsequent degradation (He and Klionsky, 2009). In the last years, it was made clear that even if autophagy can be considered a cell survival mechanism, under certain conditions its excessive enhancement may lead to a caspase-independent non-apoptotic type of cell death (type II cell death)

(Galluzzi et al., 2009). Several lines of evidence suggests that a cross-talk exists between autophagy and classical apoptosis. Important molecular players of such a connession appeared to be the anti-apoptotic genes Bcl-2, Bcl-xl and autophagy genes such as autophagy related 5 (Atg5), autophagy related 4D (Atg4D) and beclin1 (Dewaele et al., 2010).

Despite the increasing interest in understanding the mechanism of autophagy, there is limited information about the way cellular signaling molecules like kinases regulate this complex process. There are several reports that suggest a regulatory role for serine/threonine kinases involving mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), Akt, MAPKs (ERK, p38 and JNK) and PKCs in various steps of autophagy such as vesicle maturation, autophagosome inception and autophagy-related gene expression (Suffixidharan et al., 2011). It is generally accepted that PI3K/mTOR pathway primarily inhibits autophagy whereas the MAPK pathway and PKCs either positively or negatively regulate autophagy depending on the cellular context and inducers involved.

A role for autophagy in the cellular response to oxidative stress has been described. There are findings showing that increased ROS levels stimulate autophagic processes which, in turn, reduce ROS levels (Scherz-Shouval and Elazar, 2011). ~~Since excessive ROS accumulation and oxidative stress are involved in the pathogenesis of many chronic diseases, including cancer, neurodegenerative and cardiovascular diseases, understanding the complex interplay between ROS and autophagy pathways may help in developing effective clinical strategies against these pathologies.~~

It has been described the important role of lipids, including sphingolipids, in regulating autophagy. In particular, ceramides and sphingosine 1-phosphate (S1P) is considered a physiological regulator of autophagy in relation to cellular growth, survival, and aging. These two sphingolipids act as second messengers to regulate the balance between cell death and survival, however, their effect on cell fate are opposite: S1P promotes cell survival and

proliferation, whereas ceramide induces growth arrest and cell death (Harvald et al., 2015; Ogretmen and Hannun, 2004; Spiegel et al., 2003). Interestingly, it has been observed that S1P is implicated in the mitogenic response of mesenchymal cells to a low oxidative stress; conversely, high oxidative stress induces growth arrest through S1P depletion (Cinq-Frais et al., 2013).

3.2. Oxysterols and cell survival

The first evidence of the involvement of oxysterols in cell survival was provided by Berthier and colleagues, in an *in vitro* study where THP-1 human monocytic cells were challenged with a high concentration of 7K (100 μM), namely one of the most abundant oxysterols in human tissues, provided with a strong pro-apoptotic effect. The treatment of THP-1 cells with 7K induces an early and transient up-regulation of MEK/ERK signaling pathway which transiently inactivated the pro-apoptotic protein Bad, thus significantly delaying the apoptotic process initiated by 7K itself (Berthier et al., 2005).

More recently, another group performed experiments on another human promonocytic cell line (U937) with low micromolar concentration of 27OH, showing a significant stimulation of cell viability due to the phosphorylation of Akt at residue Thr^{308}, postponing apoptotic death; conversely, higher concentrations of 27OH rapidly triggered lysosomal-independent apoptosis (Riendeau and Garenc, 2009). Moreover, using low micromolar doses of another oxysterol, cholestan-3β,5α,6β-triol (Triol), Jusakul et al. demonstrated that activation of pro-survival signaling in human MMNK-1 cholangiocytes involved ERK1/2 and p38α phosphorylation (Jusakul et al., 2013).

In a very recent study, our group provided clear evidence of survival signaling by 27OH. Low micromolar concentration (10 μM) of 27OH triggered survival signals in U937 human promonocytic cell line through the activation of ERK and PI3K/Akt, with consequent

phosphorylation of the pro-apoptotic protein Bad; this reaction prevents the loss of transmembrane mitochondrial potential, thereby delaying cell apoptotic death. In addition, activation of both survival pathways appeared dependent upon a transient ROS increase, mediated by the up-regulation of NOX2; thus, the prooxidant effect of 27OH resulted to be a key early mechanism in the survival signaling operated by the oxysterol, at least on U937 cells and at low concentration (Vurusaner et al., 2014) (**Figure 4**).

In agreement with our results, another oxysterol, 7βOH, was shown to be anti-apoptotic and to induce cell proliferation when added at low concentrations (below 20 μg/ml) to human umbilical-vein endothelial cells (HUVEC); this effect was dependent on the activation of MEK/ERK cascade, but apparently independent from ROS production (Trevisi et al., 2009). However, at higher concentrations, 7βOH led HUVEC to apoptosis, showing then a dual effect on endothelial cell viability, depending on the concentration.

Also oxLDL displayed a dual effect on cell viability and proliferation of endothelial cells (Galle et al., 2001). At low oxLDL concentrations (5-10 μg/mL) a proliferative effect was observed, while at high concentrations (50-300 μg/mL) cells underwent apoptotic death. Interestingly, a NOX-dependent increase of ROS appeared involved in both conditions (Galle et al., 2001; Heinloth et al., 2000).

With regard to 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 activation of both ERK1/2 and Akt as well as subsequent phosphorylation of Bad and IkBα (Hundal et al., 2001). In another study, THP-1 monocytic cells were challenged with 50 μg/ml oxLDL, and such a treatment attenuated staurosporine-induced apoptosis through the up-regulation of ERK but not PI3K/Akt signaling (Namgaladze et al., 2008). Of note, similarly to oxLDL, both 27OH and 24OH were shown to antagonize staurosporin mediated apoptosis in human neuroblastoma SH-SY5Y cells (Emanuelsson and Norlin, 2012).

While it is well established that oxLDL activate pro-survival signaling pathways promoting macrophage proliferation and survival, little is known regarding the upstream signaling events, and the pattern recognition receptors involved. ~~The only available study by~~ Riazy et al. excludes a role for endocytic pattern recognition receptors, scavenger receptor A and CD36 in the oxLDL-mediated survival of bone marrow derived macrophages (Riazy et al., 2011). Moreover, oxLDL-induced proliferation of SMCs appeared to be partly dependent on the bioactive sphingolipid S1P, via activation of sphingomyelinase, ceramidase, and sphingosine kinase (Augè et al., 1999; Levade et al., 2001). Furthermore, by using specific inhibitors, it has been also demonstrated that sphingomyelin/ceramide pathway was involved in 7k and α-epoxycholesterol-induced SMC proliferation (Liao et al., 2010). Nevertheless, there are few results about the link between oxysterols and the pro-survival pathway involving S1P.

It appears that both MEK/ERK and PI3K/Akt signaling pathways would play a critical role in the pro-survival effect inducible by modified lipoproteins, whereby the balance between anti-apoptotic pathways (ERK, Akt) and stress-activated pro-apoptotic pathways (JNK, p38) would determine the final effect: either cell survival or apoptosis.

Another important aspect regarding pro- and anti-apoptotic signaling by oxysterols is the likely modulation of cytosolic free Ca^{2+}. Lizard and colleagues demonstrated that 7K-induced apoptosis in THP-1 cells was triggered by a sustained influx of extracellular Ca^{2+} followed by activation of calcineurin and dephosphorylation of Bad (Berthier et al., 2004). Moreover, activation of calcineurin was proved to be induced by the translocation of transient receptor potential calcium channel 1, a component of the store-operated Ca^{2+} entry channel, into lipid raft domains of PM. Notably, the same group showed that 7K, in parallel to the pro-apoptotic signaling, exerted a Ca^{2+}-dependent activation of MEK/ERK survival pathway via Ca^{2+}-sensitive proline rich tyrosine kinase 2 (Berthier et al., 2005). A net increase of intracellular

Ca^{2+} was shown to be required also for the pro-survival effect inducible by oxLDL (Matsumura et al., 1997). More recently, to clarify the down-stream pathways that are activated by intracellular Ca^{2+} increase, bone marrow derived macrophages were challenged with oxLDL; these oxidized micelles prevented macrophage apoptosis through mobilization of intracellular Ca^{2+}, that in turn activated the Ca^{2+}-sensitive kinase eukaryotic elongation factor 2 (Chen et al., 2009).

An increasing bulk of evidence points to the redox regulation of several transcription factors such as Nrf2, NF-κB, Jun/activator protein-1 (AP-1) and p53, whose modulation may lead to cell cycle changes and integration of pro- and anti-apoptotic signals (Trachootham et al., 2008).

While AP-1 and p53 are mostly involved in the induction of cell death, up-regulation of Nrf2 and NF-κB rather promotes cell proliferation and survival.

At present, little is known about oxysterol-mediated survival effects operated at the transcriptional level. Here we will briefly review this matter focusing on the redox sensitive Nrf2 pathway which plays a critical role in protecting cells against oxidative/electrophilic stress following the exposure to a variety of exogenous and endogenous stimuli and promotes cell survival.

The activation of Nrf2 by components of oxLDLs in vascular cells suggests the involvement of this transcription factor and its related antioxidant response in the signaling pathway sustaining atherosclerosis progression (Ishii et al., 2004). Moreover, oxLDLs were shown to activate Nrf2 much strongly in murine macrophages than in smooth muscle cells (SMCs), while 4-hydroxynonenal (HNE), a major product of lipid peroxidation, stimulated Nrf2 nuclear translocation in both cell types. These findings suggest that Nrf2 modulation could depend on the type of the cells and of the chemical inducers.

In a very recent study, C6 glioma cells were challenged with either 10 or 20 μM 27OH to study its role in Nrf2 modulation in neurodegenerative diseases, including Alzheimer's disease. At the concentration employed, 27OH resulted to be pro-oxidant and neurotoxic, being able to significantly down-regulate the expression of Nrf2 and of its down-stream antioxidant genes HO-1, NQO-1 and γ-glutamyl-cysteine synthetase, both at gene and protein level (Ma et al., 2015).

Nrf2-target genes, such as HO-1 and NQO-1, are critical in the cellular response against pro-oxidative stimuli such as oxLDLs and oxidized phospholipids, in macrophages, endothelial cells, and SMCs (Araujo et al., 2012). In this relation, the oxidized 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine (oxPAPC) was shown to induce HO-1, NQO-1 and glutamate-cysteine ligase modifier subunit expression in endothelial cells in a Nrf2-dependent manner (Jyrkkänen et al., 2008). Similarly, HO-1 induction via Nrf2 signaling was observed in vascular SMCs in response to moderately oxLDLs (Anwar et al., 2005).

Moreover, Nrf2 nuclear translocation is modulated by ERK1/2, p38, PKCs and PI3K/Akt pathways, but the activation of these upstream signals depends on the type of the inducer and of the targeted cells. As far as it regards, Papaiahgari and colleagues showed that, in pulmonary epithelial cells, in response to hyperoxia, a ROS-dependent ERK and Akt activation up-regulated Nrf2-mediated gene expression (Papaiahgari et al., 2004, 2006). Further, the survival signaling pathway PI3K/Akt-dependent was demonstrated to be responsible for Nrf2 protein increase and HO-1 expression in PC12 pheochromocytoma cells (Martin et al., 2004). Also HNE-mediated induction of HO-1 expression and synthesis in macrophages and epithelial cells was suggested to be dependent on the activation of ERK survival pathway (Iles et al., 2005; Li et al., 1996).

Inconsistent results were obtained in hepatoma cells challenged with 15-deoxy-delta 12,14-prostaglandin J2, where induction of HO-1 synthesis was not quenched by using the ERK1/2

inhibitor PD98059. This evidence suggests that the up-regulation of this heme catabolism-related enzyme might depend on the activation of more than a single signaling pathway (Liu et al., 2004). Conversely, the anti-apoptotic effect of 15-deoxy-delta 12,14-prostaglandin J2 was confirmed in rat pheochromocytoma cells through the involvement of ERK/Akt-dependent Nrf2 activation (Kim et al., 2008b).

Therefore, based on the presently available data, the activation of Nrf2 by different types of inducers, including lipid oxidation products, leads to cell survival and protection against oxidative stress, a condition that does not necessarily imply positive outcome, since Nrf2-dependent delay or quenching of apoptotic cell death might also result in tumorigenesis and drug resistance (Niture and Jaiswal, 2012).

There is now growing evidence indicating that ER stress is activated by oxidized lipids and modulates the balance between survival and apoptotic effects induced by lipid oxidation products. Sanson et al. demonstrated that oxLDLs trigger ER stress induction that can be prevented by the ER-associated chaperone oxygen-regulated protein 150 in human endothelial cells (Sanson et al., 2009). Moreover, 7K and HNE induced ER stress in vascular cells, characterized by the detection of ER stress markers (phospho Ire1 and PERK) and activation of their down-stream pathways. Interestingly, ER stress is involved in the apoptotic effect of oxLDLs through the Ire1/JNK pathway. Moreover, the antioxidant N-acetylcysteine prevented the ER stress induced by oxLDLs, 7K and HNE, suggesting that oxidative stress is involved in the activation of ER stress. In agreement with these findings, Pedruzzi et al. showed that 7K induced ER stress and apoptosis in aortic SMCs via the activation of Nox-4 (Pedruzzi et al., 2004). Increased ER stress also occurs in unstable plaques; in coronary artery SMCs and THP-1 monocytes, 7K induced ER stress and apoptosis (Myoishi et al., 2007).

In human aortic endothelial cells, oxidized phospholipids induced UPR activation and up-regulated the expression of inflammatory genes (Gargalovic et al., 2006). The ER stress

induced by oxLDLs in endothelial cells was prevented by AMP-activated protein kinase (AMPK) (Dong et al., 2010). Low doses of 7K induced a more subtle ER stress and activated pattern recognition receptors (PRRs) which alter the balance between survival and death in macrophages (Devries-Seimon et al., 2005; Seimon et al., 2006). Another study showed that lipid oxidation products, including oxysterols, present in advanced atherosclerotic plaques, contribute to trigger ER stress (Tabas, 2010). Altogether, these studies indicate that ER stress is induced by oxysterols and oxLDLs, and that it could play a key role in the progression of atherosclerosis.

A prolonged ER stress has been also identified as a pathogenic mechanism in diseases associated with neurodegeneration, such as Alzheimer's disease. In retinal pigment cells, 27OH increased the level of ER stress, together with amyloid-β (Aβ) production and oxidative stress (Dasari et al., 2010). Furthermore, it has been shown that 27OH activated CHOP thus down-regulating leptin, a hormone which reduces Aβ production and tau phosphorylation in neuroblastoma cells (Marwarha et al., 2012). In the given mechanism, CHOP was shown as a negative regulator of C/EPα, a transcription factor which regulates leptin expression. Another oxysterol, namely 24OH, has been shown to suppress Aβ production via amyloid precursor protein down-regulation, which occurred through the expression of GRP78 ER chaperone (Urano et al., 2013). According to these results, it can be suggested that the up-regulation of the nontoxic ER chaperon inducers, such as 24OH, may represent a therapeutic target for AD. Additional studies are needed to fully understand the relation between oxysterols and ER stress that may suggest new therapeutic implications in ER stress-driven pathologies.

It is now well established that autophagy appears stimulated in advanced atherosclerotic plaques by inflammation and oxidized lipids (Martinet and De Meyer, 2009). The protective role of autophagy in atherosclerosis involves the removal of damaged organelles in response

to mild oxidative stress (Kiffin et al., 2006). In an *in vitro* pharmacological study, 7K-stimulated autophagy was shown to attenuate SMCs apoptosis induced by low concentrations of lipophilic statins (Martinet et al., 2008). Most likely, autophagy up-regulation in statin-treated cells allowed to quench the release of cytohrome c into the cytosol and caspase activation, by this way limiting the extent of apoptotic cell death (Gozuacik and Kimchi, 2004; Martinet et al., 2008).

Further, oxidized lipids, such as oxysterols and HNE, are able to stimulate autophagy in atherosclerosis. Signs of autophagy, such as autophagolysosomes and increase of LC3-II, were detectable when epithelial cells were treated with oxLDLs but not with native LDLs (Nowicki et al., 2007). Moreover, 7K-induced autophagy was demonstrated in SMCs, in terms of myelin figure formation, intense protein ubiquitination, and LC3-II increase (Martinet et al., 2004). Interestingly, cultured SMCs treated with aggregated LDLs showed up-regulation of death-associated protein kinase (DAPK), a Ser/Thr death kinase that regulate membrane blebbing and autophagic (type II) programmed cell death (Inbal et al., 2002; Martinet et al., 2002). Indeed, DAPK was confirmed to induce survival in SMCs, since the antisense depletion of this kinase promoted caspase-dependent apoptosis (Jin and Gallagher, 2003).

Several studies focused on lectin-like oxLDL scavenger receptor 1 (LOX-1), a major receptor responsible for binding, internalization and degradation of oxLDLs, with a primary role in atherosclerosis development (Sawamura et al., 1997). In an *in vitro* study, autophagy activation in HUVECs challenged with oxLDLs was proven to be mediated by a ROS/LOX-1 pathway (Ding et al., 2013a). Such an autophagic response would contribute to the degradation of oxLDLs, thus favoring cell survival. In a similar way, treatment of vascular SMCs with a relatively low concentration of oxLDLs was shown to trigger autophagy with increased beclin-1and Atg5 expression, as well as LC3-II/LC3-1; on the contrary, high levels

of oxLDLs induced SMC apoptosis and, under this experimental condition, autophagy was suppressed (Ding et al., 2013b).

Thus, autophagy may protect vascular cells against apoptosis depending on cell types, oxidant concentration and time of exposure (Perotta and Aquila, 2015) and 7K could be one of the triggering factors of autophagy itself. The same oxysterol was suggested to be primarily involved in another pro-survival mechanism involving autophagy, namely the up-regulation of a mitochondrial enzyme, proline oxidase, in oxLDLs challenged cancer cells,. Activation of this enzyme would increase intracellular ROS and, subsequently, cell autophagic response, by this way counteracting cancer cell apoptosis (Zabirnyk et al., 2010).

No published papers concerning the potential stimulation of pro-survival autophagy by oxysterols other than 7K are presently available, even if the involvement of 27OH at low micromolar concentrations appears very likely (Vurusaner et al., unpublished data). On the other hand, a relatively high concentration (50 uM) of 7K, but also of 7βOH, and 24(S)OH, led 158N murine oligodendrocytes to a type of death termed oxyapoptophagy (Nury et al., 2013). Once again, it seems that at least certain oxysterols may modulate the actual outcome of cell autophagic response mainly depending upon their concentration, being able to trigger pro-survival autophagy only when present in low amount.

By reviewing the available literature on oxysterol-induced pro-survival signals we are developing the opinion that, in general terms, this is a complex phenomenon depending on cell types, environmental factors, cell senescence, oxysterol concentration and exposure time. Notably, when relatively low and not directly toxic concentrations of oxysterols are administered in a biologically representative mixture, they tend to have a “Trojan-horse”-like behaviour (Biasi et al., 2004; Leonarduzzi et al., 2004). Namely, instead of killing cells directly, oxysterols might delay its irreversible damage, in the meantime initiating pro-inflammatory and pro-fibrogenic pathways. On the contrary, relatively higher amounts of the

same compounds induce earlier and direct cell death (Leonarduzzi et al., 2002). Moreover, delayed macrophage apoptosis would favour growth and destabilization of advanced atherosclerotic plaques (Martinet et al., 2012). Thus, the elucidation of the molecular mechanisms underlying oxysterol-induced pro- and anti-apoptotic signaling might contribute to a better understanding of the pathogenesis of several oxysterol-associated disease processes.

4. Conclusions

Oxysterols have been shown to contribute to the modulation of a wide variety of signaling pathways, thus influencing a number of transcription factors. Further, especially oxysterols of enzymatic origin have been recognized to be primary ligands of key nuclear receptors. As repeatedly stated in this review, the type of cells, the cellular and tissue environment and, above all, the actual concentration of the cholesterol oxides, resulted to be the main conditioning factors as far as their final effects are concerned.

We deemed of particular interest trying to comprehensively analyse the potential role of various oxysterols at least, with regard to the modulation of survival signaling. Oxysterols appear as primarily involved in the pathogenesis of inflammation-driven chronic pathologies like cancer, atherosclerosis, neurodegenerative diseases, thus possibly useful is to draw the attention to the high potentiality of oxysterols in triggering and sustaining survival response in cells challenged with the various stressors.

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Table 1 - Origin of plasma and tissue oxysterols

Oxysterols of not enzymatic origin

Deriving from the diet

Mainly formed by autoxidation of foodstuff containing cholesterol (meat, cheese, milk, dairy products, etc.) as induced by heat, light exposure, refrigeration, freeze-drying.

Generated in the body by:

Oxidation driven by reactive oxygen species and the leukocyte/H2O2/HOCl system, the most frequent source being the inflammatory processes; attack by peroxyl and alkoxyl radicals.

The quantitatively most represented compounds of this subgroup are:

7ketocholesterol, 7β-hydroxycholesterol, 5α,6α-epoxycholesterol,

5β,6β-epoxycholesterol, 3β,5α,6β- trihydroxycholestane

Oxysterols of enzymatic origin

Generated in the body by:

cholesterol 27-hydroxylase (CYP27A1) (various tissues)

cholesterol 25-hydroxylase (various tissues)

cholesterol 24-hydroxylase (CYP46A1) (mainly brain)

cholesterol 7α-hydroxylase (CYP7A1) (liver, prostate)

27-hydroxycholesterol, 25-hydroxycholesterol, 24-hydroxycholesterol,

7α-hydroxycholesterol

Legends to figures

Fig. 1 Cholesterol structure and its main oxidation sites (in red).

Fig. 2 Transcription factors activated by oxysterols.

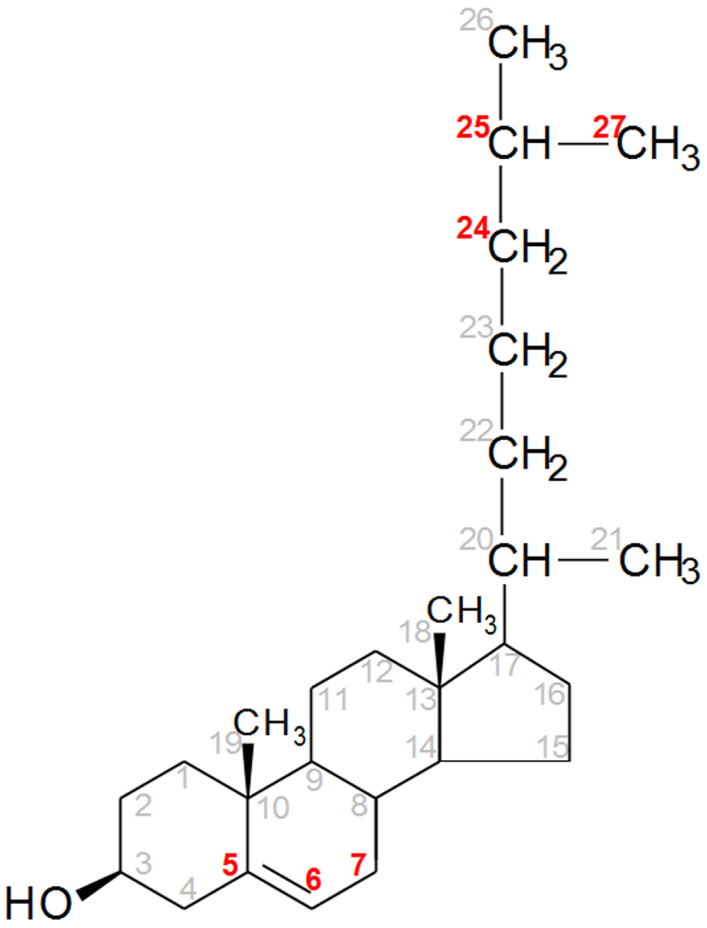
ER: estrogen receptor; IκB: inhibitory kappa B; INSIG: insulin induced gene 1; LXR: liver X receptor; MAF: v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog; MYD88: myeloid differentiation primary response gene 88; NF-κB: nuclear factor κB; NOX: NADPH oxidase; Nrf2: nuclear erythroid 2-related factor 2; PPAR: peroxisome proliferator-activated receptor; ROR: retinoic acid receptor-related orphan receptor; ROS: reactive oxygen species; RXR: retinoid X receptor; SCAP: SREBP cleavage activating protein; SREBP: sterol regulatory element binding protein; TLR4: Toll like receptor 4.

Fig. 3 Main signal transduction pathways involved in cell response to various stimuli.

Ca: Calcium; DAG: diacylglycerol; ERK1/2: extracellular signal-regulated kinase; IP3: inositol 1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; MEK1/2: mitogen-activated protein kinase ERK kinase 1/2; MEKK1/4: mitogen-activated protein kinase kinase 1/4; MKK: MAP kinase kinase; MLKs: mixed-lineage kinases; PDK1: 3-phosphoinositide-dependent protein kinase 1; PI3K: phosphatidylinositol 3-kinase; PIP3: phosphatidyl inositol triphosphate; PKB, PKC: protein kinase B and C; PLC γ: phospholipase C-γ

Fig. 4 Anti-apoptotic signaling cascade operated by low micromolar concentration of 27-hydroxycholesterol in U937 promonocytic cells.

27-OH: 27-Hydroxycholesterol; DPI: diphenyleneiodonium chloride, inhibitor of NADPH oxidases; LY294002: inhibitor of PI3K; PD98059: inhibitor of MEK; pAkt: phosphorylated Akt; pBad: phosphorylated anti-apoptotic Bad protein; pERK: phosphorylated ERK kinase; NAC: N-acetylcysteine; Nrf2: nuclear erythroid 2-related factor 2.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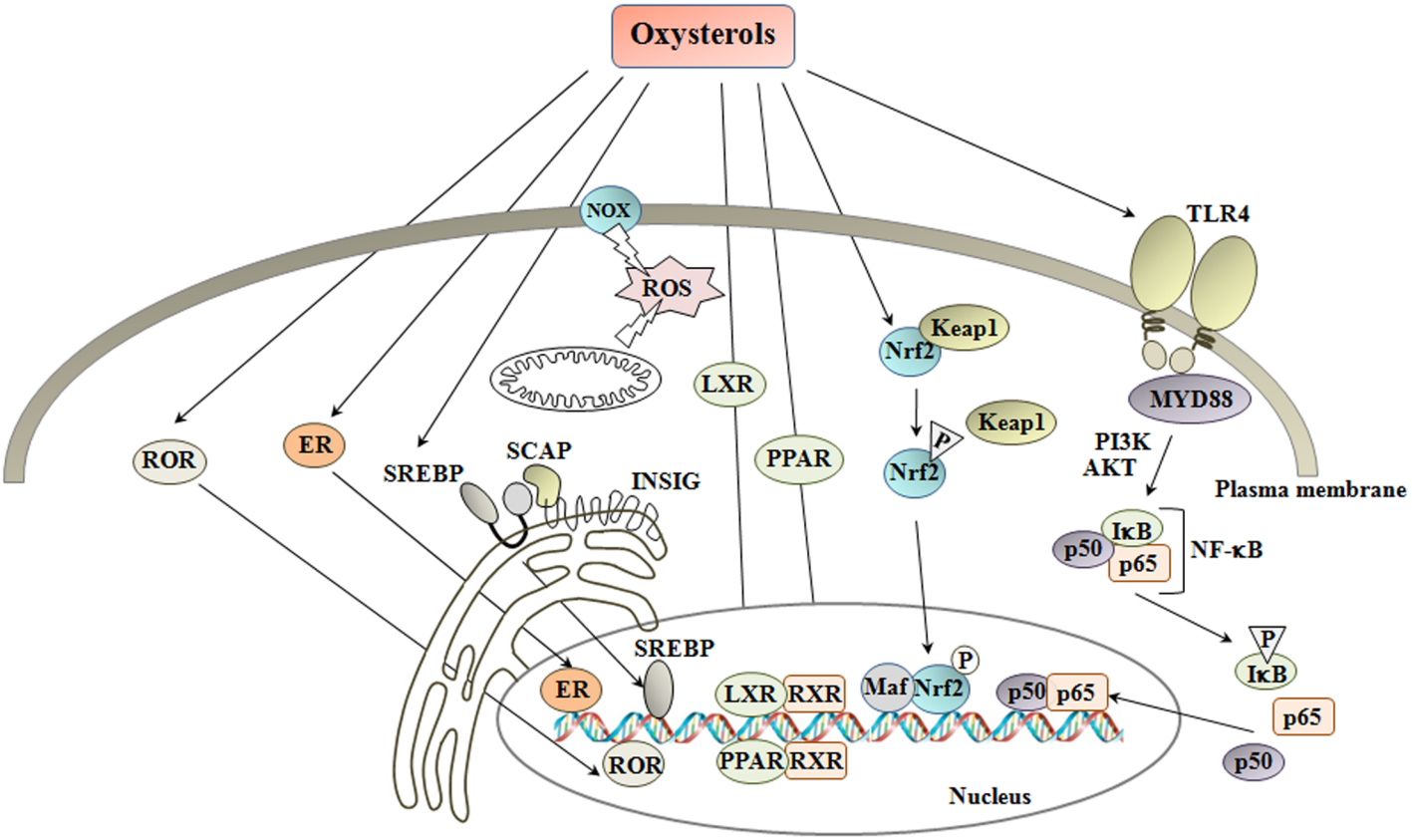
Oxysterols
NOX
ROS
TLR4
Keap1
Nrf2
LXR
MYD88
Keap1
P
Nrf2
PI3K
AKT
ROR
ER
SREBP
SCAP
INSIG
PPAR
Plasma membrane
IκB
p50
p65
NF-κB
SREBP
P
IκB
ER
LXR
RXR
Maf
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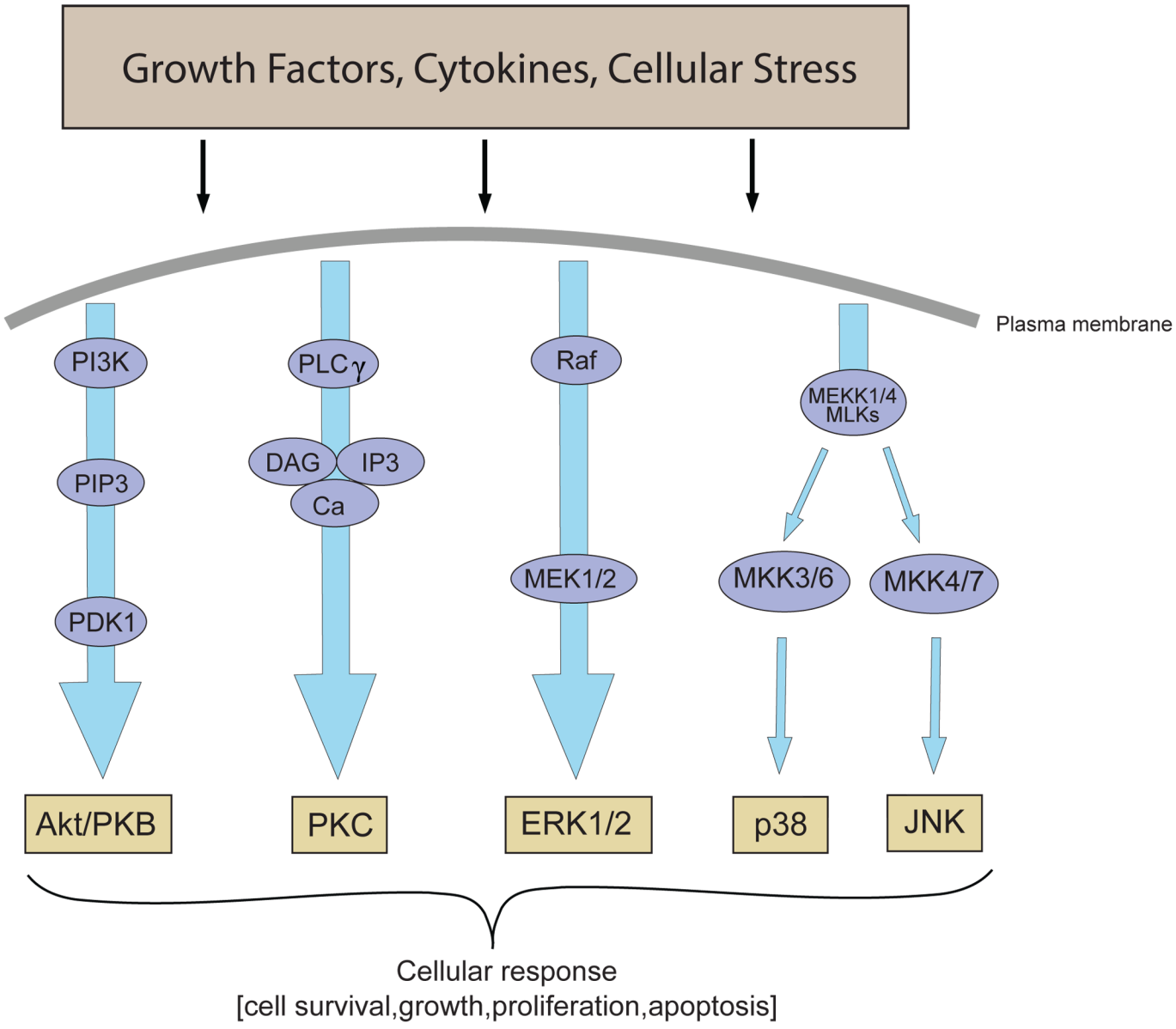
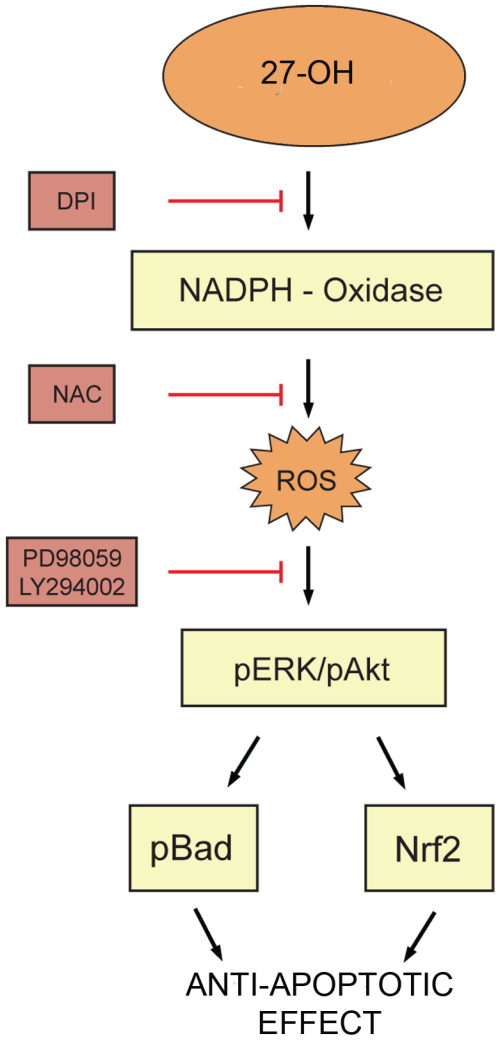

Growth Factors, Cytokines, Cellular Stress
Plasma membrane
PI3K
PIP3
PDK1
PLCγ
DAG
IP3
Ca
Raf
MEK1/2
MEKK1/4
MLKs
MKK3/6
MKK4/7
Akt/PKB
PKC
ERK1/2
p38
JNK
Cellular response
[cell survival,growth,proliferation,apoptosis]


27-OH
DPI
NADPH - Oxidase
NAC
ROS
PD98059
LY294002
pERK/pAkt
pBad
Nrf2
ANTI-APOPTOTIC
EFFECT