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Abstract

The oxysterol 27-hydroxycholesterol (27-OH) is increasingly considered to be involved in a variety of pathophysiological processes, having been shown to modulate cell proliferation and metabolism, but also to exert pro-inflammatory and pro-apoptotic effects. This study aimed to elucidate the molecular pathways whereby 27-OH may generate survival signals in cells of the macrophage lineage, and to clarify whether its known pro-oxidant effect is involved in that process. A net up-regulation of survival signaling, involving the extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/Akt phosphorylation pathways, was observed in U937 promonocytic cells cultivated over time in the presence of a low micromolar concentration of the oxysterol. Interestingly, the up-regulation of both kinases was shown to be closely dependent upon an early 27-OH-induced intracellular increase of reactive oxygen species (ROS). In turn, stimulation of ERK and PI3K/Akt both significantly quenched ROS steady-state and markedly phosphorylated Bad, thereby determining a marked delay of the oxysterol's pro-apoptotic action. The 27-OH-induced survival pathways thus appear to be redox modulated and, if they occur within or nearby inflammatory cells during progression of chronic diseases like cancer and atherosclerosis, they could significantly impact the growth and evolution of such diseases.

Keywords: oxysterols, ROS, survival signaling

Abbreviations

Bcl-2, B-cell lymphoma 2; CYP27A1, 27-hydroxylase; DHE, dihydroethidium; ERK, extracellular signal-regulated kinase; HRP, horseradish peroxidase; JC-1, 5,5',6,6'-tetrachloro1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; 7K, 7-ketocholesterol; LXR, liver X receptor; M-CSF, macrophage colony-stimulating factor; MEK, mitogen-activated protein kinase ERK kinase;

NAC, N-acetyl cysteine; Nox-2, NADPH oxidase type 2; Nrf2, nuclear factor erythroid 2 p45-related factor 2; 27-OH, 27-hydroxycholesterol; PAGE, SDS-polyacrylamide gel electrophoresis; PI3K, phosphoinositide 3-kinase; PMA, phorbol myristate acetate; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; Triol, cholestan-3β,5α,6β-triol.

27-Hydroxycholesterol (27-OH) is a prominent member of the oxysterol family, a group of 27-carbon molecules originating from cholesterol oxidation and, compared to cholesterol, containing an additional hydroxy, epoxide or ketone group in the sterol nucleus, and/or a hydroxyl group in the side chain [1,2]. 27-OH is generated enzymatically by cholesterol 27-hydroxylase (CYP27A1), a mitochondrial cytochrome P450 oxidase that is present in various tissues and cells, particularly in the liver and macrophages [3,4].

27-OH is one of the most common oxysterols in the peripheral blood of healthy individuals [5]; it is now considered to play an important physiological role that is not limited to bile acid and steroid hormone biosynthesis from cholesterol [6,7]. It has been shown to act as a selective estrogen receptor modulator [8]; like other side chain oxysterols, it is also a very good ligand of liver x receptors (LXRs) [9,10], nuclear receptors that function as master transcription factors, in cell metabolism, cell proliferation, inflammation and immunity [11,12].

Conversely, as is the case of several key biomolecules including glucose, cholesterol, and ascorbic acid, 27-OH and other oxysterols may, under certain conditions, exert pathologic effects. A variety of cholesterol oxides display marked pro-apoptotic and pro-inflammatory reactions [1,2,13,14], and their potential contribution to the pathogenesis and progression of major chronic inflammation-associated diseases is supported by a growing body of evidence [15].

The first demonstration that oxysterols of pathophysiological interest may trigger both beneficial and detrimental events within cells. in particular generating both survival and death signals, was provided by Lizard's group, in an *in vitro* study challenging human monocytic cells (THP-1) with 7-ketocholesterol (7K), a major oxysterol with a strong pro-apoptotic effect. Before eventually leading the cells to apoptotic death, this compound activated the extracellular signal-regulated kinase (ERK) signaling pathway, thereby leading to a transient but net inhibition of the pro-apoptotic protein Bad, thus actually quenching and postponing the pro-apoptotic action of 7K itself [16]. More recently, in another human promonocytic cell line (U937), a low micromolar concentration of 27-OH was

shown to activate an Akt-dependent survival pathway that delayed apoptotic death, while a higher concentration of the same oxysterol exerted an earlier and direct pro-apoptotic effect [17].

The present study aimed to investigate in depth: i) the modulation of both mitogen-activated protein kinase ERK kinase (MEK)/ERK and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways in cells of the macrophage lineage, cultivated over time in the presence of a low micromolar concentration of 27-OH; ii) the effect of such a signaling modulation on Bad protein phosphorylation; iii) the possible involvement of the pro-oxidant effect of 27-OH in generating not only death signals but also survival signals in cells thus treated.

Indeed, in U937 promonocytic cells cultivated over time in the presence of 10 μM 27-OH, both MEK/ERK and PI3K/Akt signaling pathways appeared transiently but markedly up-regulated, allowing a net phosphorylation of Bad protein, respectively at Ser75 and at Ser99, with a consequent significant delay in the oxysterol's pro-apoptotic effect. Importantly and unexpectedly, a reciprocal interaction between the pro-oxidant effect of 27-OH and its ability to generate survival signals was unequivocally demonstrated, as reported below in detail.

Materials and methods

Cell culture and treatments

The human promonocytic cell line U937 was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin/streptomycin (Pan Biotech GmbH, Aidenbach, Germany) at 37 °C with 5% CO_2. The cells were dispensed at 1×10^6/ml and made quiescent through overnight incubation in serum-free medium; they were then placed in RPMI-1640 medium with 2% fetal bovine serum and treated with 27-OH (Steraloids Inc., Newport, RI, USA) dissolved in ethanol. In some experiments, cells were pre-treated (45 min) with PD98059 (40 μM), a

selective inhibitor of MEK1/2, or with LY294002 (25 μM), a selective inhibitor of PI3K (Calbiochem, EMD Millipore Corporation, Billerico, MA, USA) or with N-acetyl cysteine (NAC) (100 μM), an antioxidant compound (Sigma, Darmstadt, Germany). Final concentrations and incubation times for all experiments are reported in the figure legends.

Cell death, viability and proliferation assays

Apoptotic cell death was determined by an Annexin V affinity assay. U937 cells seeded in 12-well plates were treated as indicated, transferred to flow cytometry tubes, and harvested by centrifugation at 300 g for 5 min. The cells were then resuspended in 1 ml of cold PBS and again centrifuged at 300 g for 5 min. After removal of supernatant, the cells were incubated in Annexin V buffer (140 mM HEPES, 10 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) (Sigma) containing 1% (v/v) Annexin V (FITC) (Alexis Biochemicals, Enzo Life Sciences, Inc. Farmingdale, NY) for 15 min in the dark. Cells were analyzed by FACS (FACSCanto, Becton Dickinson, Franklin Lakes, NJ).

In order to detect cell viability and proliferation, exposure to 27-OH was determined by WST-1 (Cell Proliferation Reagent) following the manufacturer's instructions (Roche Diagnostics, GmbH, Penzberg, Germany). Briefly, cells in 96-well plates were treated as indicated and 10 μl of WST-1 reagent was added to each well, after which the plates were incubated for 4 h at 37 °C. Absorbance was measured with a microtiter plate reader (Bio-Rad, CA, USA) at a test wavelength of 450 nm and a reference wavelength of 655 nm. Results were expressed as percentage of cell viability versus controls, taken as 100%.

Quantification of 27-OH in U937 cells by mass spectrometry

Cells where incubated with 10 μM 27-OH up to 72 h. At different incubation times, cells were centrifuged, washed with PBS and resuspended in 1 ml NaCl 0.9%. The deuterium labeled 27-OH-

d7 (Avanti PolarLipids, Alabaster, AL, USA) was added as internal standard and lipids were extracted with chloroform-methanol (2/1,v/v). The intracellular concentrations of the oxysterol were quantified by isotope dilution mass spectrometry essentially as previously described [18]. The mass spectrometer was set to the selected ion monitoring mode; the ions used for analysis were as follows: [$^2H_7$]27-OH 463 m/z, 27-OH 456 m/z. Quantification of the oxysterol was made by the internal standard ratio method.

Protein extraction and immunoblotting

Cells were treated as indicated and harvested by centrifugation at 300 g for 30 sec. Following resuspension in 1 ml of ice-cold PBS and transfer to 1.5-ml microfuge tubes, cells were spun at 2,640 g for 30 sec. The pellet was lysed by incubation for 30 min in 200 µl of cold cell lysis buffer containing 50 mM Tris/HCl (pH 8), 150 mM NaCl, 1 mM phenylmethanesulfonyl, protease and phosphatase inhibitor cocktails (Roche, F. Hoffman-La Roche Ltd., Basel, Switzerland), and 1% Nonidet P-40 (v/v) (Sigma). After centrifugation at 2,640 g for 10 min, the supernatant containing the total protein extract was removed and stored at -80 °C. Protein concentrations were determined by the DC protein assay (Bio-Rad, Munich, Germany). Proteins (30 µg) were mixed with loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris/HCl pH 6.8) (Sigma) and separated on 10-15% SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Freiburg, Germany). The membranes were blocked with 5% blocking reagent (Amersham) in PBS-Tween20 and incubated with appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology Inc., Beverly, MA) in 5% blocking reagent. After the required washes with PBS1x-Tween20, proteins were finally analyzed using an enhanced chemiluminescence detection system and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech). All critical immunoblotting experiments were repeated at least three times.

Measurement of intracellular ROS

The overproduction of reactive oxygen species (ROS), mainly superoxide anion (O_2^-), was detected by di dihydroethidium (DHE) fluorescence staining (Sigma). After treatment with 27-OH, in the presence or absence of selective inhibitors, the cells were washed and resuspended with RPMI-1640 medium (+ 2% fetal bovine serum) and incubated for 30 min in the dark with 5 μM DHE at 37 °C. Fluorescence was immediately detected on glass base dishes by a laser scanning confocal microscope (Zeiss LSM 510; Carl Zeiss S.p.A., Arese, Milan, Italy) (plan neofluar lens 40×/0.75) setting the exciting laser band to 543 nm, and using a 560-615 nm band-pass emission filter. All images were processed using LSM 510 Image Examiner software (Carl Zeiss S.p.A.).

Hydrogen peroxide determination

To measure hydrogen peroxide (H_2O_2) production, cells were lysed for cytosolic protein analysis in ice-cold buffer containing 20 mM Hepes pH 7.9, 0.35 M NaCl, 20% glycerol, 1% Igepal CA-630, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, and protease inhibitors. Levels of H_2O_2 were determined in the cytosolic fractions by monitoring the HRP-dependent oxidation of acetylated ferrocytochrome c, as described by Zoccarato *et al.*[19] Ferrocytochrome c (0.8 ml of 50 μM), HRP (2 μl of 40 μg/ml), and p-hydroxyphenylacetic acid (100 μl of 50 μM) were added to 100 μl cytosolic samples. The oxidation of acetylated ferrocytochrome c was monitored spectrophotometrically at 550 minus 540 nm, after 1, 2, and 3 min of incubation, using an absorption coefficient of 19.9 mM^{-1} cm^{-1}.

Measurement of transmembrane mitochondrial potential

5,5',6,6'-Tetrachloro1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Life Technologies, Monza, Italy) is a cationic dye that exhibits membrane-potential-dependent accumulation in mitochondria as J-aggregates that, in depolarized mitochondria, are converted to JC-1 monomers; this is indicated by the fluorescence emission shift from red to green. Before incubation with oxysterols and specific inhibitors, U937 cells were incubated for 10 min with 10 µg/ml JC-1 in RPMI-1640 medium with 1% fetal bovine serum. After treatments, cells were observed through a LSM 510 confocal laser microscopy system (Carl Zeiss S.p.A.). The images were processed using LSM 510 Image Examiner software (Carl Zeiss S.p.A.).

Statistical analysis

All the illustrated results represent one of at least three independent experiments with similar outcomes. All numerical data are presented as means ± standard deviation (S.D.). Statistical significance of the results was analyzed by the Student's t-tail test.

Results

Dose-dependent pro-apoptotic effect of 27-OH on U937 promonocytic cells

Aliquots of U937 cells were incubated for up to 72 h in the presence or in the absence of different final concentrations of 27-OH. As illustrated in Fig. 1A, 27-OH-induced apoptosis, evaluated by the Annexin V test, showed different kinetics depending on its final concentration. In particular, at the final concentration of 10 µM, significant induction of apoptosis only occurred after 72 h, while at the intermediate concentration used (50 µM), it already doubled the number of Annexin V positive cells present versus the control after 24 h cell treatment. Only at the end of the interval

considered (0-72 h), 10 µM 27-OH caused a slight though significant reduction of cell viability and proliferation, as measured in terms of WST-1 tetrazolium salt reduction by cellular dehydrogenases (Fig. 1B). Successively, focusing on the lowest oxysterol concentration, i.e. 10 µM, a time course of its pro-apoptotic effect was determined. Under the experimental conditions applied here, the number of apoptotic cells was about 20% of the total after 3 days, 40% after 5 days and about 60% after 7 days of treatment (Fig. 1C).

Measurement of 27-OH actual concentrations in 27-OH treated U937 promonocytic cells

In order to quantify the 27-OH amount actually present over time within cells, U937 cells where incubated with a single dose of 10 µM 27-OH up to 72 h. After different incubation times, cell aliquots were taken from the same cell suspension and 27-OH intracellular amount was quantified as described in the "Methods Section". As illustrated in Fig. 1D, from the very beginning the amount of 27-OH ranged between 18 and 26% of the total oxysterol amount added to the cells, showing a steady concentration over the experimental time with minor and non significant variations.

Low micromolar concentration of 27-OH produces a transient but robust stimulation of ERK1/2 and Akt phosphorylation

Since the well-known apoptotic effect of 27-OH was actually delayed by almost 3 days when promonocytic cells were incubated in the presence of a 10 µM concentration, the possible prevalence of survival signals over death signals during this interval was investigated. As reported in Fig. 2A, a marked up-regulation of two well-known cell survival signals, ERK1/2 and Akt phosphorylation, was consistently observed in U937 cells treated with 10 µM 27-OH; this occurred with a maximum

between 6 and 24 h incubation with regard to ERK, and between 6 and 48 h incubation in the case of Akt, phosphorylated at Thr308.

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

Because activation of the PI3K/Akt signaling pathway culminates in phosphorylation of the B-cell lymphoma (Bcl)-2 family member Bad, and since it thus inhibits Bad's pro-apoptotic effect [20], phosphorylation of this protein was investigated in U937 cells treated with 10 μM 27-OH. Bad was phosphorylated at the level of Ser99 at some point between 3 and 48 h cell incubation, likely in connection with Akt phosphorylation; this key apoptotic protein also appeared to be phosphorylated at its Ser75 residue, at some point between 3 and 72 h cell incubation, likely depending on 27-OH-induced MEK/ERK phosphorylation (Fig. 2B).

Inhibition of ERK- or Akt-dependent Bad phosphorylation markedly anticipated the pro-apoptotic effect 27-OH

The dependence of Bad phosphorylation, and of the consequent loss of its pro-apoptotic potential, upon the activation of either ERK (Bad phosphorylation at Ser75) or Akt (Bad phosphorylation at Ser99) were confirmed by a series of experiments employing two selective metabolic inhibitors, namely the MEK/ERK inhibitor PD98059 and the PI3K/Akt inhibitor LY294002. Pre-incubation (45 min) of U937 cell aliquots with 40 μM PD98059 markedly reduced ERK1/2 phosphorylation after 6 and 12 h treatment with 10 μM 27-OH; in parallel, it fully prevented Bad phosphorylation at Ser75 (Fig. 3A). As expected, cell pre-incubation with LY294002 prevented Akt phosphorylation and Bad phosphorylation at Ser99 (Fig. 3B). The two inhibitors have been used at non citotoxic concentrations (Supplementary Fig. 1 and 2).

Prevention of ERK-Akt-dependent Bad phosphorylation anticipated the pro-apoptotic effect of the low micromolar dose of the oxysterol. In cell suspensions pre-incubated with either ERK or Akt selective inhibitors, the percentage of apoptotic cells measured by the Annexin V test was already about 20%, after 24 h challenge with 10 μM 27-OH; this is approximately the percentage of apoptosis induced by this concentration of the oxysterol when cell incubation was continued to three days in the absence of the ERK/Akt inhibitors. The impact of inhibiting ERK and Akt-dependent survival signaling on cell survival was of course more evident after 48 and 72 h of U937 cell culture (Fig. 3C).

Biphasic increase of ROS steady-state levels in U937 cells treated with 27-OH. Dependence of 27-OH-induced ERK and Akt phosphorylation on the prior ROS increase

Challenging U937 promonocytic cells with a single 10 μM dose of 27-OH confirmed the pro-oxidant effect of this oxysterol, but the resulting increase of intracellular ROS showed a peculiar trend (Fig. 4). In DHE stained cells, a marked increase of ROS steady-state level was evident until 4-5 h of cell treatment, but then disappeared between 6 and 24/48 h of cell treatment, and reappeared at longer incubation times. Of note, the first oxidative burst was demonstrated to be responsible for the transient up-regulation of ERK1/2 and Akt phosphorylation: cell pre-treatment with NAC, at a non cytotoxic concentration (10 mM) (Supplementary Fig. 3) able to impede the intracellular rise of ROS (Fig. 5A), fully prevented 27-OH-dependent up-regulation of both ERK1/2 and Akt phosphorylation (Fig. 5B).

Impact of ERK- and Akt-dependent survival signaling on ROS intracellular levels

Very interestingly, the chronology of the transient quenching of 27-OH's pro-oxidant effect corresponded to the transient prevalence of ERK- and Akt-dependent survival signaling. In the light of this finding, it was decided to investigate the potential interference of such survival signals with ROS production. The inhibition of ERK1/2 and Akt phosphorylation, by cell pre-treatment with

PD98059 and LY294002, respectively, indeed abolished the transient disappearance of 27-OH's pro-oxidant effect. A high level of intracellular ROS was also maintained after 12 h challenge with 10 μM 27-OH, provided that cells were pre-treated with MEK/ERK or PI3K/Akt selective inhibitors. The sustained elevation of the ROS steady state was proven both by DHE staining and by H_2O_2 measurement (Fig. 5C).

Cellular sources of ROS enhanced in 27-OH treated promonocytic cells

On the basis of findings pointing to oxysterols' capacity to impair mitochondrial membrane potential (Δψm) and up-regulate Nox-2, thus increasing the intracellular ROS steady-state [21,22], derangement of Δψm and activation of Nox-2 were checked in 27-OH-treated cells, after 12 h incubation, in the presence or absence of PD98059 and LY294002 pharmacological agents. Data reported in Fig. 5C show that 12 h challenge of U937 cells with 27-OH alone did not cause any ROS increase versus controls, unless the contemporary up-regulation of ERK and Akt signaling was inhibited. In agreement with this, JC-1 (a cationic dye that only enters into mitochondria with high Δψm, when its color changes from green to red) displayed an intense red fluorescence only in control and 27-OH treated cells; conversely, it showed diffuse green fluorescence, indicating Δψm depression, when cells were challenged with 27-OH in the presence of PD98059 and LY294002 (Fig. 6A). Nox-2 is an additional source of ROS in 27-OH-treated promonocytic cells; its net activation was proved by a significant increase of membrane translocation of its component $p47^{phox}$ after 12 h incubation with 27-OH, only when MEK/ERK and PI3K/Akt survival signaling was inhibited (Fig. 6B).

Discussion

27-OH, an oxysterol of increasing interest in pathophysiology, is known to induce LXR-dependent pathways that are functional for cells [9-12]. Here, it is also shown to trigger survival

signaling, at least in cells of the monocyte/macrophage lineage; this signaling certainly involves the MEK/ERK and PI3K/Akt phosphorylation pathways.

As mentioned in the Introduction, the first evidence of survival signaling by oxysterols was reported by Berthier *et al.* [16] who treated THP-1 monocytic cells for up to 48 h in the presence of 7K at the final concentration of 0.04 mg/ml (~100 μM). In that cell system, 7K caused a marked but transient increase of ERK1/2 phosphorylation (between 1 and 6 h of treatment), which in turn set back the pro-apoptotic effect of the oxysterol by stimulating the phosphorylation of Bad at residue Ser75 [16]. Unlike our findings on U937 promonocytic cells treated with 27-OH, in that study, 7K at the same time up-regulated ERK1/2 phosphorylation and down-regulated Akt phosphorylation at Thr308. This discrepancy with our findings, likewise on monocytic cells, could be ascribed to the relatively greater cytotoxicity of 7K than 27-OH [14].

In our opinion, the fact that 27-OH and 7K, respectively very good and very poor ligands of LXRs [9,10], both stimulate pERK-dependent survival signaling rules out a primary involvement of this nuclear receptor family in this kinase up-regulation.

In another interesting study, after phorbol myristate acetate (PMA) differentiation, the same cell line U937 was challenged with a 27-OH final concentration of up to 0.01 mg/ml (~28 μM). A significant induction of cell viability occurred, which the study authors ascribed to the marked increase in the Akt level and to phosphorylation at residue Thr308, operated by the oxysterol [17]. The observation time frame of that study was limited to 24 h, while in the present research cell challenge with oxysterols was in most cases continued to 72 h.

A third oxysterol, namely cholestan-3β,5α,6β-triol (Triol), has very recently been reported to activate pro-survival signaling involving ERK phosphorylation in the human cholangiocyte MMNK-1 cell line [23]. On the one hand, as for 7K, Triol is not considered a good LXR ligand; on the other hand, its concentration, at least in human blood, is certainly negligible [5].

Taking the few relevant reports together with the present findings, it may be said that, at least in cells of the macrophage lineage, 27-OH shows a remarkable ability to trigger two distinct survival signaling pathways, depending on the activation of MEK/ERK and PI3K/Akt. Of interest, macrophages are known to express high levels of CYP27A1, i.e. the enzyme that catalyses the production of 27-OH [24]. Moreover, this enzymatic process is now a hot topic drawing the attention of scientists from different fields, in particular immunology and oncology, because of the potential impact of 27-OH-dependent up-regulation of survival and anti-apoptotic signals on immune-system modulation, and on cell neoplastic transformation and growth [25-28].

It has long been accepted that the MEK/ERK-PI3K/Akt axis makes a significant contribution to the carcinogenic process and cancer resistance to chemotherapy [29,30]; its targeting is considered a very promising new anti-neoplastic tool [31,32]. The present data indicate that 27-OH might be an endogenous trigger of this pro-survival intracellular axis.

Notably, although 27-OH was added to promonocytic cell suspension at the final concentration of 10 μM, only 20-25% of that amount was recovered within cells and throughout the experiment (Fig. 1D). In addition, the amount of this and other oxysterols recovered in aortic and carotid atherosclerotic lesions was calculated to be above 1-2 x 10^{-6}M [33-35], i.e. at least an order of magnitude higher than the amount of 27-OH measured in the human serum [5], also taking into acount the difficulty of measuring the actual concentration of the oxysterol in a heterogenous material such as the atheroma.

With regard to the mechanism underlying the enhancement of ERK and Akt phosphorylation observed in 27-OH-challenged promonocytic cells, the fact that pre-treatment with the antioxidant NAC inhibited pERK1/2 and pAkt levels confirms the pro-oxidant activity of this oxysterol [36] and demonstrates a causative role of up-regulated ROS in the phosphorylation process itself, and in the related survival effect (Fig. 4 and 5).

Both a Nox-2 dependent ROS increase, and the ROS-dependent up-regulation of Akt phosphorylation and consequent survival signaling, have already been shown in a different contest,

i.e. in macrophage colony-stimulating factor (M-CSF)-stimulated human monocytes/macrophages; in that study the two events were abrogated both by knocking-out the Nox-2 component $p47^{phox}$ and by similar pre-treatment with NAC (20 mM) [37]. In comparison to M-CSF, 27-OH was here demonstrated to activate ROS generation, also deranging the mitochondrial membrane potential of promonocytic cells (Fig. 6).

Besides demonstrating that the combined activation of ERK and Akt phosphorylation in 27-OH-treated promonocytic cells was ROS-dependent, another very interesting finding was that up-regulation of the ERK/Akt axis was, in turn, able to quench the oxidative impairment provoked by the oxysterol itself. As depicted in Fig. 4, in cells challenged with 27-OH, ROS steady-state levels transiently dropped to control values, coinciding with the entire period of ERK-Akt stimulated phosphorylation, i.e. between 6 and 48 h of cell incubation. Notably, the pro-oxidant effect of 27-OH no longer showed a biphasic pattern when enhancement of ERK-Akt phosporylation was prevented by means of selective inhibitors (Fig. 4 and 5C).

With regard to the quenching, by ERK-Akt up-regulation, of the oxidative imbalance exerted in 27-OH challenged cells, one likely explanation for this effect is the induction of antioxidant defense systems. Indeed, activation of these phosphorylation pathways has repeatedly been demonstrated to induce the nuclear factor erythroid 2 p45-related factor 2 (Nrf2) and its antioxidant response [38-41].

It thus appears that 27-OH's pro-oxidant effect is the key mechanism whereby it displays its pro-apoptotic properties, in agreement with the recognized ability of oxidative stress to trigger pro-apoptotic signals [42-44]; however, at the same time, it may generate survival signals that counteract that pro-apoptotic action, as schematically reported in Fig. 7. The pro-survival effect of 27-OH reported here was evident only when relatively low, and not directly toxic, concentrations were applied; this is, incidentally, a more realistic approach, which made it possible to decipher a sort of "Trojan horse" action exerted by this, and likely other, oxysterols [45]. Instead of killing the cell directly, 27-OH might delay the irreversible damage it can cause, meanwhile initiating for instance pro-inflammatory and pro-fibrogenic pathways. In this connection, another oxysterol, 7β-

hydroxycholesterol, has been shown to induce proliferation and to prevent apoptosis when added at relatively low micromolar concentrations (1-10 μM) to human umbilical-vein endothelial cells; this effect is dependent on ERK phosphorylation, but independent of ROS overproduction induced by the oxysterol [46]. Be this as it may, delayed macrophage death would favor growth and destabilization of advanced atherosclerotic plaques [47].

Conclusions

The findings reported here provide insight into the mechanisms underlying ROS-mediated regulation of survival/death signaling. 27-OH was shown to trigger and sustain survival signal transduction in macrophagic cells involving ERK and PI3K/Akt kinases. Activation of these phosphorylation pathways depended upon an increased production of ROS operated by the oxysterol, and was abrogated by NAC. Whereas on the one hand the latter finding restates that exogenous addition of antioxidants may be a two-sided procedure, on the other hand it conclusively demonstrates that 27-OH's mechanism of action implies redox changes within cells. Of particular interest, and rather unexpected, was the quenching of ROS intracellular levels operated by the activated survival signaling. ERK/Akt phosphorylation must either down-regulate ROS production or up-regulate endogenous antioxidant defenses, and elucidation of this complex mechanism is now in progress.

The property of generating intracellular survival signals through the combined modulation of cellular redox state and ERK/Akt phosphorylation, as well as the ERK/Akt quenching effect on oxidative stress, here reported for an amount of 27-OH of pathophysiological relevance, are most certainly worth investigating also for other lipid oxidation derived products implicated in the pathogenesis of inflammation-driven chronic diseases.

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Supplementary material

Supplementary data associated with this article can be found in the online version.

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Figure captions

Fig. 1. The pro-apoptotic effect of 27-hydroxycholesterol (27-OH) is dose- and time-dependent. (A) Different kinetics of 27-OH-induced apoptosis in U937 cells, depending on the oxysterol's final concentration. U937 cells were treated with increasing concentrations of 27-OH (10, 50 and 100 µM) for 24 h, 48 h, and 72 h, stained with FITC-Annexin V and subjected to FACS analysis. Untreated cells (Control) were used as controls. Relevant histograms represent the mean values ± S.D. of all four independent experiments; *$p < 0.01$, **$p < 0.005$ and ***$p < 0.001$ vs. control at each time-point. (B) Effect of 27-OH (10 µM) on U937 cell viability. Viability of U937 cells was analyzed by the WST-1 assay, following treatment with 10 µM 27-OH for times between 6 and 72 h. Untreated cells (Control) were taken as controls. Average absorption values versus untreated cells are displayed after multiplication by 100. Assay and results represent the mean values ± S.D. of all three independent experiments with six repeats; *$p < 0.05$ vs. control. (C) Time course of the pro-apoptotic effect of a low micromolar amount (10 µM) of 27-OH. Cells were treated for 1 to 7 days with 27-OH at the final concentration of 10 µM and cell death analyzed by flow cytometry. Untreated cells (Control) were taken as controls. Histograms represent the mean values ± S.D. of all four independent experiments; *$p < 0.01$ and **$p < 0.001$ vs. control at each time-point. (D) 27-OH amount within U937 cells. Cells were treated up to 72 h with a single dose of 10 µM 27-OH and the oxysterol

concentrations were measured by mass spectrometry. Histograms represent the percentage of 27-OH present in the cells compared to the total oxysterol administered (100%) at each time-point.

Fig. 2. Phosphorylation of ERK1/2, Akt, and down-stream Bad, induced by 27-hydroxycholesterol (27-OH): a time-course analysis. U937 cells were treated with 10 μM 27-OH for 3 to 72 h. Untreated cells (Control) were taken as controls. (A) Levels of phosphorylated ERK1/2 and phosphorylated AKT, and (B) phosphorylated Bad (Ser75 and Ser99) proteins were analyzed by Western blotting. Actin was used as loading control. One blot representative of three experiments is shown for each protein. Histograms represent the mean values ± S.D. of all three independent experiments; densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control values; *$p < 0.05$ and **$p < 0.01$ and ***$p < 0.001$ vs. control.

Fig. 3. Inhibition of MEK/ERK and PI3K/Akt signaling pathways anticipates the apoptotic effect of 10 μM 27-OH. U937 cells were either treated with 27-OH (10 μM) alone or pre-incubated with PD98059 (40 μM) or LY294002 (25 μM) 45 min before 27-OH treatment. Untreated cells were taken as controls. (A) Levels of phosphorylated and non-phosphorylated ERK1/2, phosphorylated Bad (Ser75), and (B) phosphorylated Akt and phosphorylated Bad (Ser99) proteins were analyzed by Western blotting. Actin was used as loading control. (C) One blot representative of three experiments is shown for each protein. FACS analysis was performed by harvesting and FITC-Annexin V staining the cells at 24, 48 and 72 h, to analyze the effect of inhibitors on cell death response. Histograms represent the mean values ± S.D. of all four independent experiments; *$p < 0.01$ and **$p < 0.001$ vs. control at each time-point; #$p < 0.005$ and ##$p < 0.001$ vs. 27-OH at each time-point.

Fig. 4. Pro-oxidant effect of 10 μM 27-hydroxycholesterol (27-OH). Intracellular generation of ROS was run with dihydroethidium (DHE) in U937 cells incubated with the oxysterol for 1 to 72 h. Untreated cells (Control) were taken as controls.

Fig. 5. Modulation of 27-hydroxycholesterol's (27-OH) pro-oxidant effect by N-acetyl cysteine (NAC) and pERK1/2 and pAkt selective inhibitors. (A) Protection exerted by NAC pre-treatment on ROS generation. U937 cells were treated for 3 h with 10 μM 27-OH. Untreated cells (Control) were taken as controls. Other cells were pre-incubated with 100 μM NAC for 1 h and then treated with 27-OH for 3 h. (B) Effect of NAC pre-treatment on 27-OH-dependent ERK and Akt phosphorylation. Cells were treated for 48 h with 27-OH at the final concentration of 10 μM. Untreated cells were taken as controls. Other cells were pre-incubated (1 h) with 100 μM NAC and then treated with 27-OH for 48 h. The levels of pERK1/2 and pAkt were analyzed by Western blotting (blot representative of three experiments). (C) Effect of MEK and Akt inhibitors on ROS generation and H_2O_2 production. U937 cells were incubated with l0 μM 27-OH for 12 h. Untreated cells (Control) were taken as controls. Some cells were pre-incubated with PD98059 (40 μM) or LY294002 (25 μM) for 45 min and then treated with 27-OH for 12 h. Other cells were treated only with PD98059 or LY294002 as internal controls. The over-production of ROS was detected by dihydroethidium (DHE) fluorescence staining, while H_2O_2 production was measured spectrophotometrically. Histograms represent the mean values ± S.D. of three experiments; *$p < 0.05$ vs. 27-OH.

Fig. 6. Both mitochondrial depolarization and Nox-2 activity contribute to the pro-oxidant effect of 27-hydroxycholesterol (27-OH). U937 cells were treated for 12 h with l0 μM 27-OH. Untreated cells (Control) were taken as controls. Other cells were preincubated with PD98059 (40 μM) or LY294002 (25 μM) for 45 min and then treated with 27-OH for 12 h. (A) The transmembrane mitochondrial potential was detected by JC-1 staining, and (B) Nox-2 activation by Western blotting.

Fig. 7. Schematic flow-sheet of 27-hydroxycholesterol-induced redox modulated survival signaling.

Supplementary material captions

Supplementary Fig. 1. Evaluation of PD98059 cytotoxicity. Cells were incubated for 24 h in the presence or absence of the indicated concentrations of PD98059, a selective MEK1/2 inhibitor, and Annexin V stained cells were measured by FACS analysis.

Supplementary Fig. 2. Evaluation of LY294002 cytotoxicity. Cells were incubated for 24 h in the presence or absence of the indicated concentrations of LY294002, a selective PI3K inhibitor, and Annexin V stained cells were measured by FACS analysis.

Supplementary Fig. 3. N-acetylcysteine (NAC) cytotoxicity. Cells were incubated for 24 h in the presence or absence of the indicated concentrations of the antioxidant NAC and Annexin V stained cells were measured by FACS analysis.

Fig. 1

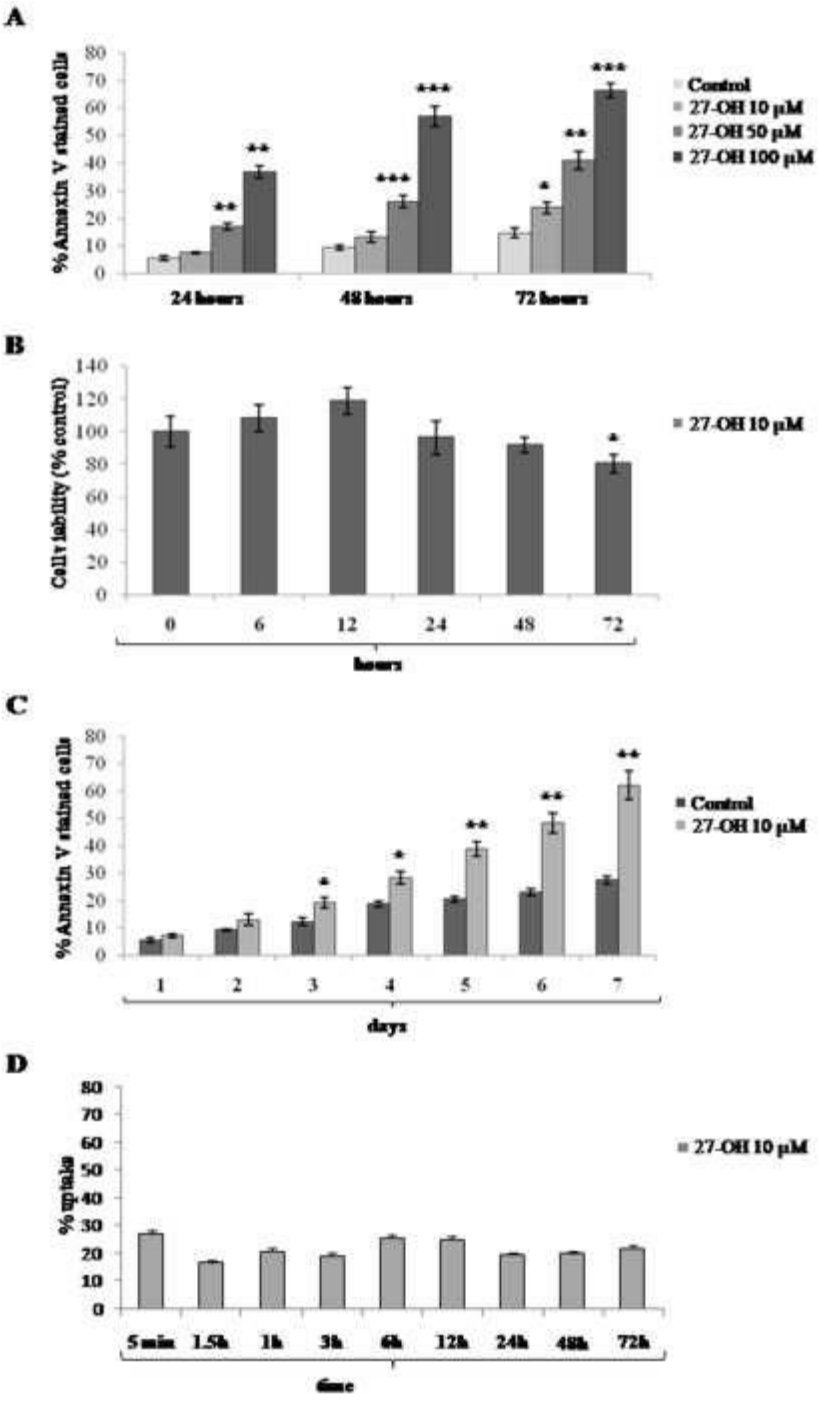

A
% Annexin V stained cells
Control
27-OH 10 µM
27-OH 50 µM
27-OH 100 µM
24 hours
48 hours
72 hours
B
Cell viability (% control)
27-OH 10 µM
hours
C
% Annexin V stained cells
Control
27-OH 10 µM
days
D
% uptake
27-OH 10 µM
5 min
1.5h
1h
3h
6h
12h
24h
48h
72h
time

Fig. 2

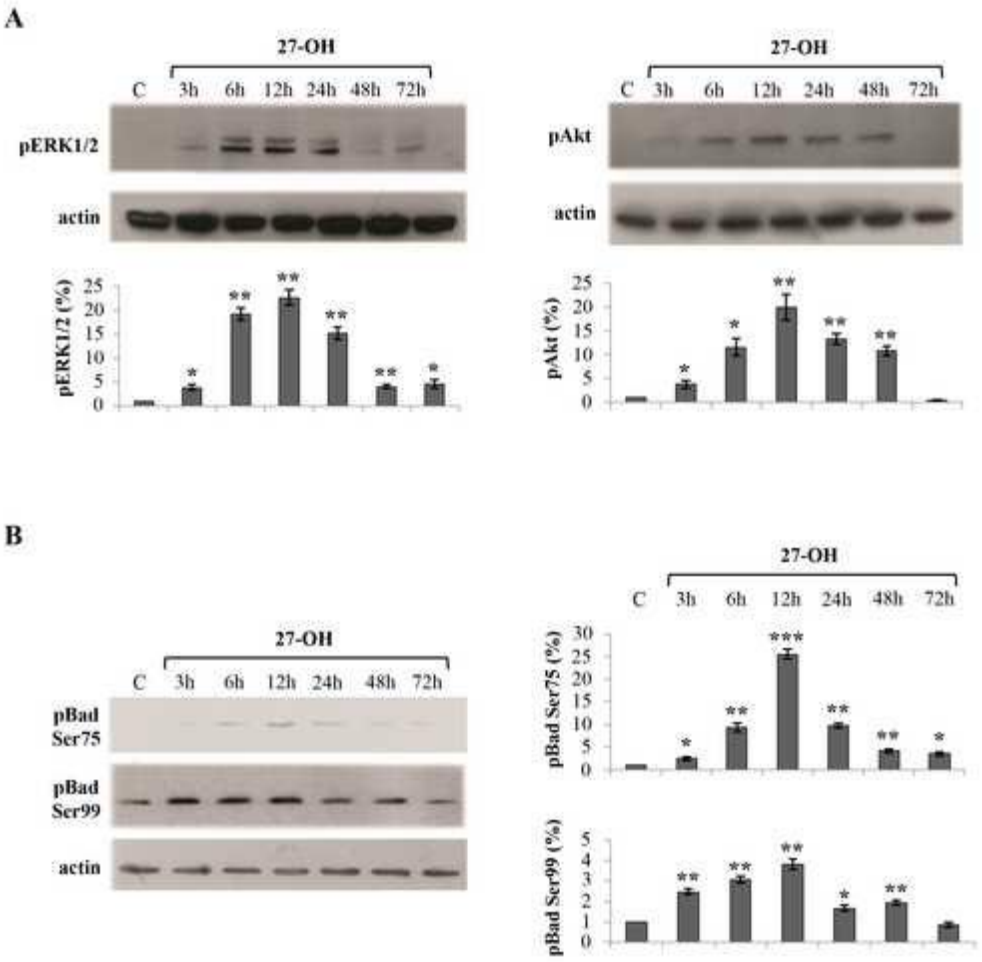

A
27-OH
C 3h 6h 12h 24h 48h 72h
pERK1/2
actin
pERK1/2 (%)
pAkt
pAkt (%)
B
pBad Ser75
pBad Ser99
pBad Ser75 (%)
pBad Ser99 (%)

Fig. 3

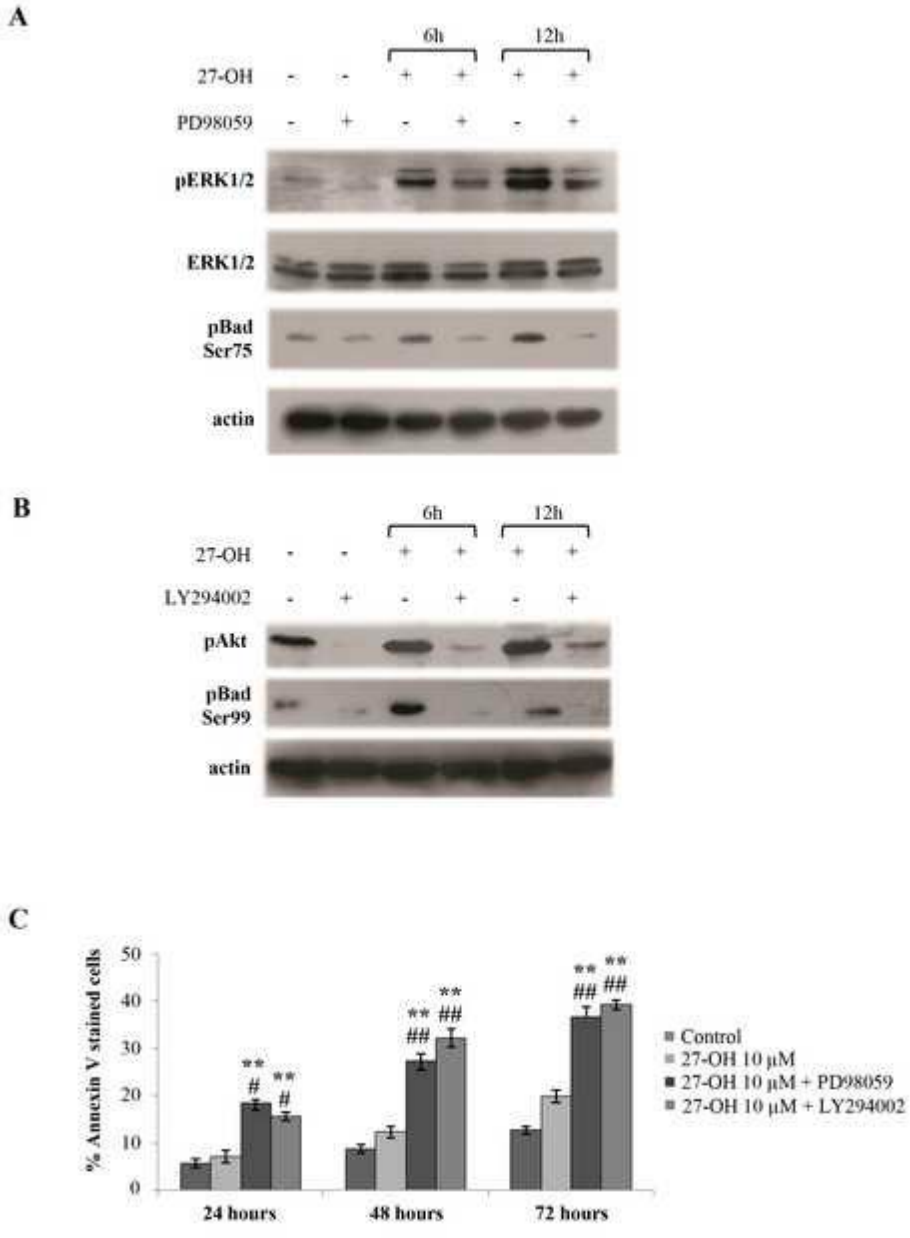

A
6h
12h
27-OH
PD98059
pERK1/2
ERK1/2
pBad Ser75
actin
B
6h
12h
27-OH
LY294002
pAkt
pBad Ser99
actin
C
% Annexin V stained cells
24 hours
48 hours
72 hours
Control
27-OH 10 μM
27-OH 10 μM + PD98059
27-OH 10 μM + LY294002

Fig. 4

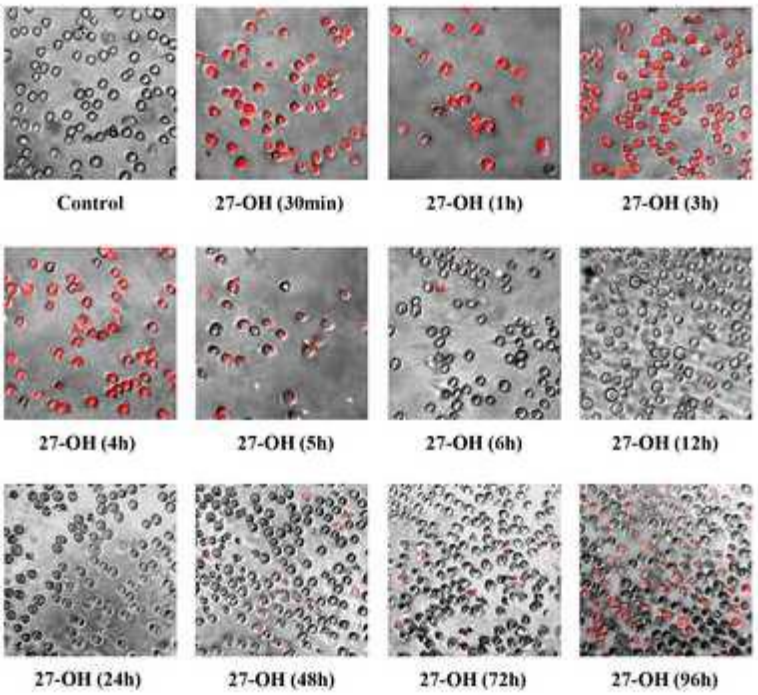

Control
27-OH (30min)
27-OH (1h)
27-OH (3h)
27-OH (4h)
27-OH (5h)
27-OH (6h)
27-OH (12h)
27-OH (24h)
27-OH (48h)
27-OH (72h)
27-OH (96h)

Fig. 5

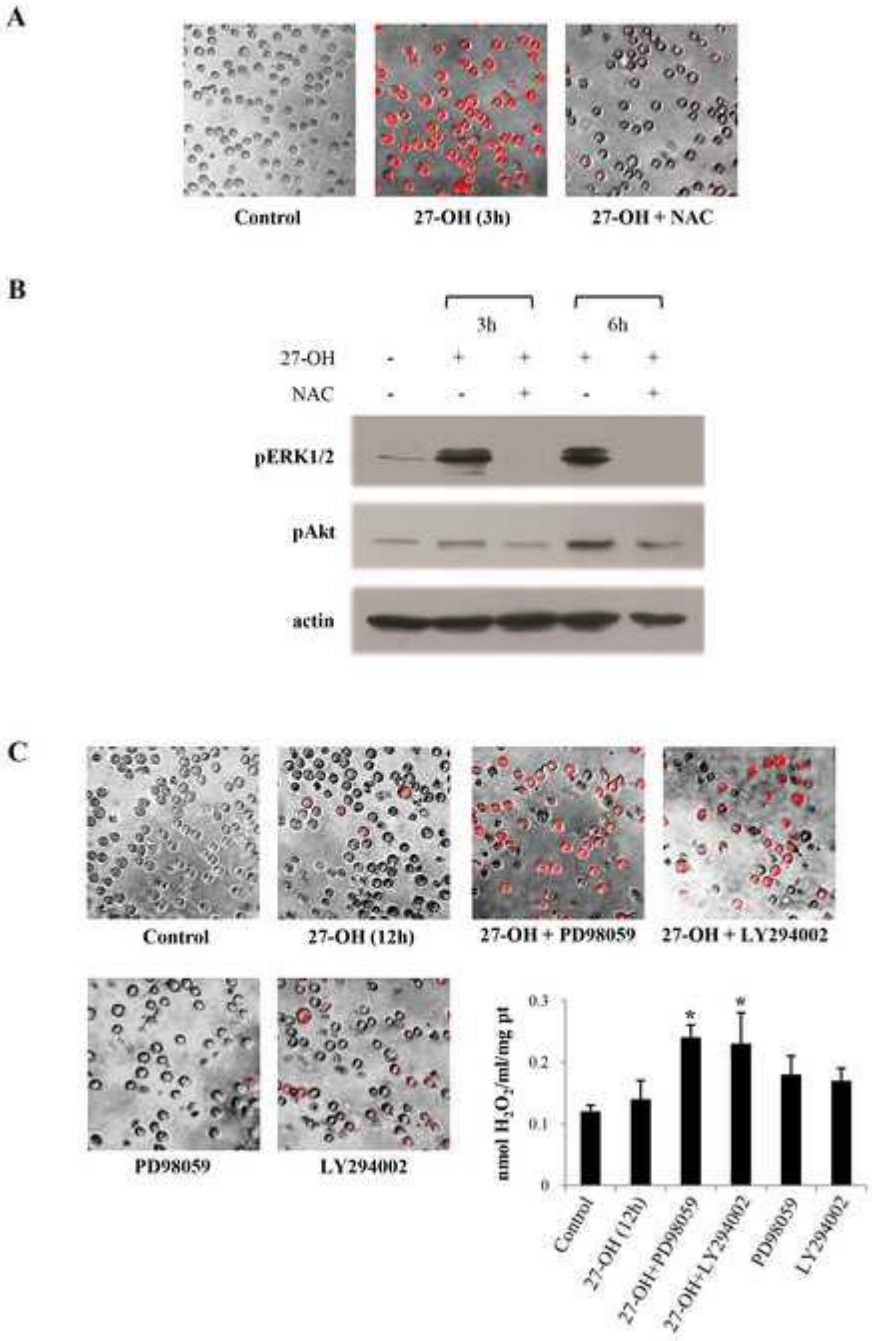

A
Control
27-OH (3h)
27-OH + NAC
B
3h
6h
27-OH
NAC
pERK1/2
pAkt
actin
C
Control
27-OH (12h)
27-OH + PD98059
27-OH + LY294002
PD98059
LY294002
nmol H2O2/ml/mg pt
0.3
0.2
0.1
0
Control
27-OH (12h)
27-OH+PD98059
27-OH+LY294002
PD98059
LY294002

Fig. 6

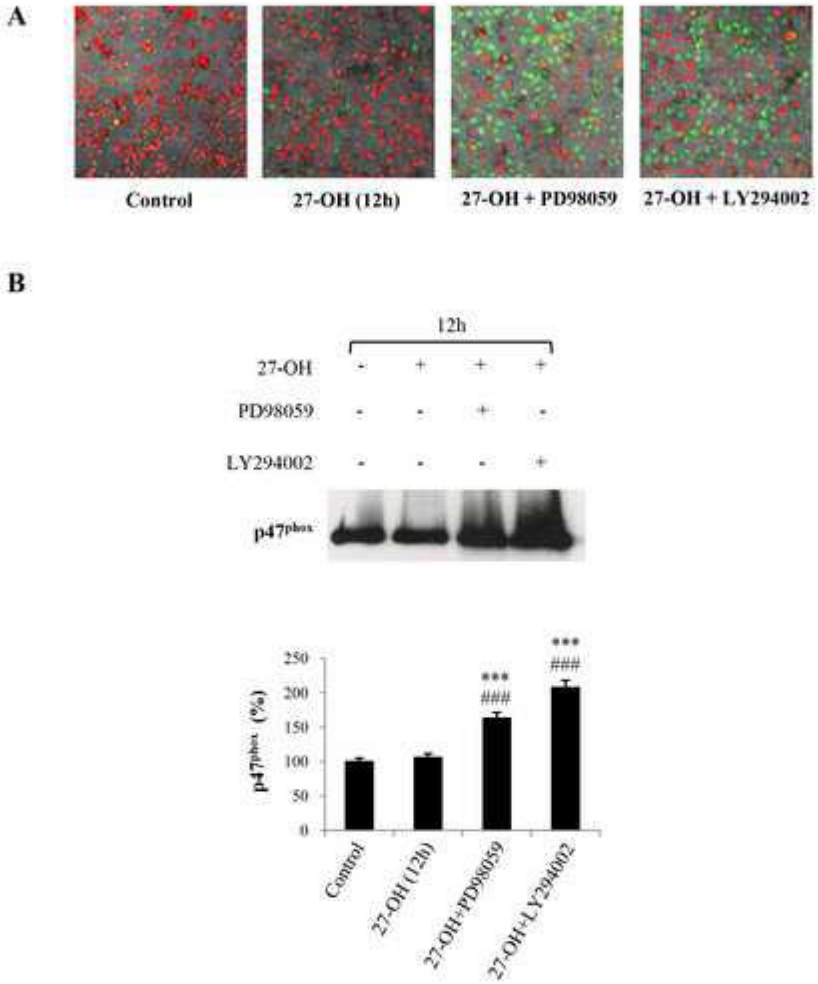
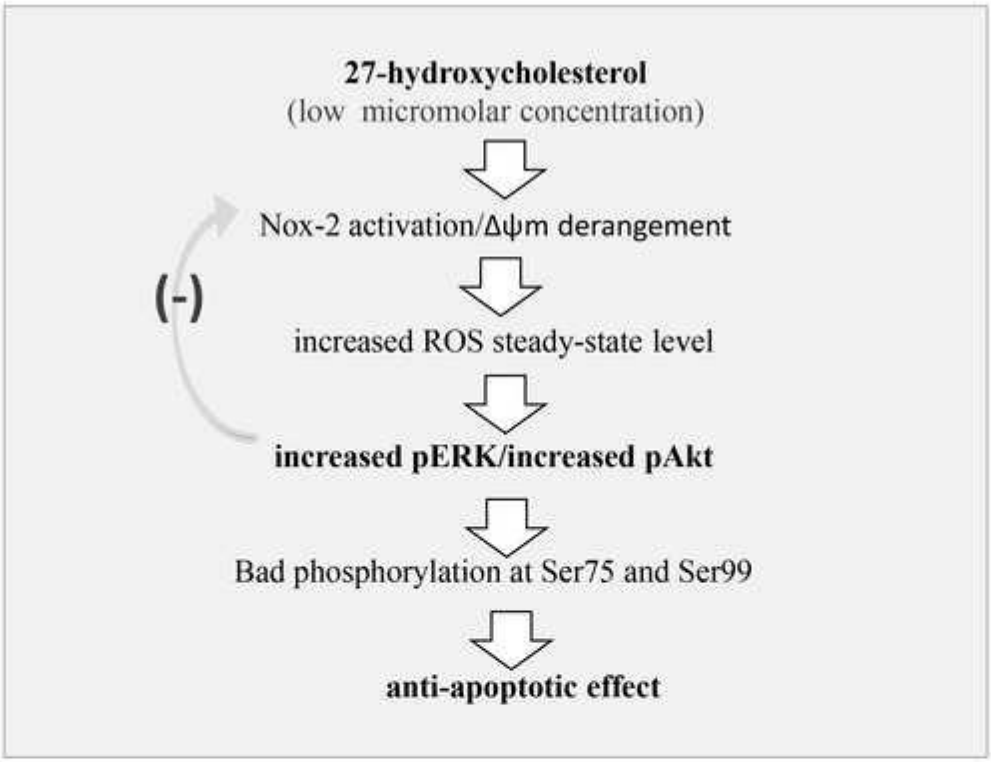
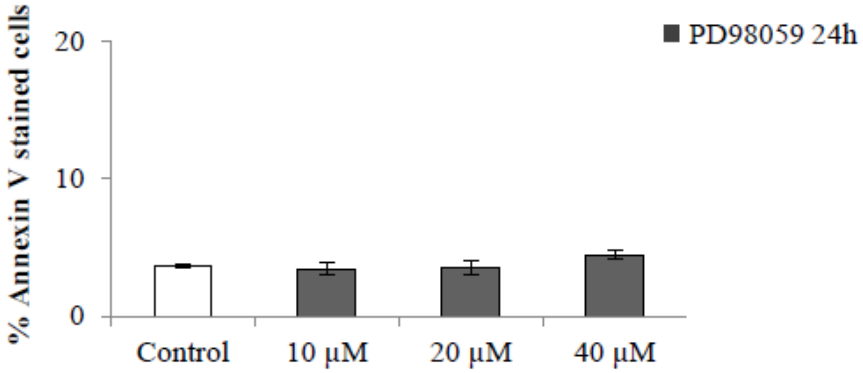

A
Control
27-OH (12h)
27-OH + PD98059
27-OH + LY294002
B
12h
27-OH
PD98059
LY294002
p47phox
p47phox (%)
250
200
150
100
50
0
***
###
Control
27-OH (12h)
27-OH+PD98059
27-OH+LY294002

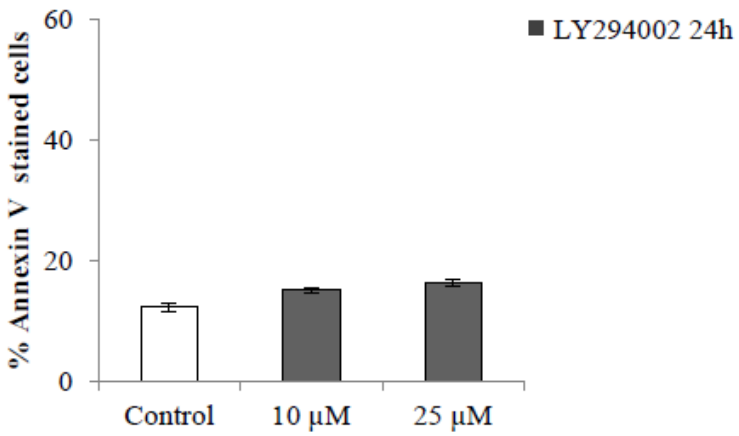
Fig. 7


27-hydroxycholesterol
(low micromolar concentration)
Nox-2 activation/Δψm derangement
(-)
increased ROS steady-state level
increased pERK/increased pAkt
Bad phosphorylation at Ser75 and Ser99
anti-apoptotic effect

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3