Nrf2 antioxidant defense is involved in survival signaling elicited by 27-hydroxycholesterol in human promonocytic cells.

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Abstract

Cholesterol oxidation products such as oxysterols are considered critical factors in the atherosclerotic plaque formation since they induce oxidative stress, inflammation and apoptotic cell death. 27-hydroxycholesterol (27-OH) is one of the most represented oxysterols in atherosclerotic lesions. We recently showed that relatively low concentrations of 27-OH generated a strong survival signaling through an early and transient increase of cellular ROS level, that enhanced MEK-ERK/PI3K-Akt phosphorylation, in turn responsible of a sustained quenching of ROS production. It remains to identify the link between ERK/Akt up-regulation and the consequent quenching effect on ROS intracellular level that efficiently and markedly delay the pro-apoptotic effect of the oxysterol. Here we report on the potent activation of Nrf2 redox-sensitive transcription factor by low micromolar amount of 27-OH added to U937 promonocytic cells. The 27-OH-exerted induction of Nrf2 and subsequently of the target genes, HO-1 and NQO-1, was proved to be: i) dependent upon the activation of ERK and Akt pathways, ii) directly responsible for the quenching of intracellular oxidative stress and by this way iii) ultimately responsible for the observed oxysterol-induced pro-survival response.

Highlights

27-hydroxycholesterol in the low micromolar range activates a Nrf2-dependent antioxidant response in human promonocytic cells

Nrf2 activation depends on the up-regulation of MEK/ERK and PI3K/Akt pathways as a result of an early and transient ROS increase provoked by the oxysterol.

Nrf2 plays a key role in the 27-OH-induced survival signaling

Keywords

Oxysterols, 27-hydroxycholesterol, Nrf2, ROS, survival signaling

Abbreviations

ARE, antioxidant response element; CYP27A1, 27-hydroxylase; DAPI, 4,6-diamidino-2-phenylindole; DPI, diphenyleneiodonium chloride; ERK, extracellular signal-regulated kinase; GPx, glutathione peroxidase; GSR, glutathione reductase; HO-1, heme oxygenase-1; HRP, horseradish peroxidase; JNK, c-Jun NH_2-terminal kinase; Keap1, Kelch-like ECH-associated proein 1; LDL, low density lipoprotein; LXR, liver X receptor; MEK, mitogen-activated protein kinase ERK kinase; NAC, N-acetyl cysteine; NOX-2, NADPH oxidase type 2; NQO-1, NAD(P)H:quionone oxireductase; NRF2, nuclear factor erythroid 2 p45- related factor 2; PAGE, SDS-polyacrylamide gel electrophoresis; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; RT-PCR, reverse transcription–polymerase chain reaction; SERM, selective estrogen modulator; 27-OH, 27-hydroxycholesterol; 7K, 7-ketocholesterol; 7β-OH, 7β-hydroxycholesterol.

Introduction

Oxysterols are a family of 27-carbon molecules originated from cholesterol oxidation by both enzymatic and non-enzymatic mechanisms [1-4]. In addition to being intermediates in cholesterol degradation, oxysterols are regulatory molecules involved in various signaling pathways [1]. The oxysterol 27-hydroxycholesterol (27-OH) is generated by 27-hydroxylase (CYP27A1), a key enzyme of bile acid biosynthesis, and is an abundant primary metabolite of cholesterol, by far the most represented oxysterol in human blood [5]. This oxysterol has been shown to be a very good ligand of liver X receptor (LXR) [6,7] and of estrogen receptors [8], two classes of nuclear receptors recognized to be involved in macrophage survival.

The cytotoxicity of 27-OH and other oxyterols appears to be mainly related to the induction of apoptosis so it is important to elucidate in full the involvement of oxysterols in the signaling cascades implied in apoptotic cell death [2,9,10]. A number of oxysterols of pathophysiologic relevance display a remarkable pro-apoptotic and pro-inflammatory effect, at least sustained by their ability of being pro-oxidant through a net activation of cellular NAD(P)H oxidases [2,10,11]. Apparently, these compounds are able to trigger not only pro-apoptotic but also anti-apoptotic signals in targeted cells [12]. It has been demonstrated that oxysterols such as 27-OH, 7-ketocholesterol (7K) and 7β-hydroxycholesterol (7β-OH) have dual effects on cell viability depending on their concentration [9,13,14]. Thus, while oxysterols can provoke the pro-apoptotic or cytotoxic activity at high concentrations, they may exert cytoprotective or anti-apoptotic effects at relatively low concentrations, via a mechanism that involves ROS [9,12,15].

Very recently, we showed that a low micromolar concentration of 27-OH (10 μM) was able to generate in U937 promonocytic cells a survival signaling, that was involving the extracellular signal-regulated kinase (ERK) and the phosphoinositide 3-kinase (PI3K)/Akt phosphorylation pathways [16]. Notably, the observed induction of the two signaling pathways was triggered by an early increase of ROS cellular level operated by 27-OH through the impairment of the mitochondrial membrane potential and the up-regulation of NADPH oxidase type 2 (Nox-2) activity. Besides the observation that the 27-OH-induced survival signaling was ROS-dependent, another interesting finding was that the upregulation of the ERK/Akt axis clearly appeared, in turn, able to quench the oxidative impairment provoked earlier by the oxysterol itself. The most likely explanation for that quenching effect was the

induction somehow of endogenous antioxidant defense systems. In relation to this, up-regulation of ERK/Akt phosphorylation pathways was known to be able to activate the nuclear factor erythroid 2 p45-related factor 2 (Nrf2) and its antioxidant response [17-20].

Nrf2, a member of the Cap'n'Collar family of b-Zip transcription factors, has been identified as a key transcriptional activator of the antioxidant genes through the antioxidant response element (ARE) [21]. Under resting conditions, Nrf2 is sequestered in the cytoplam by binding to Kelch-like ECH-associated protein 1 (Keap1), an actin-binding cytoskeletal protein [22]. Under oxidative stress Nrf2 dissociates from Keap1, then also from cytoskeleton, and migrates into the nucleus where it binds to ARE sequences, codifying for antioxidant enzymes [22,23]. Some of these enzymes include aldoketoreductase, glutathione peroxidase (GPx), glutathione reductase (GSR), heme oxygenase-1 (HO-1), and NAD(P)H:quinone oxyreductase (NQO-1) [24–26]. It is clearly established that the protective adaptive response to ROS is mediated by enhanced expression of these phase II detoxifying enzymes [27,28]. However, the specific upstream signal transduction pathways used to activate transcription of phase II genes such as HO-1 and NQO-1 are poorly defined.

Increasing evidence supports that several protein kinase pathways including the mitogen-activated protein kinases (MAPK) ERK 1/2, c-Jun NH_2-terminal kinase (JNK)I and p38, and protein kinase C (PKC), are involved in phosphorylation and stabilization of Nrf2 to facilitate its nuclear translocation and binding to ARE sequences of target genes [29,30].

Pursuing the elucidation of the mechanisms underlying the survival signaling elicited by 27-OH [16], we thus aimed to verify if the sustained quenching of the oxysterol's prooxidant effect, resulting in a prolonged cell survival, was somehow involving Nrf2 and its antioxidant response. The data here reported conclusively demonstrate that 27-OH in the low micromolar range is able to markedly up-regulate, at least in cells of the macrophage lineage, both intracellular levels and nuclear translocation of Nrf2, by this way determining a strong stimulation of HO-1 and NQO-1 expression and levels. Nrf2 seems definitely involved in the 27-OH-induced redox modulated survival signaling as a key regulator of cellular antioxidant defense.

Materials and Methods

Cell culture and treatments. The human promonocytic cell line U937 was cultured in RPMI-1640 medium and the human umbilical vein endothelial cell line HUVEC was cultured in DMEM medium, both 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 or DMEM medium with 2% fetal bovine serum and treated with 27-OH (Steraloids Inc., Newport, RI, USA) dissolved in ethanol. In some experiments, cells were pretreated (45min) 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-acetylcysteine (NAC) (100 μM), an antioxidant compound (Sigma, Darmstadt, Germany) or with diphenyleneiodonium chloride (DPI) (50 μM), an inhibitor of NADPH oxidase (Sigma, St.Louis, MO, USA). Final concentrations and incubation times for all experiments are reported in the figure legends.

Protein extraction and immunoblotting. Cells were treated as indicated and harvested by centrifugation at 300g for 30 s. Following resuspension in 1 ml of ice-cold PBS and transfer to 1.5-ml microfuge tubes, cells were spun at 2640g for 30 s. The pellet was lysed by incubation for 30 min in 200 μl of cold cell lysis buffer 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 2640g for 10 min, the supernatant containing the total protein extract was removed and stored at -80°C. For nuclear and cytoplasmic extracts, following treatments and cold centrifugation, cell pellets were resuspended in hypotonic T1 buffer (10 mM HEPES pH 7.6, 2 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF , 1% NP-40 and protease inhibitors) and incubated on ice for 20 min. After the cold incubation, tubes were centrifuged at max speed for 1 minute and supernatants containing cytoplasmic proteins were stored at -80°C. Nuclear protein isolation was carried out by incubation for 20 min on ice in a cold saline T2 buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 650 mM NaCl, 25% glycerol (v/v), 1 mM DTT, 0.5 mM PMSF and protease inhibitors). After cold centrifugation at 13,000g for 20 min, supernatants containing nuclear proteins were 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-Tween 20 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 PBS 1X-Tween 20, 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.

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

Real-time RT-PCR. Singleplex real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed on 30 ng of cDNA using TaqMan gene expression assay kits prepared for human NFE2L2 (Nrf2), HO-1, NQO-1 and β-actin, and TaqMan Fast Universal PCR master mix, and analyzed by a 7500 Fast real-time PCR system (Applied Biosystems, Life Technologies). The oligonucleotide sequences are not revealed by the manufacturer because of proprietary interests. The cycling parameters were as follows: 20 s at 95 °C for AmpErase UNG activation, 3 s at 95 °C for AmpliTaq Gold DNA polymerase activation, and 40 cycles of 3 s each at 95 °C (melting) and 30 s at 60 °C (annealing/extension). The fractional cycle number at which fluorescence passes the threshold in the amplification plot of fluorescence signal vs. cycle number was determined for each gene considered. The results were then normalized to the expression of β-actin, as housekeeping gene. Target gene expression was quantified relatively with a mathematical method proposed by Livak & Schmittgen [31].

siRNA transfection. Small interfering RNA (siRNA) was used for transient gene knockdown studies. The siRNAs used were NFE2L2 s9493 (Nrf2) and siRNA #1 for the negative control

(scramble siRNA) (Applied Biosystems, Life Technologies). Negative control corresponds to a siRNA with nonspecific sequence. Transfection of Nrf-2-specific and control siRNAs was performed following the manufacturer's instructions. Briefly, 50 nM of siRNAs was mixed with 25 μL of transfection reagent solution (NeoFX, Applied Biosystems, Life Technologies) and left at room temperature for 10 min in RPMI medium with 1% fetal bovine serum and without antibiotics. After 24 h of reverse transfection, the cells (4×10^4 500 μL^{-1}) were incubated with 27-OH for 24 h. For gene expression analysis, total RNA was isolated from the cells and used for quantitative RT-PCR as described above. The transfection efficiency, validated by quantitative RT-PCR, was approximately 87% (data not shown).

Measurement of intracellular H_2O_2. Cell aliquots were lysed for cytosolic protein analysis in ice-cold buffer containing 20 mM Hepes, pH 7.9, 0.35 M NaCl, 20% glycerol, 1% Igepal CA-630, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, and protease inhibitors. Levels of H_2O_2 were determined in the cytosolic fractions by monitoring the HRP-dependent oxidation of acetylated ferrocytochrome c, as described by Zoccarato et al. [32]. Ferrocytochrome c (0.8 ml of 50 μM), HRP (2 μl of 40 μg/ml), and p-hydroxyphenylacetic acid (100 μl of 50μM) were added to 100 μl cytosolic samples. The oxidation of acetylated ferrocytochrome c was monitored spectrophotometrically at 550 minus 540 nm, after 1, 2, and 3 min of incubation, using an absorption coefficient of 19.9 mM^{-1} cm^{-1}.

Measurement of intracellular ROS. The overproduction of reactive oxygen species (ROS), mainly superoxide anion (O_2^-), was detected by dihydroethidium (DHE) fluorescence staining (Sigma). At defined time intervals after treatment with 27-OH, 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 LSM510; Carl Zeiss S.p.A., Arese, Milan, Italy) (plan neofluar lens 40x/0.75) setting the exciting laser band to 543nm, and using a 560–615nm band-pass emission filter. All images were processed using LSM510 Image Examiner software (Carl Zeiss S.p.A.).

Cell death. The rate of apoptosis was evaluated through 4,6-diamidino-2-phenylindole (DAPI) staining. To identify apoptotic nuclei, cells were fixed and permeabilized with 95% cold ethanol for 5 min at room temperature, and then washed twice with 0.1 M PBS. Slides

were then incubated for 15 min at room temperature in DAPI solution. After rinsing in PBS, cells were observed and photographed under a Zeiss Axiovert 200 M fluorescence microscope.

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

Results

Increased levels of ROS in U937 promonocytic cells treated with 27-hydroxycholesterol

The remarkable but transient pro-oxidant effect of a single dose of 27-OH (10 μM) added to U937 cell aliquots, then incubated up to 72 h at 37°C, was confirmed by monitoring H_2O_2 production. As shown in Fig. 1A, a significant increase in ROS generation was evident until 6 h incubation, but then it disappeared at longer incubation times. As we previously reported [16], in the time frame corresponding to the up-regulation of phosphorylation of ERK and PI3K/Akt induced by 27-OH, i.e. between 6 and 24 h after cell treatment, ROS production resulted to be quenched. This finding suggested the ability of ERK and Akt survival signaling to modulate the level of cellular ROS.

Induction of Nrf2 expression, total cellular levels and nuclear translocation by 27-OH

With regard to the quenching of ROS intracellular levels by ERK/Akt phopshorylation in U937 cells, one likely explanation for this effect was the induction of the antioxidant defense system. As reported in Fig. 1B, a marked up-regulation of Nrf2 mRNA was detectable in U937 cells since the first few hours after addition of 10 μM 27-OH to the incubation medium. Consistently, a net increase of Nrf2 total cellular levels was observed between 1 and 3 h in U937 cells (Fig. 1C). Even more important, the evaluation by Western blotting of this transcription factor levels in nuclear and cytoplasmic fractions, demonstrated a marked stimulation of Nrf2 nuclear translocation already after 1 h cell challenge with the oxysterol (Fig. 1D). However, Nrf2 activation by 27-OH appeared to be transient, since, after

3 h challenge, the nuclear level of the transcription factor in the treated cells showed a decreasing trend (Fig. 1D).

Induction of HO-1 and NQO-1 expression and synthesis by 27-OH

It has been clearly established that Nrf2 plays a central role in controlling the expression of detoxyfying genes including HO-1 and NQO-1 whose protein products are involved in the elimination of reactive oxidants [23]. With the aim of demonstrating HO-1 and NQO-1 up-regulation by 27-OH in human cells of the macrophage lineage, HO-1 and NQO-1 mRNA and protein levels were measured in promonocytic U937 cells, following incubation with the oxysterol at the concentration of 10 μM. The effect of 27-OH on HO-1 and NQO-1 mRNA expression was checked by quantitative RT-PCR. Real-time RT-PCR showed a significant increase of the expression of both antioxidant genes (Fig. 2A). HO-1 gene was increased up to 6 h oxysterol treatment, then it decreased thereafter. On the other hand, NQO-1 gene was rapidly increased with a maximum increment after 3 h treatment and then the specific mRNA level returned to the basal value after 24 h treatment. Further, a marked rise of both HO-1 and NQO-1 protein levels in U937 cells treated with 27-OH was clearly detectable by Western blotting (Fig. 2B). As reported in Fig. 2B, 27-OH increased HO-1 protein level between 1 and 72 h treatment with a maximum occuring at 12 h, thereafter protein level slightly decreased. With regard to NQO-1, the addition of 10 μM 27-OH to U937 cell suspension strongly up-regulated its protein levels from 3 h up to 72 h treatment. Statistical analysis of all data obtained after 1-72 h treatment showed that HO-1 and NQO-1 synthesis was significantly increased in 27-OH-treated cells compared with control cells (Fig. 2B).

Impact of PI3K/Akt and ERK signaling on 27-OH induced Nrf2 activation

The dependence of the induction of Nrf2 total cell level and nuclear translocation in U937 cells challenged with 10 μM 27-OH upon the activation of either ERK or Akt by this oxysterol was proved by a series of experiments employing selective inhibitors of these kinases. Pre-treatment of U937 cells with the MEK/ERK inhibitor PD98059 (40 μM) or with the PI3K/Akt inhibitor LY294002 (25 μM) effectively prevented the 27-OH-mediated upregulation of total cell level of Nrf2, as monitored after 1 and 3 h oxysterol treatment (Fig.

3A). Further, as reported in Fig. 3B, the inhibition of ERK and Akt signaling with PD98059 or LY294002 respectively, afforded a net prevention of the 27-OH-induced nuclear translocation of Nrf2 observed after 1 h treatment with the oxysterol. Taken together, these results suggest that PI3K/Akt and ERK are upstream activators of Nrf2 and stimulate the nuclear accumulation of Nrf2 in U937 promonocytic cells incubated in the presence of low micromolar amount of 27-OH.

Effect of 27-OH induced PI3K/Akt and ERK signaling pathways on HO-1 and NQO-1 protein level

Pre-incubation (45 min) of U937 cell aliquots with 40 μM PD98059 significantly reduced HO-1 protein levels after 6 and 12 h treatment with 10 μM 27-OH (Fig 4A). As expected, cell pre-incubation with 25 μM LY294002 substantially prevented HO-1 expression after 6 and 12 h treatment. Morevover, the effect of these selective inhibitors was also determined for NQO-1 protein after 24 and 48 h treatment with the oxysterol (Fig 4B). It has been clearly demonstrated that pre-incubation with Erk/Akt inhibitors remarkably prevented 27-OH-induced NQO-1 expression in U937 cells.
These data indicate that ERK and PI3K/Akt survival pathways are involved in 27-OH-induced upregulation of HO-1 and NQO-1 detoxyfying genes in U937 promonocytic cells.

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

Accumulated evidence shows that mitochondrial ROS play a critical role in activation of downstream protective mechanisms, including Nrf2/ARE pathway [33]. In our previous report we showed that ROS were generated through the activation of Nox-2 and derangement of mitochondrial membrane potential in 27-OH treated human promonocytic cells [17]. In the light of these findings, we investigated whether mitochondrial and Nox-2 sourced ROS could play a critical role in mediating 27-OH-induced Nrf2 up-regulation. Therefore, U937 cells were treated with or without the antioxidant NAC prior to 3 h challenge with 10 μM 27-OH, and Nrf2 cell protein levels were analyzed by Western blotting (Fig. 5A). Indeed, the 45 min cell pre-treatment with 100 μM NAC was able to significantly but partially prevent 27-OH-dependent increase of total Nrf2 content. Statistical analysis showed that cell Nrf2 content was more than halved in NAC + 27-OH treated cells as to cells treated with the oxysterol

alone (Fig. 5A). Moreover, cell pre-treatment with NADPH oxidase inhibitor, DPI, gave a similar result. In fact, as shown in Fig. 5B, pre-incubation of cells with 50 μM DPI nearly suppressed the induction of Nrf2 after 3 h oxysterol treatment.

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

Once demonstrated that 27-OH-induced ERK/Akt survival signaling was in turn involving the activation of Nrf2, we investigated the actual role of the latter event in delaying, as previously reported [17], the oxysterol's pro-apoptotic effect. Indeed, cell transfection with specific Nrf2 siRNA determined a marked increase in the number of apoptotic cells already after 24 h incubation with 10 μM 27-OH, when the cell incubation with the oxysterol alone did not show any difference as to the control (Fig. 6).

Neither MEK/ERK, Akt and Nrf2 survival signaling nor remittance of ROS production in U937 cells was observed at high 27-OH dose

When a high 27-OH concentration (100 μM), was added to cell incubation medium, the marked quenching of ROS production induced by 10 μM oxysterol was not any more observed and an unremitting oxidative stress took place (Fig. 7A). Further, the single addition of 100 μM 27-OH to U937 cells did not stimulate, actually in part inhibited, the phosphorylation of MEK/ERK and Akt as observed with the low dose of the oxysterol (new Fig. 7B). Consistently, the high dose of 27-OH did not show any significant modulation of both total cellular Nrf2 levels and Nrf2 nuclear translocation at 1 and 3 h treatment (Fig. 7C and Fig. 7D, respectively).

Discussion

27-OH is the most abundant oxysterol in the human circulation as well as in advanced atherosclerotic lesions [34]. The sterol 27-hydroxylase (CYP27A1) is found in most tissues and cells including macrophages, where it catalyzes the addition of a hydroxyl group to cholesterol in position C27 producing 27-OH. New studies targeting this enzymatic process for the treatment of cancer indicate that 27-OH is an agonist of the estrogen receptors and

increases the risk of breast cancer, therefore 27-OH dependent up-regulation of anti-apoptotic signals on immune-system modulation should be investigated in detail [35–37].

In the current study, we reported that activation of Nrf2 and Nrf2-dependent antioxidant defense plays a key role in 27-OH induced anti-apoptotic response in U937 promonocytic cells. To our knowledge, while enhanced phosphorylation of both ERK1/2 and Akt by 27-OH was very recently reproduced in another monocytic cell line, namely THP-1 [38] the up-regulation of Nrf2 by low micromolar amounts of this oxysterol has not been previously shown. Not 27-OH but oxLDLs, that by the way act as the vehicle of 27-OH in human circulation, were proved to effectively activate Nrf2 in vascular cell lines thus pointing to the involvement of this redox-sensitive transcription factor in the signaling pathway sustaining atherosclerosis progression [39]. In the same study oxLDLs were shown to activate Nrf2 much strongly in murine macrophages than in smooth muscle cells, while 4-hydroxynonenal, a major product of oxidized lipids was equally stimulating Nrf2 nuclear translocation in both cell types, by this way suggesting that modulation of Nrf2 response could much depend on the type of chemical inducer and the type of cell.

Specific cell behaviour should indeed explain the contradictory findigs achieved in C6 glioma cells challenged with 10 or 20 μM 27-OH with regard to the modulation of Nrf2 system. In fact, this oxysterol was found to rather inhibit both expression and level of Nrf2 and its down-stream genes HO-1, NQO-1 and γ-GCS [40]. Also supporting a cell specificity is the fact that the up-regulation of Akt phosphorylation of Thr308 residue observed in U937 cells challenged with low micromolar amounts of 27-OH was not reproducible in primary human aorta smooth muscle cells [13]. Moreover, with regard to both Nrf2 expression and synthesis in HUVEC treated with either 27-OH 10 μM or 27-OH 100 μM neither any significant oxysterol-mediated enhancement nor any difference between the two concentrations was observed (Supplementary Fig.1).

As depicted in Fig. 2, in U937 cells challenged with 27-OH, HO-1 and NQO-1 protein levels linearly increased between 1 and 12 h of cell incubation, this coinciding with ERK-Akt phopshorylation period. The decrease in HO-1 induction after 48 h 27-OH exposure was consistent with the slow disappearance of the protection against ROS window where ROS reappered at longer incubation times (between 72 and 96 h) as previously reported [16]. However, NQO-1 keeps high levels at 48 and 72 h of cell incubation suggesting that HO-1

seems more directly involved in quenching ROS generated by 27OH-induced Nox and by mitochondrial derangement.

HO-1 and NQO-1, among Nrf2-target genes, result to be expressed in all main cell types present in mouse and human atherosclerotic lesions, such as macrophages, endothelial cells, and smooth muscle cells [41]. A number of reports have addressed these cytoprotective and antioxidant enzymes as critical in the cellular response against prooxidative stimuli such as oxidized LDL and oxidized phospholipids. Moderately oxidized LDL were demonstrated to up-regulate HO-1 expression in vascular smooth muscle cells via Nrf2 activation [30]. Similarly, the oxidized phopsholipid oxPAPC was shown to induce HO-1 and NQO-1 in endothelial cells in a Nrf2-dependent manner [42].

As mentioned in the Introduction, Nrf2 nuclear translocation was reported to be modulated by ERK1/2, p38 MAPK, PKC and PI3K/Akt. Moreover, it has been suggested that the intensity of these upstream signals that control Nrf2 nuclear transposition varies with the different inducers in the different cell types. For example, it has been shown that oxidative stress-dependent ERK and Akt activation mediates Nrf2 antioxidant response by favouring translocation of Nrf2 to the nucleus in pulmonary epithelial cells [20,43]. In addition, a PI3K/Akt-dependent Nrf2 protein increase was observed in PC12 pheochromocytoma cells [44]. Here we report that ERK and Akt phosphorylation is also required for the 27-OH-mediated induction of Nrf2 and of the target genes HO-1 and NQO-1. In fact, as shown in Fig. 3, Nrf2 expression was blocked by the MEK inhibitor PD and the PI3K/Akt inhibitor LY. These inhibitors were used at the concentrations reported in the literature and also tested as not being cytotoxic in the experimental model adopted [16]. Certainly all results obtained by using selective inhibitors should be taken with caution, but in this specific case, all data relevant to the modulation of Nrf2-driven survival effect with such compounds were fully consistent among them and further indirectly validated by the findings obtained using Nrf2 siRNA (Fig. 6).

Another critical observation was that 27-OH-mediated increase of HO-1 and NQO-1 synthesis was quenched or even prevented by inhibiting ERK and PI3K/Akt pathways (Fig. 4, panels A, B). In agreement with these findings, HNE was reported to induce HO-1 through activation of ERK pathway in epithelial cells [45]. Similarly, the HO-1 induction observed in human vascular smooth muscle cells as exerted by moderately oxidized LDL implied the activation of PKC and MAPK pathway [30]. In contrast, a marked activation of HO-1 synthesis by HepG2 hepatoma cells was reported to be exerted by defined metabolites of the prostaglandin J(2) series, but not quenched by the ERK1/2 inhibitor PD98059, suggesting, as

stated by the same authors, that up-regulation of this heme catabolism-related enzyme might be obtained through more than a single signaling pathway [46].

Finally, our observation that cell supplementation with NAC or DPI prevents the induction of Nrf2 signaling by 27-OH (Fig. 5) is consistent with the few specific literature available. In C10 epithelial cells the quenching of NADPH oxidase activity with DPI significantly reduced Nrf2 nuclear translocation and ARE-mediated transcription in response to hypoxia [20,43].

In summary, as schematically shown in Fig. 8 (graphical abstract) the marked up-regulation of ERK/Akt axis exerted by low micromolar amount of 27-OH is primary responsible for a net induction and activation of Nrf2 and, subsequently, for the stimulated expression and synthesis of HO-1 and NQO-1, molecular events able to transiently inhibit the pro-oxidant effect of the investigated oxysterol and, more importantly, to significantly prolonge the survival of so treated cells. Notably, relatively high micromolar amount of 27-OH did not stimulate at all the investigated survival signaling (Fig. 7), but even appeared to exert a constant prooxidant effect and much earlier stimulate apoptosis of human promonocytic cells [13,16].

The fact that 27-OH can trigger and sustain survival signals at least in cells of the macrophage lineage might be remarkable in pathophysiology, since this oxysterol is generated by an enzyme constitutively expressed in a number of cell types, in particular monocytes/macrophages [3-4], and it is the most abundant in the blood of healthy subjects as well [5]. In principle, 27-OH-mediated pro-survival reactions should appear beneficial and possibly contribute to processes related to inflammation and immunity. However, in the long run, as in the case of chronic inflammation, oxysterol-induced cell survival could have detrimental effects, like for instance favoring the survival of cancer cells. Thus, the positive or detrimental outcome of 27-OH-mediated survival signaling largely depends on different physiological or disease processes and in the latter case very much on their grade of progression. Still important to be considered and taken into account.

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Figure captions

Fig. 1. 27-hydroxycholesterol (27-OH) induces gene expression, synthesis and nuclear translocation of Nrf2 in U937 cells.

(A) Effect of 27-OH on H_2O_2 production. U937 cells were incubated with 10 μM 27-OH for 1 to 72 h. H_2O_2 production was measured spectrophotometrically. Histograms represent the mean values ± standard deviation (S.D.) of all three independent experiments. **p<0.005 and ***p<0.001 vs. control group. (B) Effect of 27-OH on the expression of Nrf2. Gene expression was quantified by real-time RT-PCR in U937 cells treated for 1 and 3 h with 10 μM 27-OH. Untreated cells were used as controls. Data, normalized to actin, are expressed as mean values ± S.D. of three different experiments. ***p < 0.001, and **p < 0.005 vs. control group. (C) Nrf2 protein level in U937 cells treated with 10 μM 27-OH for 1 and 3 h was analyzed by Western blotting. Untreated cells (c) were taken as controls. Actin was used as loading control. (D) Nrf2 expression in nuclear and cytoplasmic lysates was determined by Western blotting. Untreated cells (c) were taken as controls. Lamin A/C and actin were used as loading controls. One blot representative of three experiments is shown. Histograms represent the mean values ± S.D. of all three independent experiments; densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control values; *p<0.05 , **p<0.01 and ***p<0.001 vs. control group.

Fig. 2. Effect of 27-hydroxycholesterol (27-OH) on HO-1 and NQO-1 expression and protein levels.

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

Fig. 3. Inhibition of MEK/ERK and PI3K/Akt signaling pathways downregulates Nrf2 induction. U937 cells were either treated with 27-OH (10 μM) alone or pre-incubated with

PD98059 (40 μM) or LY294002 (25 μM) 45 min before 27-OH treatment. Untreated cells were taken as controls. (A) Nrf2 protein levels were analyzed by Western blotting. Actin was used as loading control. (B) Nrf2 levels in nuclear and cytoplasmic lysates were determined by Western blotting. Lamin A/C and actin are used as loading controls. One blot representative of three experiments is shown. Histograms represent the mean values ± S.D. of all three independent experiments; **p < 0.01 and ***p < 0.001 vs. control group; #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. 27-OH.

Fig. 4. Effects of MEK/ERK or PI3K/Akt inhibition on HO-1 and NQO-1 induction. U937 cells were either treated with 27-OH (10 μM) alone or pre-incubated with PD98059 (40 μM) or LY294002 (25 μM) 45 min before 27-OH treatment. Untreated cells were taken as controls. (A) Protein levels of HO-1 and (B) NQO-1 were analyzed by Western blotting. Actin was used as loading control. One blot representative of three experiments is shown for each protein. Histograms represent the mean values ± S.D. of all three independent experiments; *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control group; #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. 27-OH.

Fig. 5. Modulation of Nrf2 induction by N-acetyl cysteine (NAC) and diphenyleneiodonium chloride (DPI).
(A) Effect of NAC (B) DPI on 27-OH-dependent Nrf2 induction. Cells were treated for 3 h with 27-OH at the final concentration of 10 μM. Untreated cells were taken as controls. Other cells were pre-incubated (1 h) with (A) 100 μM NAC or (B) 50 μM DPI and then treated with 27-OH for 3 h. Protein level of Nrf2 was analyzed by Western blotting. Actin was used as loading control. One blot representative of three experiments is shown. Histograms represent the mean values ± S.D. of all three independent experiments; *p < 0.05 and **p < 0.01 vs. control group; #p < 0.05 and ##p < 0.01 vs. 27-OH.

Fig. 6. Evaluation of apoptosis by DAPI staining. U937 cells were transfected with a specific Nrf2 siRNA for 24 h and then incubated with 10 μM 27-OH for 24 h. Apoptotic cells were evaluated in terms of DAPI staining and examined using a fluorescence microscope (Zeiss Axiovert 200 M) with an ultraviolet filter and a 20x/0.30 lens.

Fig. 7. Effect of high concentrations of 27-Hydroxycholesterol (27-OH) on generation of intracellular ROS, activation of MEK-ERK/PI3K-Akt pathway and Nrf2.

(A) Unremitting prooxidant effect of 100 μM 27-OH. ROS generation was monitored by dihydroethidium (DHE) staining in U937 cells incubated with the oxysterol from 1 up to 24 h. Untreated cells (Control) were taken as controls**.** (B) Effect of high 27-OH concentrations on ERK1/2 and Akt phopshorylation. Levels of phosphorylated ERK1/2 and phosphorylated Akt in U937 cells treated with 100 μM 27-OH from 1 to 72 h, were analyzed by Western blotting. Untreated cells (c) were taken as controls. Actin was used as loading control (C) Effect of 100 μM 27-OH on Nrf2. Total protein levels of Nrf2 were analyzed by Western blotting. Actin was used as loading control. (D) Nrf2 levels in nuclear and cytoplasmic lysates were determined by Western blotting. Lamin A/C and actin are used as loading controls. One blot representative of three experiments is shown. Histograms represent the mean values ± S.D. of three independent experiments; densitometric measurements were normalized against the corresponding actin or lamin levels and expressed as percentage of control values; *$p < 0.05$.

Fig. 8. Schematic flow-sheet of 27-hydroxycholesterol-induced Nrf2 modulated survival signaling.

Supplementary figure 1. Effect of 27-hydroxycholesterol (27-OH) on the expression and synthesis of Nrf2.

(A) Gene expression was quantified by real-time RT-PCR in HUVEC treated for 1 and 3 h with 10 or 100 μM 27-OH. Untreated cells were taken as control. Data, normalized to actin, are expressed as mean values of two different experiments.

(B) Nfr2 protein levels were analyzed by Western blotting in HUVEC treated for 1 and 3 h with 10 or 100 μM 27-OH. Untreated cells were taken as control. Top: blot representative of two experiments. Bottom: histogram representing mean values of two different experiments. Nrf2 densitometric measurements were normalized against the corresponding actin levels and expressed as percentage of control value.

Fig. 1

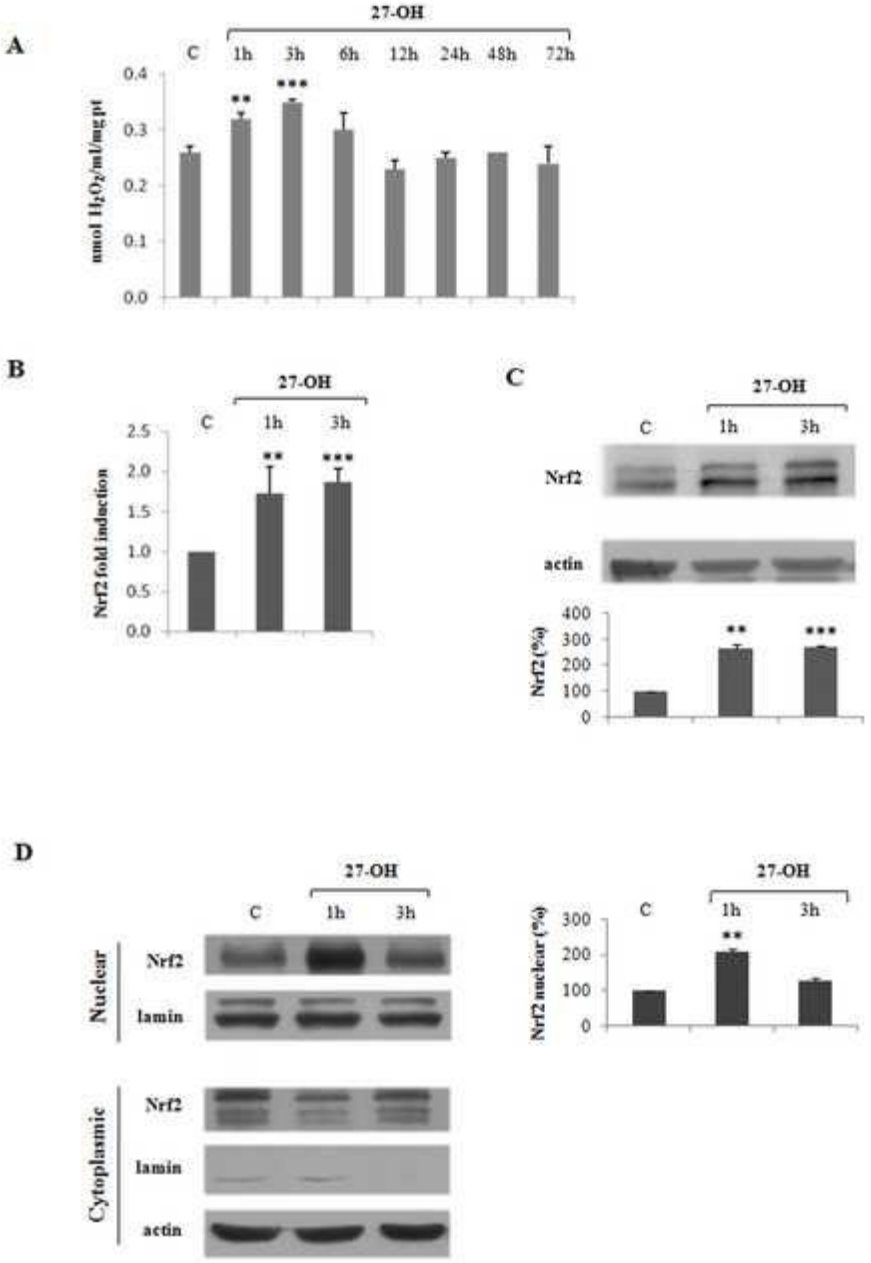
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Fig. 2

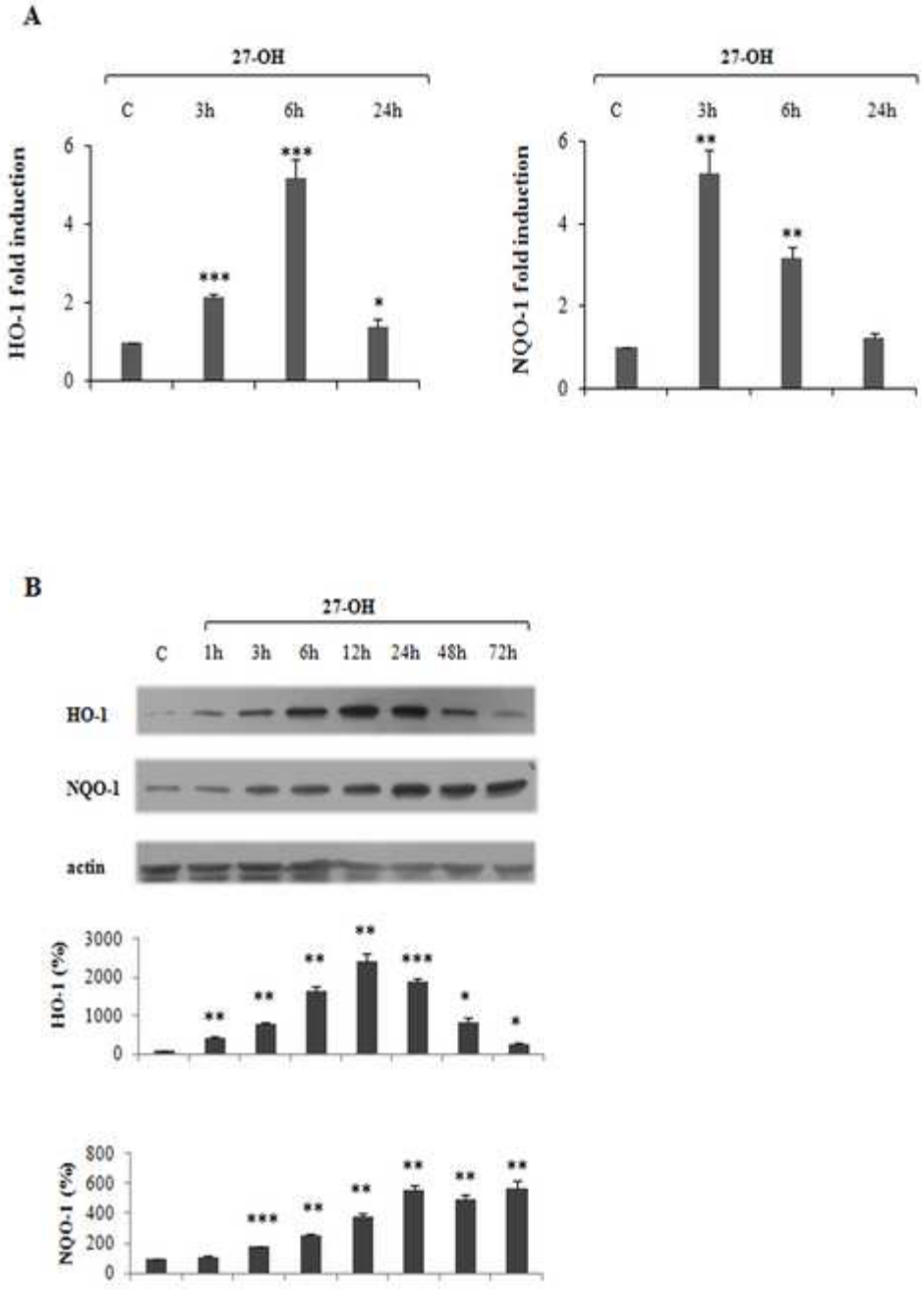
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Fig. 3

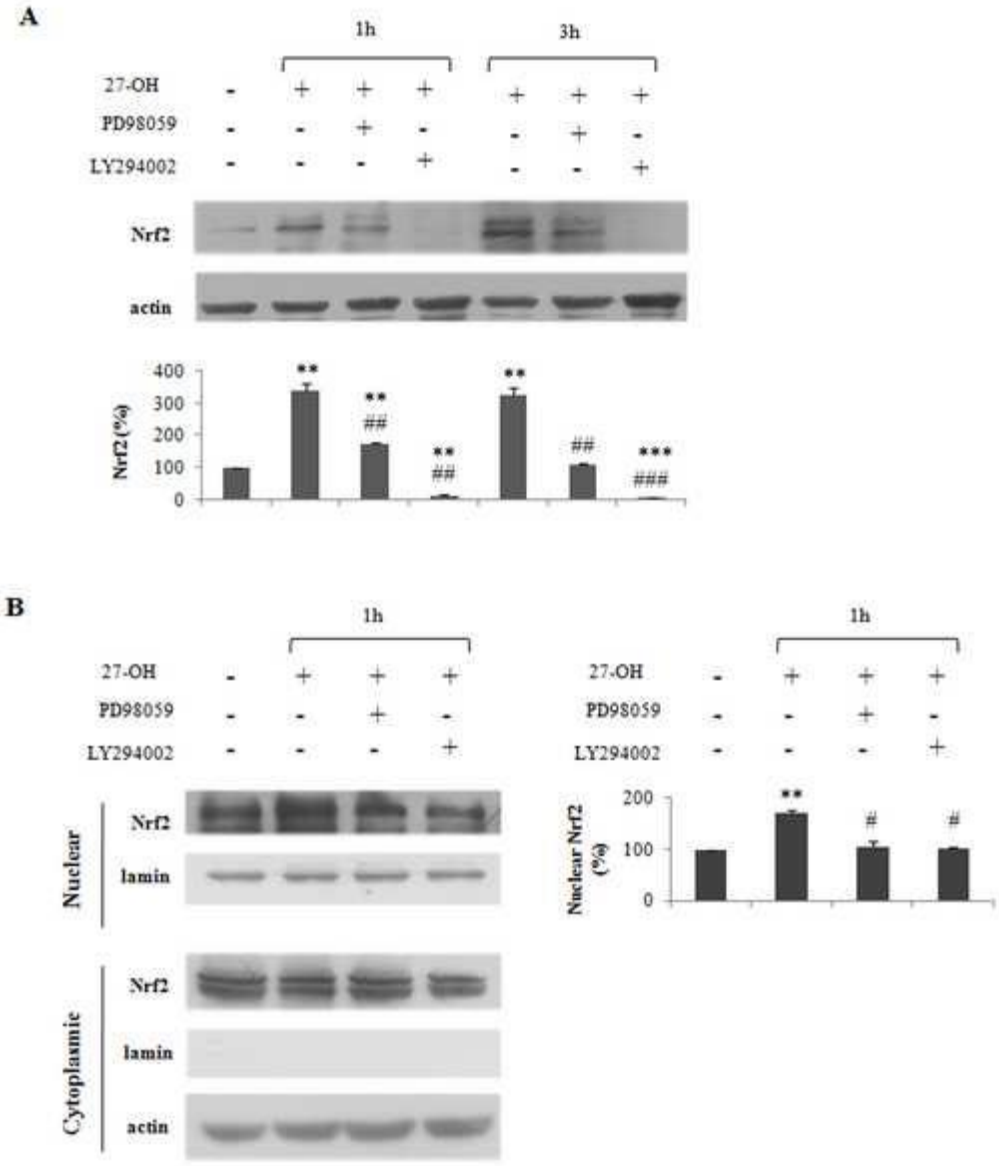
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Fig. 4

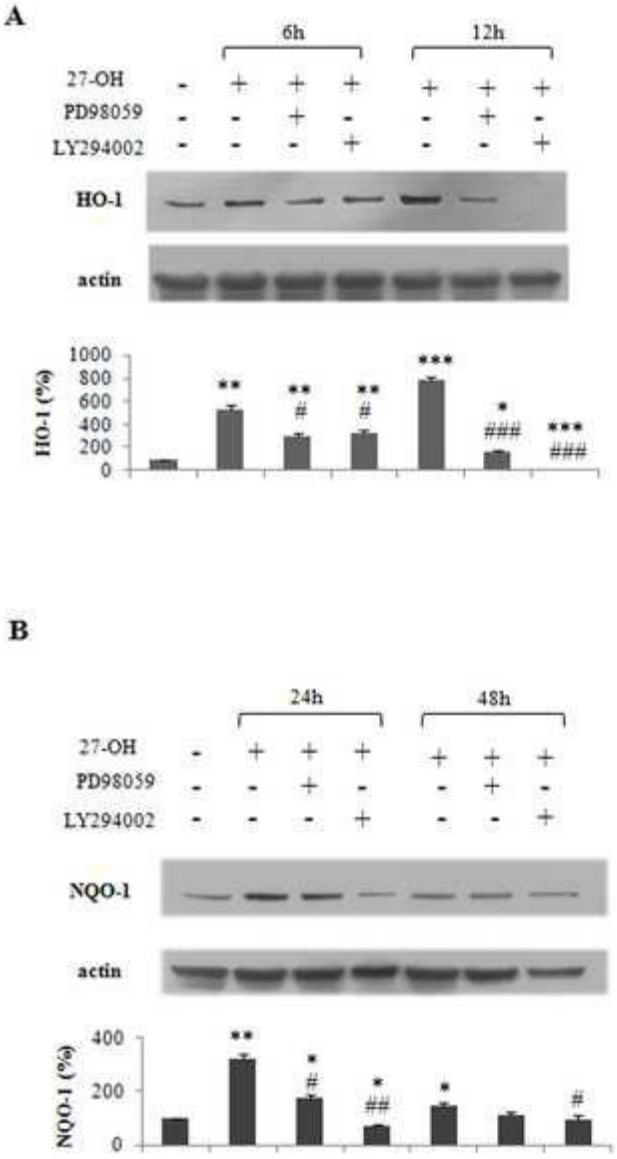
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Fig. 5

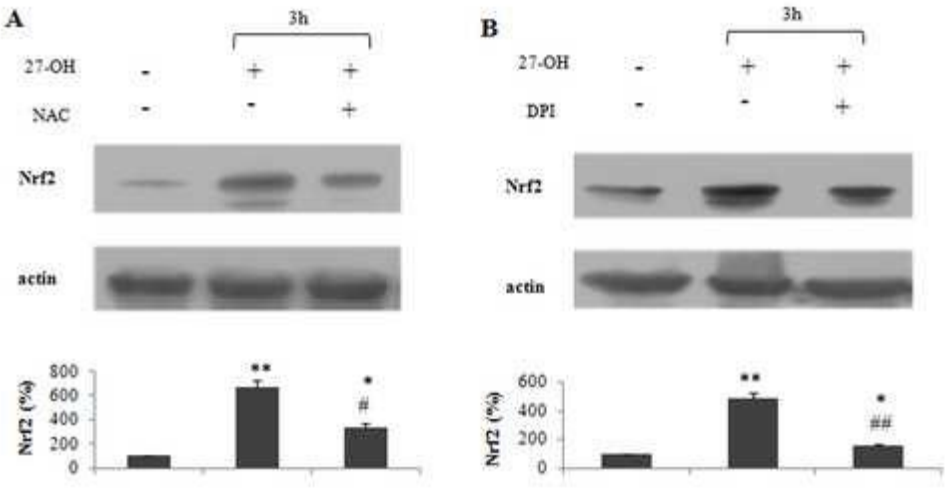
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Fig. 6

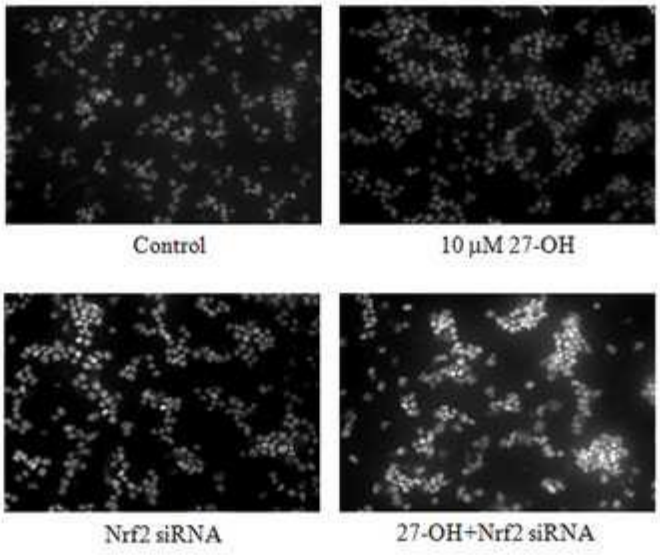
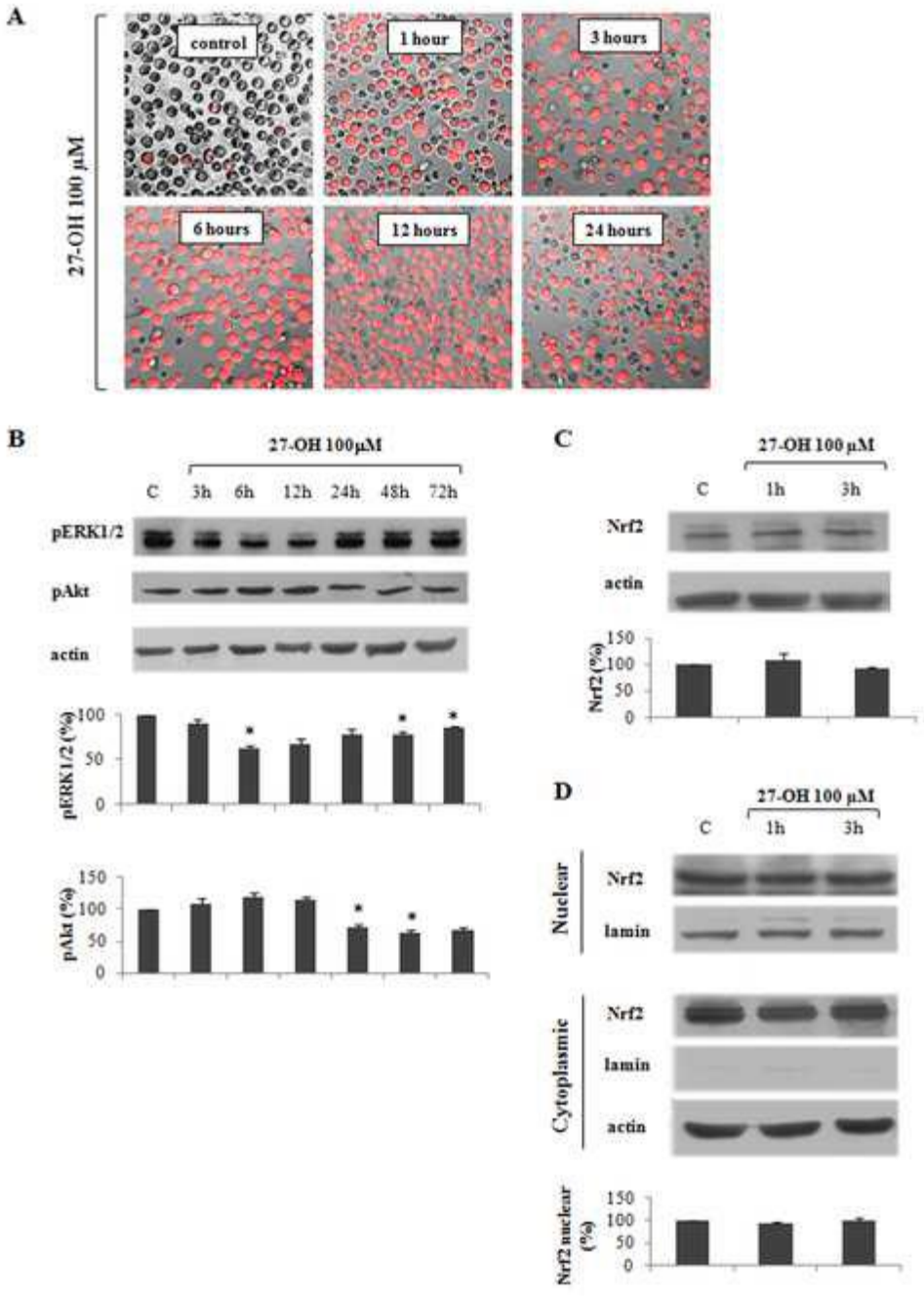


Fig. 7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actin
pERK1/2 (%)
pAkt (%)
C
27-OH 100 μM
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actin
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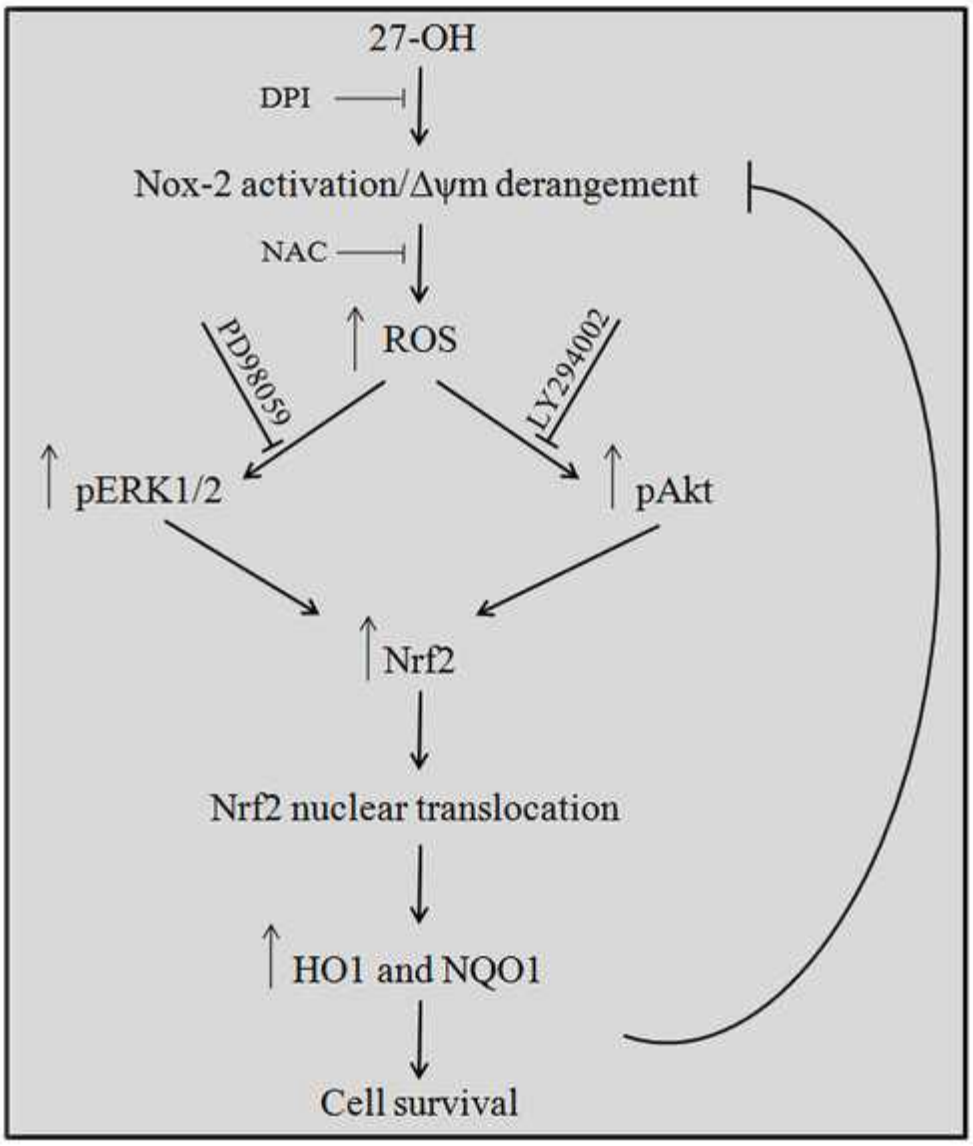
actin
Nrf2 nuclear (%)

Fig. 8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Nrf2
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HO1 and NQO1
Cell survival

Supplementary Fig. 1