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Effect of 27-hydroxycholesterol on survival and death of human macrophages and vascular smooth muscle cells

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Abstract
The objective was to compare the effect of a LXR synthetic ligand (T0901317) on cell viability and lysosomal membrane destabilization in human U937 macrophage and aortic smooth muscle cell (HASMC) incubated in the presence of cholesterol or 27-OH and to verify whether the Akt signalling pathway is involved. In U937 macrophages, cholesterol triggered cell survival while 27-OH triggered either survival (low concentration) or a lysosomal independent apoptosis (high concentration). Despite a strong effect of T0901317 on macrophage survival, its effect on cell viability is hampered in cells incubated in the presence of cholesterol or 27-OH. In these cells, cholesterol triggers the phosphorylation of Akt on the Thr308 residue. In HASMC, cholesterol induced apoptosis but no additionnal effect of T0901317 prevented apoptosis. All together, cell survival triggered by LXRs is impaired in the presence of cholesterol or high concentrations of 27-OH in human U937 macrophages and is not effective in HASMC.

Keywords: *27-Hydroxycholesterol, cellular survival/death, human macrophages, vascular smooth muscle cells, LXR*

Introduction

Cardiovascular diseases are the leading cause of mortality in industrialized countries. Atherosclerosis, a component of the cardiovascular diseases [1], is a chronic inflammatory disease associated with lipid disorders with the consequence of complex disturbances on cellular and molecular events within vascular cells. Atherogenesis is initiated by monocyte-derived macrophages that infiltrate and take place beneath the vascular wall before maturing into foam cells. These cells are characterized by an important accumulation of lipids including oxygenated metabolites of cholesterol called oxysterols [2]. However, macrophages are not the only cell types that mature into foam cells. As a balance between macrophage apoptosis and phagocytic clearance of apoptotic bodies is an hallmark of former stages of atherogenesis [3], the latter stages are characterized by chronic local inflammation in the vascular wall that leads to migration and proliferation of vascular smooth muscle cells (VSMC). Ultimately, these cells will mature into foam cells and be involved in the development of the lipid core and in the stabilization of the atherosclerotic plaque [4].

Oxysterols are bioactive derivates of cholesterol that accumulate in the necrotic core [5] of the atherosclerotic plaque and are recognized to trigger cellular perturbations within macrophages [6]. Among the different type of oxysterols, 27-hydroxycholesterol (27-OH) is the most abundant specie observed in blood circulation of healthy volunteers [7], in men at high risk to develop cardiovascular disease [8], in atherosclerotic lesions [9–11] as well as in macrophages isolated from atherosclerotic lesions [12]. This oxysterol has the ability to activate the liver

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X receptor (LXR) [13,14] that is a nuclear receptor involved in mice macrophage survival by triggerring expression of the apoptosis inhibitor factor AIM/Spα/Api6 [15,16]. The AIM/Spa/Api6 is absent in human U937 macrophages [17]. Thus, as LXR is proposed to trigger mice macrophage survival [15,16], knowledge is lacking in human macrophages. The role of Akt Signaling pathway, an antiapoptotic kinase, has been investigated in human macrophages incubated with 7-ketocholesterol (7KC), a pro-apoptotic oxysterol, that leads to an Akt degradation [18] or dephosphorylation on residues Ser473 [19] and Thr308 [20]. In contrast, CRP has been shown to enhance LXRα protein levels and Akt phosphorylation in THP-1 cells, suggesting that the CRP, a non-specific inflammation marker, can trigger simultaneously LXR and Akt signaling in human macrophages [21]. However, the role of LXR on cell viability of human vascular cells is lacking. To the best of our knowledge, the effect of LXR activation by T0901317 on the cellular viability of human macrophages and vascular smooth muscle cells incubated in the presence of lipids associated to atherosclerotic plaque formation, such as cholesterol and 27-OH, has never been tested simultaneously.

Thus, this study aims to verify whether the activation of LXRs by the synthetic ligand T0901317 has the same effect on the survival/death of human macrophages and aortic smooth muscle cells incubated in the presence of cholesterol or 27-OH and to start to characterize whether the Akt signalling pathway is involved.

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Materials and methods

Reagents

Cholesterol and 27-hydroxycholesterol were purchased from Steraloids (Newport, RI) and suspended in ethanol. Phorbol 12-myristate 13-acetate (PMA) and LXRs ligand T0901317 were purchased from Axxora, LCC (San Diego, CA) and suspended in DMSO. RPMI-1640 media was purchased from thermo Scientific Hyclone (Fisher Scientific, Ottawa, ON, Canada) and Medium 231, SMGS and SMDS were purchased from Invitrogen (Burlington, ON, Canada). All fluorescent dyes were purchased from Molecular Probes Inc (Eugene, OR). Antibodies for Akt were purchased from Cell signaling (Danvers, MA), whereas CD36 and smooth muscle cell α-actin antibodies were from Abcam (Cambridge, MA) and α-Tubulin antibody from Sigma (Oakville, ON, Canada).

U937 and HASMC cell culture and treatment

Human leukaemic monocyte lymphoma cell line U937 purchased from ATCC (American type culture Collection, Manassas, VA) were maintained in suspension culture in RPMI-1640 media supplemented with 10% foetal bovine serum in an atmosphere of 5% CO_2 at 37°C. Monocytes were differentiated into adherent macrophages using PMA (60 ng/ml) for 24 h.

Human aortic smooth muscle cells HASMC purchased from Invitrogen (Burlington, ON, Canada) were proliferated in Medium 231 supplemented with Smooth Muscle Growth Supplement (SMGS) that contained foetal bovine serum (4.9%), human basic fibroblast growth factor (2 ng/ml), human epidermanl growth factor (0.5 ng/ml), heparin (5 ng/ml), insulin (5 μg/ml) and BSA (0.2 μg/ml) in an atmosphere of 5% CO_2 at 37°C, as described by the manufacturer. HASMC were differentiated using Medium 231 supplemented with the Smooth Muscle Differentiation Supplement (SMDS) that contained foetal bovine serum (1%) and heparin (30 μg/ml) for 48 h. During differentiation, HASMC undergo cessation of growth and a change in cellular morphology from slender stellate cells to enlarged rectangular or triangular shaped cells. Cells used for differentiation underwent a maximum of 12 passages.

Differentiated human U937 macrophages and HASMC were incubated in the absence or in the presence of either cholesterol or 27-OH at various concentrations (0.002–0.04 mg/ml) [22]. As positive control, U937 macrophages were also incubated in presence of 0.01 mg/ml of 7-ketocholesterol (7KC) or 7β-hydroxycholesterol (7β-OH) for 12 hours. For U937 macrophage-differentiated cells treated with cholesterol, oxysterol and LXRs ligand, the macrophages serum free medium/MSFM (Burlington, ON, Canada) will be used to minimize interference of fatty acids found in bovine serum [23] while HASMC were cultured in fresh SMDS. Cholesterol- or oxysterol-loaded cells were then treated in the absence or presence of vehicle (DMSO, final concentration 1/10 000) or LXRs ligand (T0901317 used at 5 μM, $EC_{50} = 50$ nM) for 18 h. All experiences were performed in triplicate. As differentiated HASMC were cultured in 1% foetal bovine serum, a negligeable interference of fatty acids was expected.

Cytometry

Annexin V R-phycoerythrin conjugate (AV) and 7-Amino-Actinomycin D (7AAD) fluorescent dyes were used to determine the relative proportion of viable (AV negative and 7AAD negative), early apoptotic (AV positive and 7AAD negative) and advanced apoptotic (AV positive and 7AAD positive) cells using an Epics Elite EST flow cytometer (Beckman-Coulter) sorting on 200 000 cells. Simultaneously, according to a modified protocol described by Suarez et al. [24], the lysosomal membrane destabilization was measured using the lysosome-specific dye Lysotracker green DND-26

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(DND26) to determine the relative proportion of live cells with intact lysosomal membrane (DND26 positive and 7AAD negative), live cells with dommaged lysosomal membrane (DND26 negative and 7AAD negative) and dead cells with intact lysosomal membrane (DND26 positive and 7AAD positive). For the effect of cholesterol and 27-OH concentrations on survival/death and lysosomal membrane destabilization, data were expressed as the proportion of cells relative to control (without any treatment) set to one. For the effect of LXRs ligand, data were expressed as the proportion of cells relative to the corresponding oxysterol concentration used alone set to one.

Western blot analysis for determination of expression of CD36 and AKT/PKB

To measure protein abundance, cells were washed in PBS twice, suspended in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EGTA, 20 mM β-glycerophosphate, 1% NP-40, 1/1000 protease inhibitor, 10 mM NaF and 2 mM Na_3VO_4) and placed on ice. Total protein content was determined using BCA assay kit and read on a plaque reader 1420 multilabel counter victor V3 (Perkin Elmer, MA) at 562 nm. A total of 25 µg of protein was resolved by 10% SDS-PAGE, transfered to nitrocellulose membrane and analysed by Western blot. Blots were blocked with 5% non-fat dry milk in 10 mM Tris, pH 7.5, 150 mM NaCl, 0.004% Igepal, 0.004% Np-40 and 0.02% Tween 20, incubated with the appropriate primary antibody overnight at 4°C (in 5% dry milk or 3% BSA) for 2 h and then incubated with second horseradish-conjugated anti-mouse or anti-rabbit antibody (1/10 000 dilution) for 1 h at room temperature. Detection was performed using ECL western blotting detection reagent (Amersham, QC, Canada), detected with the Typhoon Trio Variable Mode Imager (Amersham, QC, Canada) and quantified by ImageQuant TL software. Relevant primary antibodies used were anti-Akt (1:1000 dilution) ; anti-phospho-Akt(Ser473); anti-phospho-Akt(Thr308); anti-CD36 (1:1000 dilution) as a marker of macrophage differentiation; anti-smooth muscle α-actin (1:1000 dilution) as internal control for HASMC; and anti-α-tubulin (1:1000 dilution) as internal control for U937 macrophages. Data were expressed as relative to internal controls α-tubulin (U937 macrophages) or smooth muscle cell α-actin (HASMC) set to one. Phosphorylated protein abundances were expressed as relative to unphosphorylated protein. For the effect of LXRs ligand, data were expressed as the protein abundance relative to the corresponding oxysterol concentration used alone set to one.

Statistical analysis

Statistically significant differences between control and different concentrations of oxysterols/drugs were determined by analysis of variance (ANOVA). Subsequently, *t*-test was performed to assess pair differences. Differences were considered to be statistically significant at $p < 0.05$ using S.A.S. 8.12.

Results

To validate human U937 macrophages differentiation, the protein abundance of CD36 was measured. The protein abundance of CD36 exhibited a 2.36 ± 0.16-fold increase folowing PMA differentiation as compared to its abundance in monocytes ($p = 0.01$). HASMC differentiation were confirmed by the expression of α-actin, as illustrated in Figure 6C and D.

T0901317 induces survival in unloaded human U937 macrophages

Following PMA-induced differentiation of monocytes into macrophages, 55% of U937 macrophages were viable, while 10% and 35% of them were in early and advanced apoptosis, respectively (Figure 1A). Following a treatment with the LXRs ligand T0901317 for 6, 12, 18 and 24 h, the relative proportions of viable cells were significantly increased by 62% ($p = 0.0005$), 73% ($p = 0.01$), 64% ($p = 0.0003$) and 46% ($p = 0.0009$), respectively. For the following experiences, an incubation time of 18 h was used to allow sufficient time to detect any changes in protein abundance.

7KC and 7β-OH induce cell death

In U937 macrophages treated with 7KC, the relative proportion of early apoptotic cells was significantly reduced ($p = 0.10$) in favor of advanced apoptotic cells ($p = 0.03$) (Figure 1C) with a significant increase ($\sim 20\%$) in the proportion of dead cells with intact ($p = 0.03$) or damaged ($p = 0.03$) lysosomes (Figure 1D). In presence of 7β-OH, cell survival is significantly reduced (p=0.02) in favor of advanced apoptosis ($p = 0.01$) (Figure 1C) with a reduced proportion of viable ($p = 0.01$) or dead cells ($p = 0.01$) with intact lysosomes (Figure 1D). These results confirm that in U937 macrophages, and not in U937 monocytes as previously described [6], a treatment by 7KC or 7β-OH triggers cell apoptosis with (7KC) or without (7β-OH) a destabilization of lysosomal membranes.

At low concentration, 27-OH induces cell viability, whereas at higher concentration it induces lysosomal independent cell death in human U937 cells

In contrast to cholesterol, for which the effect was concentration-independent, the effect of 27-OH on

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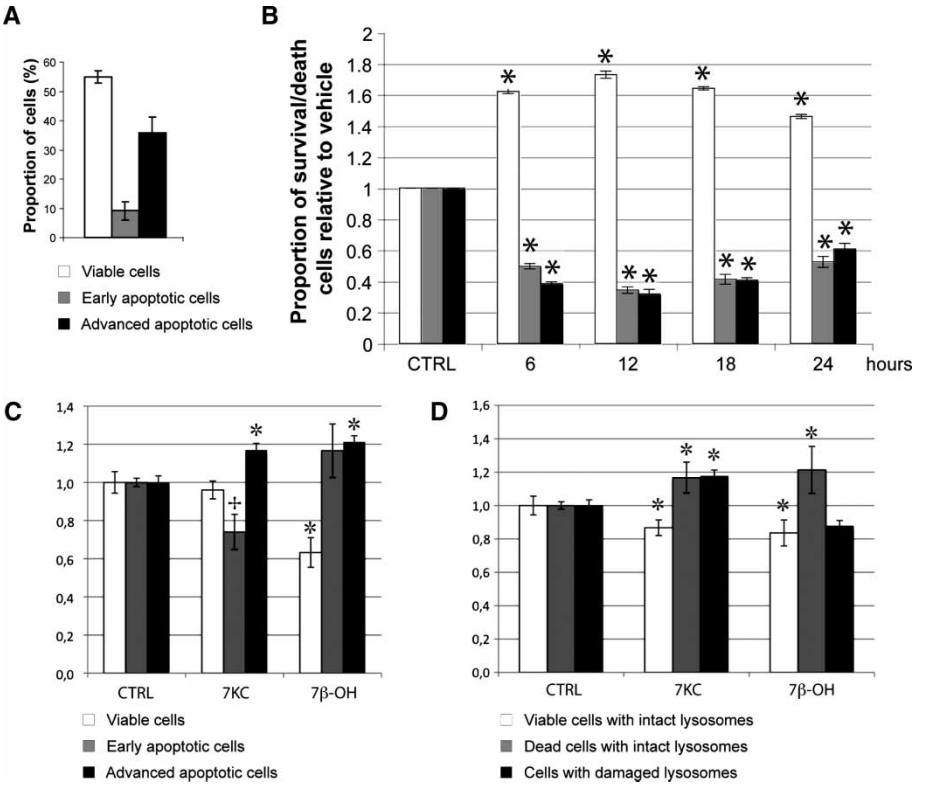

Figure 1. Effect of a 24 hours treatment of PMA (A), time on treatment with T0901317 (B) and effect of 7KC and 7β-OH on survival/death (C) and on lysosomal destabilization (D) in U937 cells. In panel A, the relative proportion of survival and apoptotic cells were measured using flow cytometry and fluorescent dyes as described in the Material and Methods. Experiences were performed in triplicate and data expressed as mean ± SEM. In panel B, C and D, the relative proportion of survival and apoptotic cells were measured as in panel A and differences relative to control set to one were tested using *t*-test, B: $0.10 < p < 0.5$; ✻: $p < 0.05$.

U937 macrophages appeared to depend on the concentrations used. Indeed, at low concentrations, the 27-OH triggered significantly cell viability (0.002 mg/ml: $p = 0.03$ and 0.01 mg/ml: $p = 0.05$) and decreased significantly advanced apoptosis (0.002 mg/ml: $p = 0.001$ and 0.01 mg/ml: $p = 0.008$),

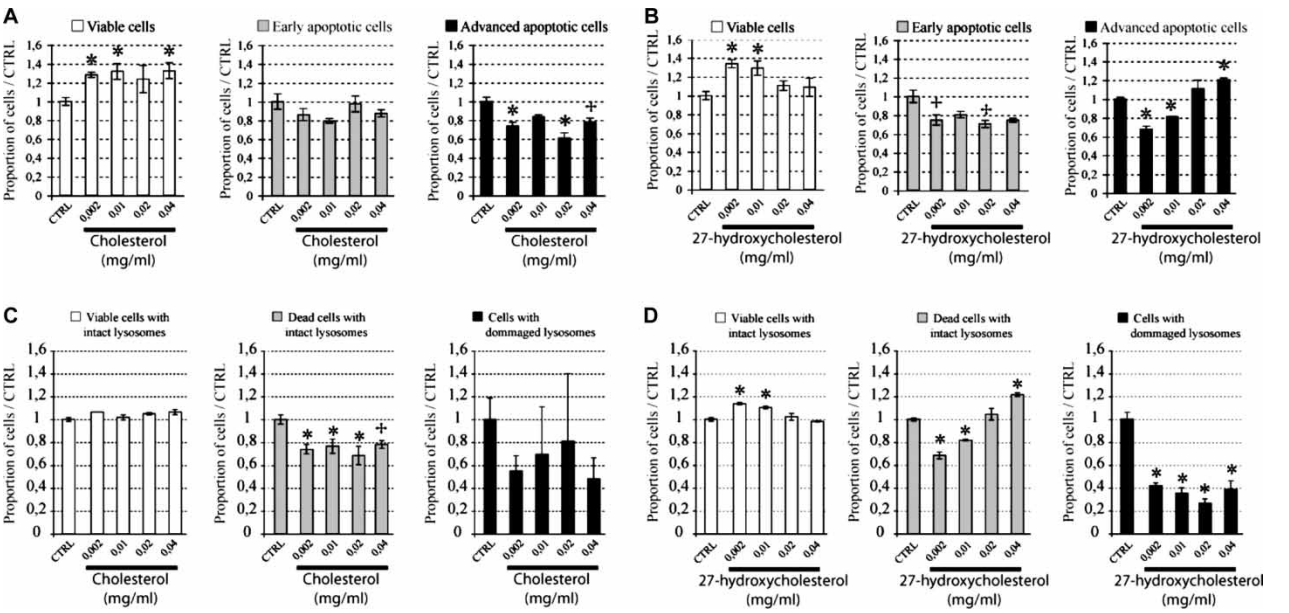

Figure 2. Relative proportion of viable and apoptotic macrophages (A and B) and of cells with destabilized lysosomal membranes (C and D) following cholesterol (A and C) or 27-OH (B and D) incubation. The relative proportion of survival and apoptotic cells as well as cells with destabilized lysosomal membranes were measured using flow cytometry and fluorescent dyes as described in Materials and Methods and expressed as relative to control set to one. Experiences were performed in triplicate and data expressed as mean ± SEM. Differences relative to control were tested using *t*-test, ✢: $0.10 < p < 0.5$; ✻: $p < 0.05$.

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while at greater concentrations it triggered significantly advanced apoptosis (0.04 mg/ml: $p = 0.002$) (Figure 2B). The effect of 27-OH on lysosomal membrane was of more importance than cholesterol itself. As a matter of fact, low concentrations of 27-OH were significantly in favour of viable cells with intact lysosomal membranes (0.002 mg/ml: $p = 0.003$ and 0.01 mg/ml: $p = 0.008$), while at greater concentrations, apoptotic cells (advanced apoaptotic cells) exhibited intact lysosomal membranes (0.04 mg/ml: $p = 0.0006$) (Figure 2D). The effect of this oxyterol on dead cells with damaged lysosomal membrane was significantly reduced by at least a factor of 2 (0.002 mg/ml: $p = 0.04$; 0.01 mg/ml: $p = 0.01$; 0.02 mg/ml: $p = 0.01$; and 0.04 mg/ml: $p = 0.009$).

In HASMC, cholesterol triggers apoptosis with destabilized lysosomal membrane while 27OH induces lysosomal independent apoptosis

In contrast to U937 macrophages, cholesterol triggered significantly early apoptosis (0.01 mg/ml: $p = 0.01$ and 0.04 mg/ml: $p = 0.08$) in HASMC (Figure 3A) that appeared to be associated with a destabilization of lysosomal membranes at high concentrations (0.04 mg/ml: $p = 0.03$) (Figure 3C). The effect of 27-OH in these cells is also very different to the effect observed in macrophages. Indeed, 27-OH triggered a significant increase in the relative proportion of advanced apoptotic cells (0.01 mg/ml: $p = 0.07$ and 0.02 mg/ml: $p = 0.02$) (Figure 3B) while the proportion of dead cells with intact lysosomal membranes was increased (0.02 mg/ml: $p = 0.06$) in favour of a decrease in the proportion of dead cells with damaged lysosomal membranes (Figure 3D). As observed in U937 macrophages incubated in the presence of 27-OH, the proportions of dead cells with damaged lysosomal membrane were significantly reduced by a factor of 2 (0.002 mg/ml: $p = 0.02$; 0.01 mg/ml: $p = 0.07$; 0.02 mg/ml: $p = 0.02$; and 0.04 mg/ml: $p = 0.07$).

Effect of an activation of LXRs on viability of human U937 macrophages incubated in the presence of cholesterol or 27-OH

The activation of LXRs by the ligand T0901317 in human U937 macrophages incubated in the presence of low or high cholesterol concentrations had no significant effect on cell viability (Figure 4A) and lysosomal membrane destabilization (Figure 4C). However, activation of LXRs in human U937 macrophages incubated in the presence of low concentrations of 27-OH increased significantly the proportion of cells in advanced apoptosis (0.01 mg/ml: $p = 0.06$) (Figure 4B) and significantly increases the proportion of dead cells with intact lysosomes (0.09 mg/ml: $p = 0.09$) (Figure 4D).

Effect of an activation of LXRs on viability of HASMC incubated in the presence of cholesterol or 27-OH

In HASMC incubated in the presence of cholesterol, the activation of LXRs by the T0901317 compound had no significant effect on cell viability (Figure 5A) and lysosomal membrane destabilization

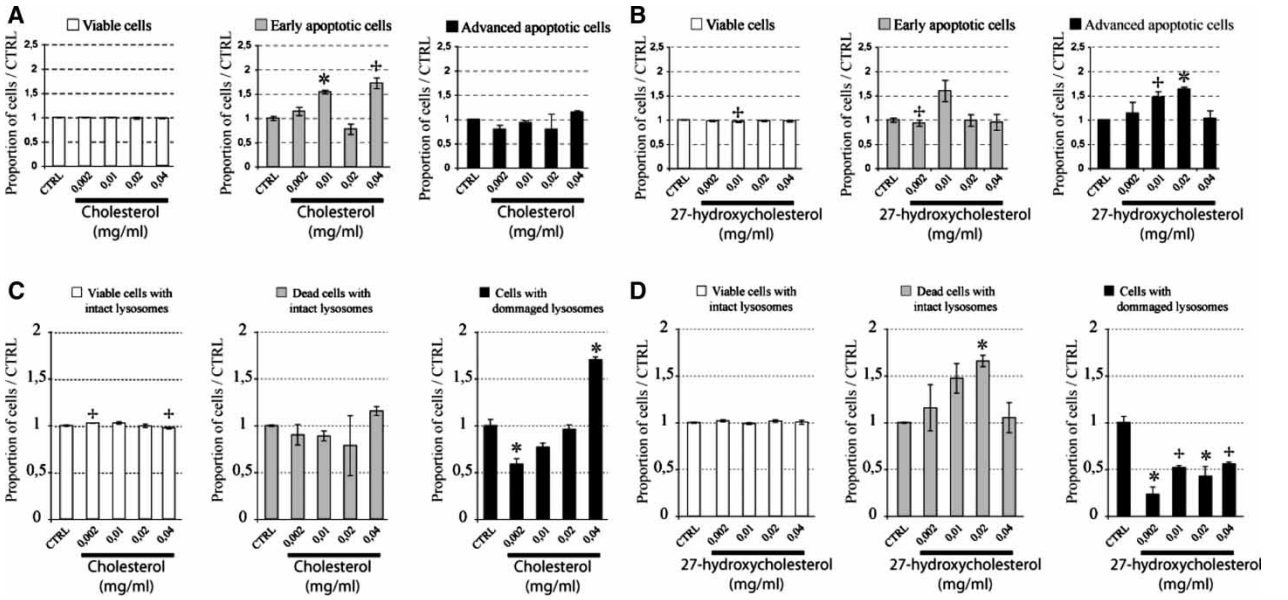

Figure 3. Relative proportion of viable and apoptotic HASMC (A and B) and of cells with destabilized lysosomal membranes (C and D) following cholesterol (A and C) or 27-OH (B and D) incubation. The relative proportion of survival and apoptotic cells as well as cells with destabilized lysosomal membranes were measured using flow cytometry and fluorescent dyes as described in Materials and Methods and expressed as relative to control set to one. Experiences were performed in triplicate and data expressed as mean ± SEM. Differences relative to control were tested using *t*-test, ⊹: $0.10 < p < 0.5$; ✻: $p < 0.05$.

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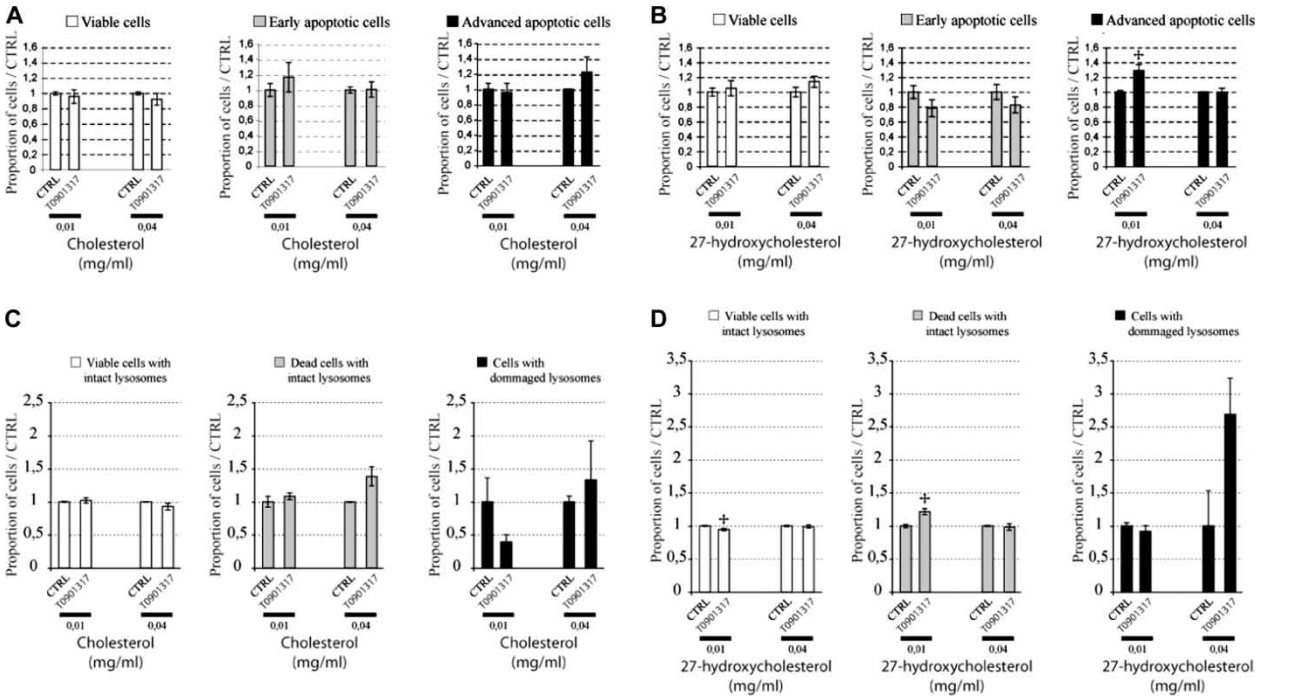

Figure 4. Relative proportion of viable and apoptotic macrophages (A and B) and of cells with destabilized lysosomal membranes (C and D) following cholesterol (A and C) or 27-OH (B and D) incubation followed by T0901317 treatment. The relative proportion of survival and apoptotic cells as well as cells with destabilized lysosomal membranes were measured using flow cytometry and fluorescent dyes as described in Materials and Methods and expressed as the proportion of cells relative to the corresponding oxysterol concentration used alone set to one. Experiences were performed in triplicate and data expressed as mean ± SEM. Differences relative to control were tested using *t*-test, ✝: $0.10 < p < 0.5$; ✱: $p < 0.05$.

(Figure 5C). However, in the presence of 27-OH, the activation of LXRs triggered significantly a 2-fold decrease in the proportion of advanced apoptotic cells (0.01 mg/ml: $p = 0.05$ and 0.04 mg/ml: $p = 0.07$) (Figure 5B) with intact lysosomal membrane (0.01 mg/ml: $p = 0.07$) (Figure 5D).

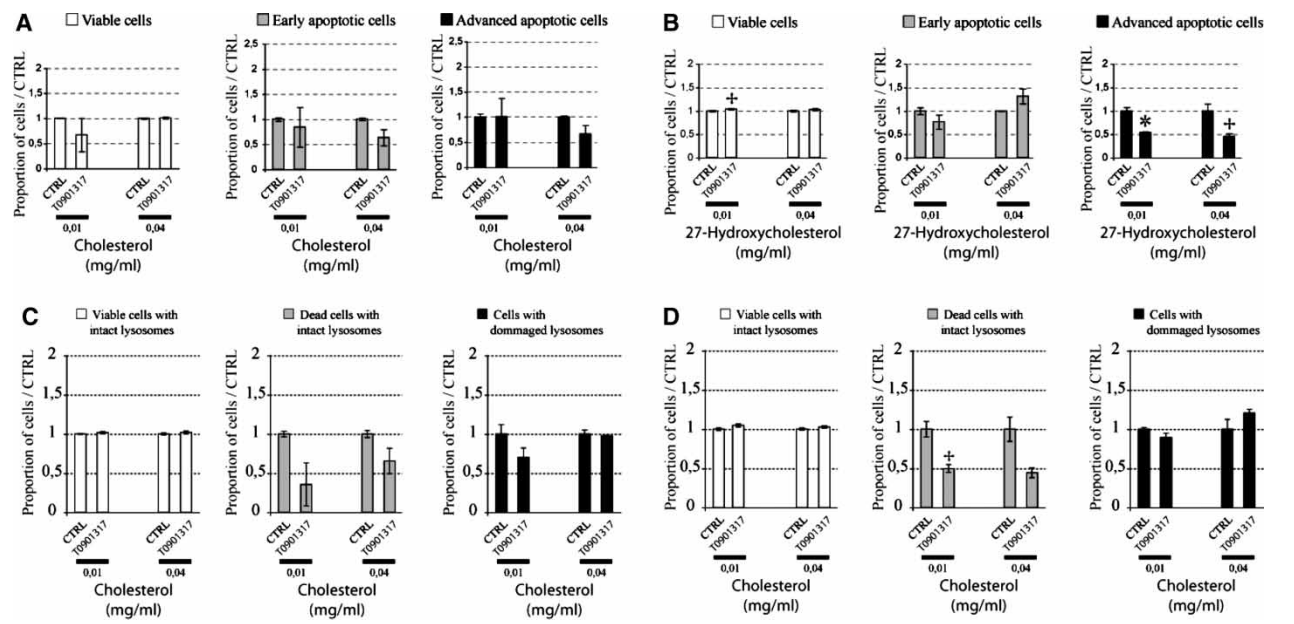

Figure 5. Relative proportion of viable and apoptotic HASMC (A and B) and of cells with destabilized lysosomal membranes (C and D) following cholesterol (A and C) or 27-OH (B and D) incubation followed by T0901317 treatment. The relative proportion of survival and apoptotic cells as well as cells with destabilized lysosomal membranes were measured using flow cytometry and fluorescent dyes as described in Materials and Methods and expressed as the proportion of cells relative to the corresponding oxysterol concentration used alone set to one. Experiences were performed in triplicate and data expressed as mean ± SEM. Differences relative to control were tested using *t*-test, ✝: $0.10 < p < 0.5$; ✱: $p < 0.05$.

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Effect of cholesterol, 27-OH and T0901317 on Akt signalling pathway

To verify whether cell viability is associated with activation of the Akt signalling pathway, protein abundance of Akt and its phosphorylation at residues Thr308 and Ser473 have been determined by western blotting. The protein abundances of Akt and its phosphorylated residue Thr308 were increased in human U937 macrophages incubated in the presence of increasing concentrations of cholesterol (Figure 6A). The activation of LXR by the ligand T0901317 had no additional affect. In the presence of 27-OH, both Thr308 and Ser473 residues were less phosphorylated, but Akt protein abundance was unchanged (Figure 6B). In the presence of 27-OH, the LXR ligand T0901317 enhanced the abundance of Akt protein and its phosphorylation at residue Thr308 only at high concentrations of 27-OH.

The protein abundance of Akt and its phosphorylated residue Thr308 did not change in HASMC incubated in the presence of increasing concentrations of cholesterol (Figure 6C), whereas the phosphorylation of Ser473 was increased in a cholesterol concentration sensitive manner. Addition of T0901317 triggered an additional increase in the phosphorylation of the Ser473 residue. In the presence of 27-OH, the Akt protein abundance and its phosphorylation residue were unchanged (Figure 6D). In HASMC incubated in the presence of 27-OH and treated with T0901317, the Akt residue Ser473 was more phosphorylated when cells were incubated in the presence of high concentrations of 27-OH.

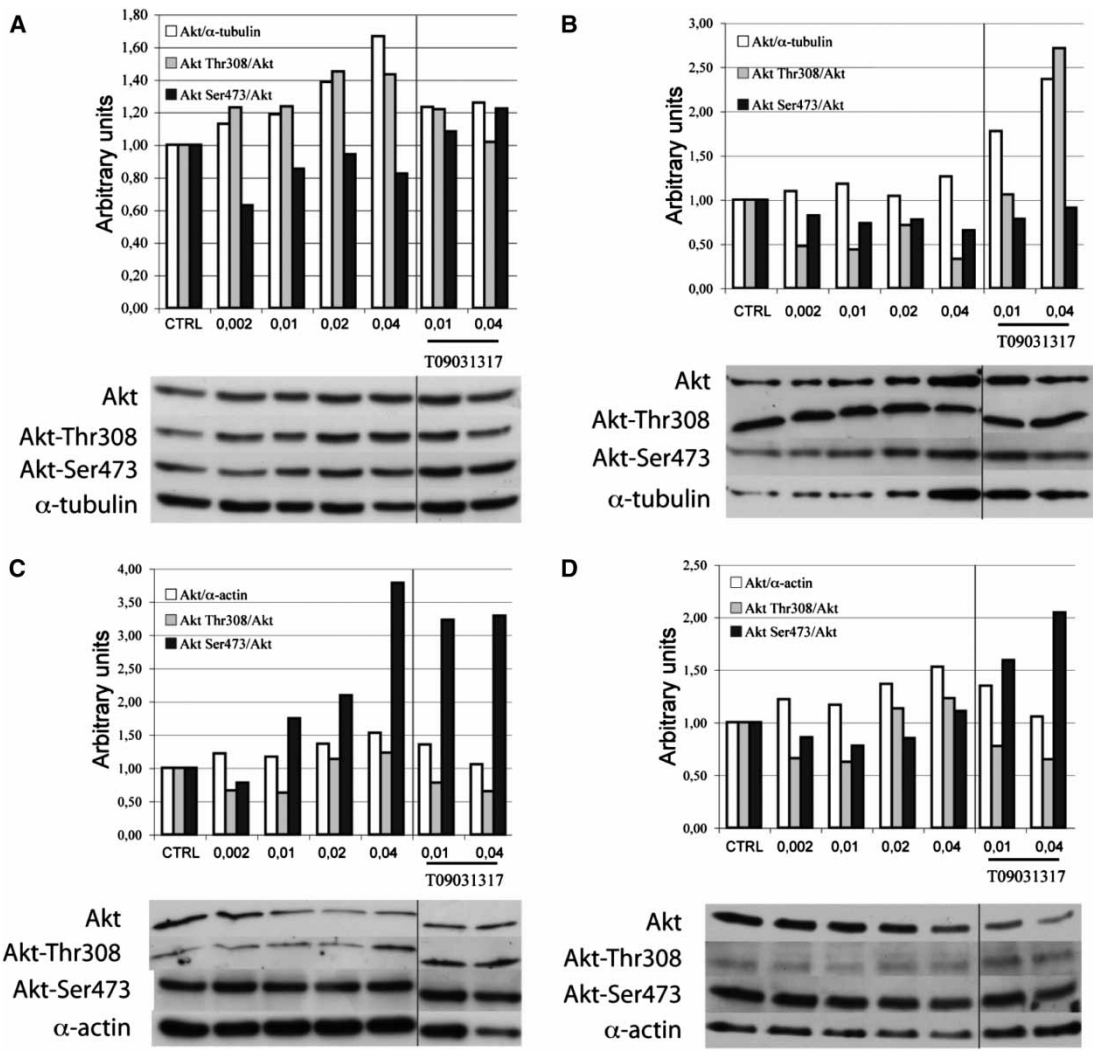

Figure 6. Protein abundance of Akt and its phosphorylation sites Thr308 and Ser473 in human U937 macrophages (A and B) and HASMC (C and D) incubated in the presence of cholesterol (A and C) or 27-OH (B and D). In addition, proteins abundances were detected in cells incubated in the presence of cholesterol or 27-OH followed by T0901317 treatment. The protein abundances were measured using Western Blot as describd in Materials and Methods and expressed as relative to control set to one. For the effect of LXRs ligand, data were expressed as the protein abundance relative to the corresponding oxysterol concentration used alone set to one.

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Discussion

In early lesions, the balance between macrophage apoptosis and phagocytosis is an important hallmark to overcome the continuous recruitment of macrophages at the atherosclerotic lesion site [3]. However, as atherogenesis progresses, the cellular insults caused by oxydative stress is increasing, leading to an increasing proportion of apoptotic cells that can no longer be cleaned-up by newly arrived macrophages within the atherosclerotic plaque [3]. The resultant effect is that newly arrived macrophages are faced with a 'cellular oxidative insult shock', mainly caused by an increase in the production and accumulation of oxysterols (mainly 27-OH) in the core [5,25] as well as in macrophages [12,26] of the atheroclerotic plaque. This increasing 'cellular oxidative insult shock' will trigger secondary necrosis of macrophages rather than apoptosis [3] and will lead to migration and recruitment of subjacent vascular smooth muscle cells that will later accumulate large amounts of cholesterol [27] and transform into foam cells [28].

The sterol 27-hydroxylase (Cyp27A1), also called 26-hydroxylase in older reports, is responsible for the one-step conversion of cholesterol into 27-OH, previously referred to as 26-OH [29]. In macrophages, intracellular accumulation of cholesterol induces the excretion of 27-OH, whereas the specific inhibition of 27-hydroxylase causes intracellular cholesterol accumulation. These observations suggest that 27-hydroxylase plays an important role in the elimination of extrahepatic cholesterol through the production of 27-OH [30]. Indeed, this oxysterol hydroxylated at position C27 provides an alternative mechanism to prevent intracellular cholesterol accumulation, as compared with the classical ATP-binding cassette family members-mediated cholesterol efflux [29,31]. Our results are contrasting with previous observations where 28 μmol/L (~ 0.01 mg/ml) of 27-OH alone had no effect on cell survival/death of U937 macrophages [2]. In the present report we observed a concentration effect of 27-OH on cell survival and death (Figure 2). This discrepancy can be explained by technical differences in the media used. While RPMI-1640 supplemented with 10% FBS have been employed by Larsson et al. [2], our results come from macrophages cultured in the macrophages serum-free medium/MSFM used to minimize interference of fatty acids found in bovine serum with T0901317 and oxysterols [23]. However, this media allowed us to mimic conditions of impaired glucose tolerance or type 2 diabetes (hyperinsulinemia and hyperglycemia) according to the concentration of insulin (500 nM) and glucose (17.5 μM) in this medium [32]. Thus, it is possible that 27-OH, the most oxysterol present in atherosclerotic plaque, has no effect on cells cultured in the presence of serum, whereas in the absence of conditions associated to hyperinsulinemia and hyperglycemia this oxysterol has an effect on survival of macrophages. As a matter of fact, it can be speculated that glucose by itself has some effect of the same target of 27-OH, since LXR has been proposed as a glucose sensor and that LXR ligand and glucose can bind LXR simultaneously [33]. Thus, the effect we observed can result from a synergic effect of 27-OH in the presence of glucose. As close to 75% of diabetic subjects (a population-based autopsy study) exhibited vascular complications associated to atherosclerosis development [34] as compared to 8% in normal population (pathology reports) [35]; investigating oxysterol effects on cells cultured in the presence of media that mimic conditions of impaired glucose tolerance is of great importance. In these conditions, we demonstrated that macrophages cultured in the presence of cholesterol are prone to survive through the activation of the Akt signalling pathway (phosphorylation of the Thr308 residue). This Akt phosphorylation was reproduced when both 27-OH and T0901317 were used to treat human macrophages. In contrast, in the presence of 27-OH, only low concentrations triggered survival in an Akt signalling pathway independent manner. At greater concentrations of 27-OH, the induction of apoptosis in macrophages without affecting the stabilization of lysosomal membranes can be explained by the fact that the balance between the beneficial (activation of survival) and adverse effects (induction of oxidative stress) of 27-OH is in favour of adverse effects. By activating LXR by its synthetic ligand, it was impossible to trigger survival at these concentrations, suggesting that adverse effects of 27-OH can't be reversed by T0901317.

In contrast to macrophages, 27-OH does not trigger survival in HASMC bur rather induces cell death without affecting the stabilization of lysosomal membranes. Previous reports demonstrated that cholesterol [36] and oxysterols [37,38] trigger death of vascular smooth muscle cells. Our report suggests that vascular smooth muscle cells located in atherosclerotic plaque in the presence of 27-OH are more prone to die by apoptosis rather than to survive. The VSMC death induction will lead to a destabilization of the atherosclerotic plaque since it will be more prone to rupture [39]. Despite the observation that the Akt signalling pathway is activated through the phosphorylation site Ser473 in the presence of cholesterol, the presence of 27-OH impairs this activation. As for macrophages, the addition of T0901317 in HASMC incubated in the presence of 27-OH no longer activates survival but rather diminishes the proportion of cells in advanced apoptosis.

Thus, treatment of T0901317 appears to be different in macrophages and HASMC incubated in the presence of the principal oxysterol found in atherosclerotic plaque (27-OH), suggesting that it is

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inefficient in macrophages but diminishes vascular smooth muscle cell death and thus improves plaque stabilization, leading to a small protective effect in later atherosclerotic stages. Interestingly, the 27-OH does not trigger lysosomal membrane destabilization, an effect attributed to oxidized low-density lipoprotein [40].

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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