Antiapoptotic and Proliferative Effects of Low Concentrations of 7 β -Hydroxycholesterol in Human Endothelial Cells via ERK Activation

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Key Words

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species

Abstract

The atherogenic potential of oxidized low-density lipoproteins (oxLDL) has been correlated to their 7 β -hydroxycholesterol (7 β OHC) content; oxLDLs have a dual effect on endothelial cell viability, inducing apoptosis or proliferation depending on the concentration. Considering that 7 β OHC is apoptotic for endothelial cells at concentrations ≥ 20 μ g/ml, a study on the effect of lower concentrations of 7 β OHC on human umbilical vein endothelial cells (HUVECs) was undertaken. 7 β OHC (1–10 μ g/ml) increased 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction of growth-factor-deprived HUVECs. This effect was due to the increased cell proliferation, determined by [3 H]thymidine incorporation, and the reduction of apoptosis, revealed by the decreased caspase-3 activation and annexin V staining. 7 β OHC also protected against staurosporine apoptosis. 7 β OHC induced an increase in intracellular ROS antagonized by N-acetylcysteine; however, HUVECs treatment with the antioxidant did not inhibit the effects of 7 β OHC. 7 β OHC produced an increase in extracellular signal-regulated kinase

(ERK) phosphorylation that was blocked by inhibitors of store-operated calcium entry 2-aminoethoxydiphenyl borate and gadolinium. MEK inhibition with PD98059 or U0126 as well as store-operated calcium entry inhibition antagonized the effect of 7 β OHC. The results suggest that 7 β OHC promotes HUVECs survival and proliferation by a mechanism independent of ROS production and involving calcium-dependent activation of ERK.

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Introduction

Oxysterols are oxidized derivatives of cholesterol, formed either by enzymatic or nonenzymatic reactions. Oxysterols represent a class of potent regulatory molecules with diverse, important biological actions. In fact, a large amount of studies have evidenced their role in cholesterol turnover, atherosclerosis, apoptosis, necrosis, inflammation and immunosuppression [reviewed in ref. 1].

It has been suggested that oxysterols play a role in the induction and development of atherosclerosis. Increased oxysterol concentrations have been found in plasma of patients at high risk of cardiovascular disease as well as in macrophage foam cells and atherosclerotic lesions [2–

4]. Furthermore, *in vitro* studies have demonstrated that oxysterols, in particular the derivatives oxidized at C7, are proapoptotic for endothelial cells, vascular smooth muscle cells and monocyte/macrophages [5–8]. The finding that 7-oxysterols such as 7 β -hydroxycholesterol (7 β OHC), 7-ketocholesterol and 7 β -hydroperoxycholesterol predominate in oxidized low-density lipoproteins (oxLDL) and the observation that apoptotic pathways evoked by oxysterols mimic those of oxLDL in various cell types suggested that vascular injury induced by oxLDLs could be due, at least in part, to the presence of 7-oxysterols [9–12].

The atherogenic potential of oxLDLs is linked to their ability to induce endothelial dysfunction and apoptosis, vascular smooth muscle cell proliferation, monocyte chemotaxis and formation of foam cells [11, 13]. Several mechanisms are involved in these effects, reflecting the complex composition of oxLDLs. Nevertheless, some pathways involved in the cytotoxicity of oxLDLs and oxysterols are dependent on reactive oxygen species (ROS) generation [13–17]. It has been demonstrated that oxLDLs have a dual effect on endothelial cell viability depending on their concentration. At low concentrations (5–10 μ g/ml), oxLDLs induce cell proliferation while at high concentrations (50–300 μ g/ml) these lipoproteins induce cell death. ROS production by NAD(P)H oxidase is involved in both effects [18, 19]. This finding is in agreement with the increasing evidence of a dual effect of ROS on cell survival and proliferation. Formerly considered as toxic agents because of their oxidant properties that induce detrimental modifications of macromolecules such as DNA, proteins, lipids and carbohydrates, ROS (at low levels) act as signaling molecules inducing protection from apoptosis, cell growth and cell proliferation [20].

Preliminary experiments performed in our laboratory showed that 7 β OHC had a dual effect on the viability of human umbilical vein endothelial cells (HUVECs): at concentrations below 20 μ g/ml, this oxysterol induced an increase in cell viability while it promoted cell death at 20 μ g/ml. Taking into account the relationship between the effect of oxLDLs and 7 β OHC in vascular cells and considering that, as mentioned above, low concentrations of oxLDLs induce endothelial cell proliferation, a study on the effect of low concentrations of 7 β OHC was undertaken. The role of ROS and ERK in the action of the oxysterol was investigated. The results show that at concentrations below 20 μ g/ml, 7 β OHC is antiapoptotic and induces cell proliferation in HUVECs. These effects are independent of ROS production and are dependent on the activation of the MEK/ERK cascade.

Materials and Methods

Chemicals

Cell culture media, fetal bovine serum (FBS), 2-aminoethoxydiphenyl borate (2-APB), gadolinium chloride, N-acetylcysteine (NAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), PD98059, SP600125, staurosporine, cholesterol, 7 β OHC, 7-ketocholesterol, 25-hydroxycholesterol were from Sigma. U0126 was from Calbiochem. Type 2 collagenase was purchased from Worthington. Human recombinant basic fibroblast growth factor (FGF) was from PreproTech. Mouse monoclonal IgG antibody to phosphorylated ERK1/2 (phos-ERK1/2) and rabbit polyclonal IgG antibody to overall ERK1/2 were provided from Santa Cruz Biotechnology. Horseradish-peroxidase-conjugated antimouse and antirabbit IgG antibodies were from Amersham.

Cell Culture and Treatments

HUVECs were isolated in our laboratory as previously described [21] from human umbilical cords obtained after cesarean sections following informed consent from all the subjects. Cells were grown at 37°C under 5% CO₂ on 1% gelatin-coated culture flasks in medium M199 supplemented with 10% FBS, 5 ng/ml FGF, 25 U/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin-G, and 100 μ g/ml streptomycin. HUVECs were used from passage 2 to 6.

Oxysterols (7 β OHC, 7-ketocholesterol and 25-hydroxycholesterol) and cholesterol were dissolved in absolute ethanol (5 mg/ml) and then diluted in complete medium without FGF to obtain the final concentrations of 1, 5, 10 or 20 μ g/ml. For the experiments with staurosporine, the cells were incubated in cell culture medium (without FGF) in the presence or not of 7 β OHC for 2 or 15 h; then staurosporine was added to the medium for another 3 h. For the experiments performed in the presence of inhibitors, the substances were added 30 min before treatment with 7 β OHC and were present during all the experiment. Control cells were treated with the appropriate solvents; maximal concentrations of ethanol and dimethylsulfoxide were 0.4 and 0.1%, respectively, and did not affect cell viability and proliferation.

MTT Assay

HUVECs (1×10^4 cells/well) were plated in 96-well culture plates and incubated with or without the oxysterol at the indicated concentrations and times. The MTT reduction assay was performed as previously described [22].

[³H]Thymidine Uptake

HUVECs (5×10^3 cells/well) were plated in 96-well culture plates and incubated with 7 β OHC and/or FGF or with cholesterol at the indicated concentrations for 48 h. Twenty-four hours before the end of the treatment, 1 μ Ci [³H]thymidine was added to each well. At the end of the treatment, the incubation medium was removed and the cells were washed with PBS. Then the wells were incubated for 30 min with 100 μ l of 1% SDS-4 mM EDTA solution. Samples were collected in vials and radioactivity was measured by liquid scintillation counting (Tri-Carb 2100 TR, Packard).

Caspase-3 Activity

HUVECs (3×10^5 cells/well) were plated in 6-well plates. After treatment, the cells were washed twice with PBS and lysed in 200 μ l of lysis buffer (100 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.01% Triton X-100). The lysate was centrifuged at 10,000 g

Fig. 1. Effect of 7βOHC on MTT reduction by HUVECs. Cells were incubated for 24 h (a) or 48 h (b) in cell culture medium without FGF-containing 7βOHC. C = Control. Data are expressed as means $\pm$ SEM of 4 independent experiments performed in sextuplicate. ** $p < 0.01$ (one-way ANOVA, Dunnett's post test).

for 15 min and the supernatant was used for the analysis. Caspase-3 activity was measured using the EnzChek Caspase-3 assay kit # 2 (Molecular Probes) according to the manufacturer's instructions. Fluorescence was measured at 485 nm (excitation) and 535 nm (emission) with a multilabel plate counter (Victor²-Wallac). Caspase-3 activity, expressed as arbitrary units of fluorescence signal, was normalized for the protein content.

Flow-Cytometric Analysis of Annexin V and Propidium Iodide Binding

HUVECs (1.5×10^5 cells/well) were plated in 12-well plates. At the end of the treatment, the cells were detached, centrifuged and suspended in 100 μ l of binding buffer (NaCl 140 mM, CaCl₂ 2.5 mM, HEPES 10 mM, pH 7.4) containing annexin V-Alexa Fluor 488 (Molecular Probes) and propidium iodide (Sigma). After incubation for 15 min at room temperature in the dark, the samples were diluted with 400 μ l of binding buffer and analyzed by flow cytometry (Epics XL, Beckman Coulter). For each sample, 10,000 events were collected. Data acquisition and analysis were done using the ExpoTM 32 ADC software (Beckman Coulter).

Intracellular ROS Levels

Intracellular ROS levels in intact cells were determined using the cell-permeable probe 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA, Molecular Probes). HUVECs (2×10^4 cells/well) were plated in 96-well plates. After overnight FGF deprivation, the cells were incubated for 30 min at 37°C with 20 μ M CM-H₂DCFDA in physiological salt solution (PSS) (140 mM NaCl, 5.9 mM KCl, 1.8 mM CaCl₂, 1.4 mM MgCl₂ · 6H₂O, 1.2 mM NaH₂PO₄, 5.5 mM glucose, 5 mM HEPES). Cells were carefully washed with PSS with 10% FBS and incubated for another 15 min at 37°C. Then, the medium was changed to fresh PSS containing 10% FBS with or without 7βOHC. Fluorescence was measured (485 nm excitation and 535 nm emission) at 37°C for 3 h, at 10-min intervals, with a multilabel plate reader (Victor²-Wallac). Fluorescence was expressed as the percentage of signal measured at time 0.

Western Blotting Analysis of ERK Phosphorylation

HUVECs were plated in 10-cm dishes and cultured until subconfluence was reached. Then the cells were incubated for 5 h in cell culture medium containing 1% FBS; the cells were treated with 1–20 μ g/ml 7βOHC for 30 or 60 min. Then the cells were lysed in 100 μ l lysis buffer (NaCl 75 mM, Tris 10 mM, EDTA 10 mM, deoxycholate 0.75%, SDS 0.5%, Triton X-100 1%, phenylmethylsulfonyl fluoride 0.5 mM, leupeptin 10 μ g/ml, aprotinin 25 μ g/ml, NaF 1.25 mM, Na₄P₂O₇ 1 mM, Na-orthovanadate 2 mM) and centrifuged at 10,000 g for 15 min. The supernatant (30 μ g) was subjected to 10% SDS-PAGE and Western blotting. Membranes were probed first for human phos-ERK1/2. After detection, the membranes were reprobed for overall ERK1/2. Bands were detected by chemiluminescence using the ECL-Advance Western blotting detection kit (Amersham).

Statistical Analysis

Results are presented as means $\pm$ standard error of the mean (SEM). Graphs and statistical analysis of the differences between experimental groups were performed using GraphPad Prism version 3.03 for Windows, GraphPad Software.

Results

Effect of 7βOHCS on HUVECs Viability

It has been suggested that oxysterols, in particular 7βOHC and 7-ketocholesterol are responsible for oxLDL cytotoxicity at the vascular wall. However, when HUVECs were incubated with 7βOHC for 24 or 48 h, it was found that oxysterol treatment had a dual effect on cell viability, depending on the concentration (fig. 1). In fact, 7βOHC induced a significant increase in the number of viable cells, determined by the MTT reduction assay, at

Fig. 2. Effect of 7βOHC and cholesterol on [³H]thymidine uptake by HUVECs. **a** Cells were incubated for 48 h in cell culture medium containing different concentrations of FGF in the presence of 0, 5 or 10 μg/ml 7βOHC; data are expressed as means ± SEM of 3 independent experiments performed in quadruplicate; ** p < 0.01, ## p < 0.01, § p < 0.05 (vs. corresponding control, incubated

without 7βOHC) (one-way ANOVA, Dunnett's post test). **b** HUVECs were incubated for 48 h in cell culture medium without FGF in the presence of 7βOHC (5, 10 μg/ml) or cholesterol (5, 10 μg/ml). C = Control. Data are expressed as means ± SEM of 3 independent experiments performed in quadruplicate; ** p < 0.01 (one-way ANOVA, Dunnett's post test).

concentrations ranging between 1 and 10 μg/ml, while promoting cell death at 20 μg/ml. This latter observation is in accordance with the notion of the atherogenic action of 7βOHC and with the findings of Lemaire et al. [5] and Lizard et al. [6] indicating that 20 μg/ml 7βOHC induces HUVECs apoptosis. It should be noted that HUVECs were treated in a medium without FGF, a condition that inhibits HUVECs proliferation and induces apoptosis [21, 23]. Thus the increase in MTT reduction by 7βOHC at 1–10 μg/ml could be explained either by an increase in cell proliferation or by the inhibition of apoptosis induced by growth factor deprivation. These two possibilities were investigated in detail.

Effect of 7βOHC on HUVECs Proliferation

The effect of 7βOHC on HUVECs proliferation in the presence or in the absence of FGF was determined by measuring [³H]thymidine incorporation (fig. 2a). 7βOHC alone was able to double [³H]thymidine incorporation. When added to increasing concentrations of FGF, 7βOHC had an additive effect on the proliferation stimulated by the growth factor. Additivity of the effect of 7βOHC on [³H]thymidine uptake was also observed when HUVECs were incubated with vascular endothelial growth factor (VEGF) or epidermal growth factor (EGF) (data not shown). At the same concentrations used for 7βOHC, cholesterol did not affect [³H]thymidine uptake in HUVECs (fig. 2b).

Effect of 7βOHC on HUVECs Apoptosis

We have previously shown that growth factors activate signaling pathways that protect HUVECs from apoptotic stimuli [23]. To evaluate whether 7βOHC can inhibit apoptosis induced by FGF deprivation, the effect of the oxysterol on two hallmarks of apoptosis such as caspase-3 activation and phosphatidylserine translocation was determined. Incubation of HUVECs with 7βOHC (5, 10 μg/ml) inhibited the increased caspase-3 activity induced by FGF deprivation (fig. 3a). This result was confirmed by measuring phosphatidylserine translocation through flow-cytometric analysis of annexin V binding. In fact, figure 3b shows that incubation without FGF for 24 h raises the number of annexin-V-positive (apoptotic) cells and that this increase is counteracted by 7βOHC treatment. The maximal antiapoptotic effect was obtained at 10 μg/ml 7βOHC. Analysis of annexin V binding of FGF-deprived HUVECs demonstrated that treatment of the cells with cholesterol (1–10 μg/ml) did not protect HUVECs from apoptosis (fig. 4a). Furthermore, incubation of HUVECs for 24 h with 10 μg/ml of three different oxysterols (7βOHC, 7-ketocholesterol or 25-hydroxycholesterol) resulted in increased cell viability only in the cells treated with the two compounds oxidized at the 7-position (fig. 4b).

The antiapoptotic activity of 7βOHC was also tested against the microbial alkaloid staurosporine, one of the most widely used apoptogenic agents [24]. Pretreatment

Fig. 3. Effect of 7βOHC on apoptosis induced by FGF deprivation in HUVECs. **a** Cells were incubated for 6 h in cell culture medium with FGF or without FGF in the presence of 7βOHC; caspase-3 activity of cell lysates was measured at the end of the treatment; data are expressed as means $\pm$ SEM of 4 experiments performed in triplicate, * $p < 0.05$, ** $p < 0.01$ (vs. control) (one-way ANOVA, Dunnett's post test). **b** HUVECs were incubated for 24 h in cell culture medium with FGF or without FGF in the presence of 7βOHC; then flow-cytometric analysis of annexin V binding was performed; data are expressed as means $\pm$ SEM of 6 independent experiments performed in triplicate, ** $p < 0.01$ (vs. control) (one-way ANOVA, Dunnett's post test).

Dunnett's post test). **b** HUVECs were incubated for 24 h in cell culture medium with FGF or without FGF in the presence of 7βOHC; then flow-cytometric analysis of annexin V binding was performed; data are expressed as means $\pm$ SEM of 6 independent experiments performed in triplicate, ** $p < 0.01$ (vs. control) (one-way ANOVA, Dunnett's post test).

Fig. 4. Effect of cholesterol and various oxysterols on HUVEC viability. **a** Cells were incubated for 24 h in cell culture medium with FGF or without FGF in the presence of different concentrations of 7βOHC or cholesterol; then flow-cytometric analysis of annexin V binding was performed; data are expressed as means $\pm$ SEM of 2 independent experiments performed in duplicate, ** $p < 0.01$ (vs. control) (one-way ANOVA, Dunnett's post test). **b** HUVECs were incubated for 24 h in cell culture medium without FGF in the presence of 10 μg/ml of 7βOHC, 7-ketocholesterol (7KC) or 25-hydroxycholesterol (25OHC); data are expressed as means $\pm$ SEM of 3 experiments performed in triplicate, ** $p < 0.01$ (vs. control) (one-way ANOVA, Dunnett's post test).

** $p < 0.01$ (vs. control) One-way ANOVA, Dunnett's post test). **b** HUVECs were incubated for 24 h in cell culture medium without FGF in the presence of 10 μg/ml of 7βOHC, 7-ketocholesterol (7KC) or 25-hydroxycholesterol (25OHC); data are expressed as means $\pm$ SEM of 3 experiments performed in triplicate, ** $p < 0.01$ (vs. control) (one-way ANOVA, Dunnett's post test).

of HUVECs for 15 h with 10 μg/ml 7βOHC was able to reduce the increased caspase-3 activity induced by 50 nM staurosporine (fig. 5a). The antiapoptotic effect was confirmed by analysis of annexin V binding (fig. 5b). A 2-hour pretreatment with 7βOHC was sufficient for the oxysterol to display its effect (see experimental condi-

tions of fig. 7c). In spite of the protection against the early phases of apoptosis afforded by 7βOHC, longer treatments (18–24 h) with staurosporine were cytotoxic for HUVECs also in the presence of the oxysterol, indicating that 7βOHC is able to delay rather than block the apoptotic program induced by staurosporine.

Fig. 5. Effect of 7βOHC on apoptosis induced by staurosporine in HUVECs. **a** Cells were incubated for 15 h in cell culture medium without FGF, in the presence of 10 μg/ml 7βOHC, then cells were treated with 50 nM staurosporine for 3 h; at the end of the incubation caspase-3 activity of cell lysates was determined; data are expressed as means ± SEM of 3 experiments performed in tripli-

cate, *** $p < 0.001$, ^{SSS} $p < 0.01$ (one-way ANOVA, Bonferroni's post test). **b** Cells were treated as indicated in **a**; then flow cytometric analysis of annexin V binding was performed; data are expressed as means ± SE of 4 independent experiments performed in triplicate, *** $p < 0.001$, ^{SSS} $p < 0.001$ (one-way ANOVA, Bonferroni's post test).

Role of ROS in the Antiapoptotic and Proliferative Effects of 7βOHC

It has been demonstrated that oxLDLs induce endothelial cell proliferation at low concentrations (5–10 μg/ml) and apoptosis at higher concentrations and that both effects are mediated by ROS production [18, 19]. Considering that 7βOHC is a component of oxLDLs, the involvement of ROS in the antiapoptotic effect of the oxysterol was studied. Time course determination of intracellular ROS showed that 7βOHC increases ROS levels in a concentration-dependent manner (fig. 6a). Basal and 7βOHC-stimulated ROS productions were suppressed when the antioxidant NAC was added to the incubation medium (fig. 6b). However, HUVECs treatment in the presence of NAC failed to inhibit the antiapoptotic and proliferative effect of 7βOHC (fig. 6c–e).

Role of ERK in the Effect of 7βOHC

Due to its central role in the regulation of cell survival and proliferation, the involvement of ERK in the effect of 7βOHC was investigated. Western blotting analysis of phosphorylated ERK levels revealed that 7βOHC (1–20 μg/ml) increases ERK activation in HUVECs (fig. 7a). ERK phosphorylation was transient, i.e. it rapidly decreased after 60 min of treatment (fig. 7b). Furthermore, inhibition of the ERK pathway using two different MEK inhibitors, PD98059 or U0126, completely blocked the antiapoptotic and proliferative effects of 7βOHC (fig. 7c,

d), indicating that the effect of 7βOHC is dependent on the activation of the MEK/ERK pathway.

ERK activation by 7-oxysterols has previously been shown in human aortic smooth muscle cells and THP-1 cells (a human acute monocytic leukemia cell line) by a mechanism dependent on the increase in intracellular calcium [7, 25, 26]. Therefore, the role of calcium in ERK activation induced by 7βOHC was investigated. Since store-operated calcium entry represents a major pathway of calcium influx during agonist stimulation in endothelial cells [27], HUVECs were treated with 7βOHC in the presence of 100 μM 2-APB or 100 μM gadolinium chloride (GdCl₃), two distinct blockers of store-operated calcium entry [28, 29]. As shown in figure 8a, the increased ERK phosphorylation induced by the oxysterol was prevented either by 2-APB or gadolinium treatment. Moreover, 2-APB and gadolinium antagonized the increased MTT reduction induced by 7βOHC (fig. 8b), suggesting the involvement of store-operated calcium entry in the mechanism of action of 7βOHC.

Discussion

The data presented show that 7βOHC is antiapoptotic in HUVECs at concentrations below 20 μg/ml. 7βOHC protects against two different proapoptotic stimuli: growth factor deprivation and staurosporine treatment

Fig. 6. Role of ROS production in the antiapoptotic and proliferative effects of 7βOHC. **a, b** Time course determination of intracellular ROS in HUVECs treated with different concentrations of 7βOHC (**a**). Effect of NAC 10 mM on intracellular ROS production induced by 10 μg/ml 7βOHC (**b**). Curves were plotted as means of at least 4 independent experiments performed in quadruplicate; increase in ROS induced by each concentration of 7βOHC is statistically significant with respect to the control ($p < 0.0001$, F test); inhibition of ROS production by NAC is statistically significant ($p < 0.0001$, F test). **c, d** Effect of NAC 10 mM on

the protective effect of 10 μg/ml 7βOHC against apoptosis induced by FGF deprivation for 24 h (**c**) or by staurosporine treatment for 3 h (**d**); cell treatments were performed as indicated in figures 3 and 5, subsequently flow-cytometric analysis of annexin V binding was performed. **e** Effect of NAC 10 mM on [3 H]thymidine uptake by HUVECs treated with 10 μg/ml 7βOHC or 5 ng/ml FGF. Data are expressed as means $\pm$ SEM of at least 2 experiments performed in duplicate, * $p < 0.05$, $^s p < 0.05$, ** $p < 0.01$, $^{ss} p < 0.01$, *** $p < 0.001$, $^{ssss} p < 0.001$ (one-way ANOVA, Bonferroni's post test).

as demonstrated by the decrease in caspase-3 activity and phosphatidylserine translocation in the cells treated with the oxysterol. Besides its antiapoptotic effect, 7βOHC stimulates cell proliferation, suggesting that the oxysterol can activate pathways commonly used by growth factors. Although the proliferative effect is significant, the efficacy of 7βOHC is lower with respect to the effect of FGF and other growth factors tested (EGF and VEGF). The antiapoptotic and proliferative effects of 7βOHC seem to be associated with the oxidation of cholesterol at the C7-position. This is suggested by the observation that 7-ketocholesterol also increases cell viability in HUVECs and by the lack of effect of cholesterol and 25-hydroxycholesterol. Although these results give an indication on the possible molecular features required for the antiapoptotic effect, more detailed studies are required to establish a structure-activity relationship.

To our knowledge, the antiapoptotic and proliferative effects of 7βOHC have not been previously described. In fact, several studies performed on various cell types, including HUVECs, have always shown the cytotoxic effect of 7βOHC and other oxysterols; however, many of these studies used higher concentrations of oxysterols. In agreement with these results, we also observed an increase in cell death at 20 μg/ml 7βOHC. Regarding the concentrations of 7βOHC used in in vitro studies, including ours, it should be noted that these concentrations are much higher than those measured in human plasma. Reported plasma levels of 7βOHC in normal subjects are extremely variable: 1–2,200 nM (corresponding to 0.4–880 ng/ml), probably due to differences in sample storage, extraction and analysis procedures [1, 2]. A recent study has demonstrated that in healthy elderly subjects, the serum levels of 7βOHC are in the range of 100 ng/ml, with a 70% increase in atherosclerotic patients [30].

Fig. 7. Role of ERK in the antiapoptotic and proliferative effect of 7βOHC. Expression of phosphorylated and overall ERK1/2 in HUVECs treated with 1–20 μg/ml 7βOHC for 30 min (**a**) or treated with 10 μg/ml 7βOHC for 30 and 60 min (**b**); C = control, FGF: 20 ng/ml FGF for 20 min; the blots shown are representative of three independent experiments. **c** Effect of MEK inhibition on the antiapoptotic action of 7βOHC: after overnight FGF deprivation, the cells were incubated for 2 h with or without 10 μg/ml 7βOHC; then the cells were treated with 50 nM staurosporine (S) for 3 h, caspase-3 activity of cell lysates was determined; where indicated, 25 μM PD98059 or 10 μM U0126 were added to the incubation

medium; data are expressed as means ± SEM of 3 experiments performed in triplicate, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, §§ $p < 0.01$ (one-way ANOVA, Bonferroni's post test). **d** Effect of MEK inhibition on cell proliferation induced by 7βOHC: HUVECs were incubated for 48 h with 10 μg/ml 7βOHC, 5 ng/ml FGF or both, where indicated 10 μM U0126 was added to the incubation medium, then [³H]thymidine uptake was measured; data are expressed as means ± SEM of 3 experiments performed in triplicate, ** $p < 0.01$, * $p < 0.05$ (vs. control cells without U0126), § $p < 0.05$, §§§ $p < 0.001$ (vs. their analogues without U0126) (one-way ANOVA, Bonferroni's post test).

Oxysterols, in particular 7-oxysterols, have been considered as the cytotoxic components of oxLDLs responsible for the ability of these lipoproteins to promote atherosclerotic plaque formation [10–12]. In vascular cells such as endothelial cells, smooth muscle cells, macrophages and lymphocytes, these compounds induce cell death by different mechanisms depending on the cell type and the particular oxysterol considered [31, 32]. However, in analogy to our observations with 7βOHC, Heinloth et al. [19] have demonstrated that oxLDLs have a dual effect on cell viability and proliferation in endothelial cells, inducing cell proliferation or apoptosis de-

pending on their concentration. These authors suggest that lysophosphatidylcholine, a component of oxLDLs, is partly responsible for the proliferative effect of oxLDLs since this compound mimics oxLDLs activity in endothelial cells. Taking into account our results, it could be hypothesized that 7βOHC also participates in the proliferative action of oxLDLs. The idea that low concentrations of oxLDLs may promote angiogenesis and protect endothelial cells from apoptosis is discussed in a recent study by Dandapat et al. [33], who found that small concentrations of oxLDLs (<10 μg/ml) induce VEGF production and capillary tube formation from endothelial

Fig. 8. Effect of inhibitors of store-operated calcium entry on 7βOHC action. **a** Expression of phosphorylated and overall ERK1/2: HUVECs were incubated for 30 min with or without 100 μM 2-APB or 100 μM GdCl₃, then 7βOHC (10 μg/ml) was added for 30 min; C = control, FGF: 20 ng/ml FGF for 20 min; the blot shown is representative of 3 independent experiments. **b** MTT reduction: cells were incubated for 24 h in cell culture medium without FGF in the presence of 7βOHC (10 μg/ml); where indicated, 100 μM 2-APB or 100 μM GdCl₃ was present in the incubation medium; FGF: 5 ng/ml; data are expressed as means ± SEM of 3 independent experiments performed in sextuplicate; *** *p* < 0.001 (vs. control without 7βOHC), ### *p* < 0.001 (vs. control with 7βOHC) (one-way ANOVA, Bonferroni's post test).

cells; nevertheless, they do not ascribe the observed effects to a particular component of oxLDLs. We did not measure VEGF production in cells treated with 7βOHC, thus it cannot be excluded that 7βOHC could stimulate VEGF production in HUVECs; however, under our experimental conditions, 7βOHC induced an early increase in ROS levels and ERK phosphorylation, suggesting a direct effect of the oxysterol.

Regarding the possible mechanism involved in the antiapoptotic and proliferative effect of 7βOHC, we first investigated the role of ROS. Several studies demonstrated

that oxidative stress participates in the cytotoxicity of oxysterols. It has been shown that ROS production by NAD(P)H oxidase is involved in the onset of 7-ketocholesterol-induced apoptosis in human aortic smooth muscle cells [15]. It has also been reported that increased ROS production and decreased glutathione levels are implicated in cell damage caused by 7-ketocholesterol in the monocytic cell line U937 [34]. It is worth noting that excessive or sustained levels of ROS are detrimental to vascular cells and are involved in the pathogenesis of various cardiovascular diseases while moderate concentrations of ROS act as signaling molecules and regulate vascular cell functions [20]. In agreement with this notion, the dual effect of oxLDLs as well as the angiogenic action of low concentrations of oxLDLs mentioned above have been correlated with the production of ROS by NAD(P)H oxidase [19, 33]. Our results show that in HUVECs 7βOHC induces a concentration-dependent increase in intracellular ROS; nevertheless, the inability of the antioxidant NAC to block both the antiapoptotic and proliferative effect of 7βOHC demonstrates that ROS are not involved in the mechanism of action of 7βOHC.

The ERK pathway regulates cell survival and proliferation by growth factors and other extracellular signals [35]. Therefore, the possibility that nontoxic concentrations of 7βOHC could activate ERK was considered. We found that in HUVECs 7βOHC induces ERK phosphorylation at both antiapoptotic (<20 μg/ml) and proapoptotic (20 μg/ml) concentrations. However, inhibition of MEK antagonizes the antiapoptotic and proliferative effects of 7βOHC in HUVECs. The fact that this antagonism is obtained with two structurally different inhibitors (PD98059 and U0126) indicates that the lack of 7βOHC effect is specifically due to MEK inhibition. ERK activation by 7-oxysterols was previously shown in human aortic smooth muscle cells and in the human monocytic cell line THP-1 at concentrations inducing cell death [7, 25, 26]. Those authors did not investigate whether ERK activation by 7βOHC is involved in apoptosis in human aortic smooth muscle cells [7]. Instead, in THP-1 cells, they found that blocking MEK with U0126 accelerated the proapoptotic action of 7-ketocholesterol [25]. Moreover, ERK activation induced by 7βOHC in THP-1 cells is not involved in cell death caused by the oxysterol, but is required for the secretion of interleukin-8 stimulated by 7βOHC [25]. Thus, it appears that 7-oxysterols can simultaneously activate both proapoptotic and antiapoptotic pathways in various cell types. The balance of antiapoptotic pathways (ERK dependent) and proapoptotic pathways (due to oxidative stress and/or sustained calcium increase) would deter-

mine the final effect: cell death or survival. It cannot be excluded that, at a certain concentration of 7 β OHC, this balance changes depending on several factors such as cell type (smooth muscle cells, endothelial cells, monocytes), cell culture origin (normal or tumoral cells), cell senescence and environmental conditions. It should be noted that exposure of endothelial cells to oxLDLs induces ERK phosphorylation and the activation of pathways that lead to cell survival and angiogenesis, as demonstrated by the reduction in capillary tube formation and the enhancement of oxLDLs cytotoxicity when ERK activation is blocked by MEK inhibitors [33, 36]. These observations are in agreement with our hypothesis that 7 β OHC takes part in the biological effects of oxLDLs.

In order to understand the mechanism through which 7 β OHC induces ERK activation in HUVECs, we investigated the role of calcium. In fact, in the studies mentioned above, ERK activation by 7-oxysterols is reported to be a calcium-dependent process. In THP-1 cells, the effects of both 7 β OHC and 7-ketocholesterol are dependent on calcium entry through L-type calcium channels [25, 26]. On the contrary, in human aortic smooth muscle cells, calcium oscillations induced by 7 β OHC are due to calcium release from the endoplasmic reticulum (ER) and are not affected by the L-type calcium channel blocker verapamil [7]. In endothelial cells, calcium influx occurs mainly through receptor-gated channels and mechanisms related to the filling degree of intracellular calcium stores [27]. For this reason, we investigated the involvement of store-operated calcium entry in ERK activation induced by 7 β OHC. We found complete inhibition of ERK phosphorylation in HUVECs treated with 2-APB or the trivalent cation Gd³⁺, two inhibitors of store-operated calcium entry [28, 29]. Furthermore, we found that both compounds were able to antagonize the effect of 7 β OHC on cell viability, thus suggesting that opening of store-operated calcium channels is essential for the antiapoptotic and proliferative actions of 7 β OHC.

Store-operated calcium entry is a common mechanism regulating calcium influx into cells. Under physiological conditions, it is activated by inositol-triphosphate-mediated depletion of ER, in response to agonists that bind to phospholipase-C-coupled receptors. Activation of calcium entry is also obtained by treatment of the cells with compounds such as thapsigargin, which inhibits the sarcoplasmic/ER Ca²⁺-dependent ATPase and consequently depletes ER calcium stores. Further investigations are needed to understand whether in HUVECs 7 β OHC acts by depleting ER calcium stores (phospholipase-C-dependent or independent) or by a direct interaction with the store-operated calcium channels. Interestingly, a recent study has demonstrated that in HUVECs, store-operated calcium entry is mediated by the proteins Stim1 and Orai1 and that knockdown of these proteins inhibits cell proliferation induced by VEGF [37].

In conclusion, our results offer evidence of an unknown effect of 7 β OHC on human endothelial cells. Although it is well established that 7 β OHC and other oxysterols promote vascular cell damage at high concentrations, our findings demonstrate that lower concentrations of 7 β OHC could activate pathways that promote cell survival and proliferation. The dual effect of 7 β OHC on endothelial cell viability should be taken into account when considering the pathophysiological role of this oxysterol in the vasculature.

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