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Original Paper

Oxysterol Mixture in Hypercholesterolemia-Relevant Proportion Causes Oxidative Stress-Dependent Eryptosis

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Key Words
Hypercholesterolemia • Human red blood cell • Oxysterols • Eryptosis • Oxidative stress

Abstract
Background/Aims: Oxysterol activity on the erythrocyte (RBC) programmed cell death (eryptosis) had not been studied yet. Effects of an oxysterol mixture in hyper-cholesterolemic-relevant proportion, and of individual compounds, were investigated on RBCs from healthy humans. ***Methods:*** Membrane phosphatidylserine (PS) externalization, calcium entry, ROS production, amino-phospholipid translocase (APLT) activity were evaluated by cytofluorimetric assays, cell volume from forward scatter. Prostaglandin PGE2 was measured by ELISA; GSH-adducts and lipoperoxides by spectrophotometry. Involvement of protein kinase C and caspase was investigated by inhibitors staurosporin, calphostin C, and Z-DEVD-FMK, respectively. ***Results:*** Oxysterols caused PS externalization and cell shrinkage, associated with PGE2 release, opening of PGE2-dependent calcium channels, ROS production, GSH depletion, membrane lipid oxidation. Addition of antioxidants prevented Ca^{2+} influx and eryptosis. Calcium removal prevented cell shrinkage, with small effect (-20%) on the PS exposure, whereas ROS generation was unaltered. Either in the presence or absence of calcium i) oxysterols inhibited APLT, ii) staurosporin, calphostin C, Z-DEVD-FMK blunted and iii) antioxidants fully prevented the oxysterol-induced PS externalization. Only 7-ketocholesterol and cholestan-3β,5α,6β-triol were individually active. Eryptosis was observed in RBCs isolated after *ex vivo* spiking of human whole blood with the oxysterol mixture. ***Conclusions:*** Oxysterols induce an oxidative stress-dependent eryptosis, involving calcium-independent mechanisms. Eryptotic activity of oxysterols may be relevant *in vivo*.

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Introduction

Cholesterol oxidation products in circulating low density lipoproteins (LDL), collectively termed oxysterols, are considered to play a critical role in the initiation and development of a number of chronic diseases, including atherosclerosis, neurodegenerative pathologies, and diabetes [1]. High levels of oxysterols (20 to 30 μM) have long been known to occur in hypercholesterolemic subjects [2-5], and increased concentrations in plasma and cerebrospinal fluid are correlated to enhanced risk of cardiovascular and Alzheimer's diseases, respectively [6-9]. Recent literature now supports that certain oxysterols exert pathological effects by induction of apoptotic cell death. This has been shown with different cell types of the vascular compartment, namely smooth muscle cells [10, 11], endothelial cells [11, 12] and monocyte-macrophages [11, 13, 14], in intestinal epithelium cells [15, 16], and in oligodendrocytes [17]. Similarly to nucleated cells, RBCs incur in suicidal death or eryptosis, characterized by cell shrinkage and membrane scrambling with phosphatidylserine (PS) appearance at the RBC surface [18]. Eryptosis is to be considered a physiological event, leading to disposal of aged RBCs by macrophages, however growing evidences suggest that this process may contribute to the patho-physiology of various clinical disorders. Enhanced eryptosis is observed in chronic uremia [19], sickle cell disease [20], thalassemia [21] and diabetes [22]. Externalization of PS at the RBC surface may activate coagulant enzymes [23] and thus cause thrombosis and thrombo-occlusive disease [20, 23-26]. Moreover, eryptotic RBCs may adhere to the vascular wall [25, 27], contributing to the inflammatory process of endothelial tissue leading to atherosclerosis. RBCs are continuously exposed to circulating lipoproteins, with rapid transfer of cholesterol and derivatives between particles and cells [28], however eventual toxicity of oxysterols on these cells has not been studied yet. This work explored the eryptotic activity of oxysterols on isolated human healthy RBCs, and investigated mechanistic aspects associated. The major plasma oxysterols, i.e. 7-ketocholesterol (7-KC), cholestan-3β,5α,6β-triol (TRIOL), 5α,6α-epoxy-cholesterol (α-epox), and 5β,6β-epoxy-cholesterol (β-epox), 7α-hydroxy-cholesterol (7α-OH), 7β-hydroxy-cholecholesterol (7β-OH), at the concentrations occurring in hyper-cholesterolemic subjects [5, 29, 30], have been assayed either individually or in a mixture, and role of calcium and oxidative stress as initiating factors in the oxysterol-induced eryptotic transduction investigated. The oxysterol toxicity on RBCs has finally been evaluated after *ex vivo* spiking of normal blood with the mixture.

Materials and Methods

7-KC, TRIOL, α-epox, β-epox, 7α-OH, 7β-OH and fluorescent-labeled phosphatidylserine,1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadia- zol-4-yl)amino]lauroyl}-sn-glycero-3-phosphoserine (NBD-PS), were purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA); calphostin C was from Calbiochem (Merck Millipore, Darmstadt, Germany). All other reagents and chemicals were from Sigma Chemical Co (St. Louis, MO), unless indicated.

Cells and incubation conditions

Blood was drawn from five healthy volunteers, with informed consent, and RBCs isolated by a 20 min centrifugation at 2,000 g, 4 °C, over Ficoll (Biochrom KG, Berlin, Germany). RBCs (0.4 % hematocrit) were incubated at 37°C, 5% CO_2 and 95% humidity, in Ringer solution containing (mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH, 5 glucose, 1 $CaCl_2$, pH 7.4, for 48 h. For the nominally calcium-free solution, $CaCl_2$ was replaced by 1mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). 7-KC, TRIOL, α-epox, 7α-OH, 7β-OH and β-epox, at their final concentration of 7 μM, 2 μM, 4 μM, 1 μM, 2 μM and 4 μM, respectively, were added individually or in mixture. Oxysterols were delivered to the cells dissolved in a final 0.1% (v:v) tetrahydrofuran (THF) concentration. Preliminary experiments showed that THF did not have any effect under this condition, therefore control RBCs were incubated with THF. Where indicated, COX inhibitor acetylsalicylic acid (ASA, 50 μM), pan-caspase inhibitor Z-DEFD-FMK (100 μM), protein kinase C (PKC) inhibitor staurosporin (1.0

μM), calphostin C 0.5 μM), N-acetyl-L-cysteine (NAC, 10 μM), vitamin E (α-T, 20 μM), or the calcium chelating agent 1,2-bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetracetic acid, tetracetoxymethyl ester (BAPTA-AM) (50 μM) were added into the incubation medium 1 h before adding the oxysterols. α-T was delivered to the cells in a final ethanol concentration 0.1% (v:v). Control RBCs were incubated with ethanol and THF in these assays.

Measurement of phosphatidylserine (PS) externalization and forward scatter

RBCs were washed once in Ringer solution and adjusted at $1.0x10^6$ cells/mL with combining buffer. Cell suspension (100 μL) was added to a new tube and incubated with 5 μL Annexin V-FITC (eBioscience Inc., San Diego, CA, USA), at room temperature in the dark for 15 min. Then samples of at least $1x10^4$ cells were subjected to fluorescence-activated cell sorting (FACS) analysis by Epics XL™ flow cytometer, using Expo32 software (Beckman Coulter, Fullerton, CA). Cells were analysed by forward scatter, and annexinV-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of hemolysis

RBCs were centrifuged (3 min, 400 *g*) and the supernatants harvested. Concentration of hemoglobin (Hb) in the supernatant was determined by the absorbance at 408 nm (Soret's band). The absorption of the supernatant from analogous erythrocytes lysed in distilled water was defined as 100% hemolysis.

Measurement of cytosolic calcium

Intracellular Ca^{2+} concentration was measured using fluo-3 AM as a fluorescent Ca^{2+} probe, whose intensity is directly representative of the ion concentration. Fluo-3/AM (2μM final concentration), was added into the cell medium 40 min before the end of treatment. After centrifugation (2,000 g, 5 min), cells were washed with 0.9% NaCl in 5 mM phosphate buffer, pH 7.4 (PBS) and suspended in 500 μL PBS. The fluorescent intensity was analyzed by FACS analysis in at least $1x10^4$ cells for each sample.

Measurement of prostaglandin E2 (PGE2) production

RBCs ($1x10^9$ cells/mL) were incubated for 48 h either in the presence or in the absence of oxysterols as indicated above. PGE2 secretion in extracellular medium was quantified in pg/ml using a Prostaglandin E2 Enzyme Immunoassay Kit (Cayman Chemical Corporation, Inc. Ann Arbor, MI) in accordance with the manufacturer's protocol.

Measurement of intracellular ROS

The ROS level was monitored by measuring fluorescence changes resulting from oxidation of dichloro-dihydro-fluorescein diacetate (DCFDA). DCFDA, at 10 μM final concentration, was added to the cell medium 30 min before the end of treatment. Cells were collected by centrifugation (2,000 g, 4°C, 5 min), washed, suspended in PBS and subjected to FACS analysis. At least $1x10^4$ cells were analyzed for each sample.

Measurement of glutathione (GSH) in red blood cells

GSH was measured in cells from 3.0 mL incubation mixture, precipitated (2,000 g, 4°C, 5 min) and hemolysed with 0.5 mL H_2O, by titration with DTNB and spectrophotometric quantitation at 412 nm, using a molar extinction coefficient of 13,600 [31].

Measurement of membrane lipid hydroperoxides

Ghosts were prepared by three 30 min washing-centrifugation cycles (20,000 g, 4°C) with excess hypotonic PBS, and finally suspended in 1 mL PBS. Conjugated diene (CD) lipid hydroperoxides were extracted from 500 μl of the suspension with 3ml of a $CHCl_3$:CH_3OH (2:1) mixture. The organic extract was evaporated under a nitrogen stream, re-suspended in cyclohexane and quantified spectrophotometrically at 234 nm, using a molar extinction coefficient of 27,000 [32].

Measurement of APL-translocase (APLT) activity

RBC suspensions (0.5 mL) were incubated at 37°C with 0.5 mM NBD-PS fluorescent probe, added from a 1mmol/L stock solution in HEPES-buffer. After 60 min, 5 μl of the sample was added to 250 μl HEPES

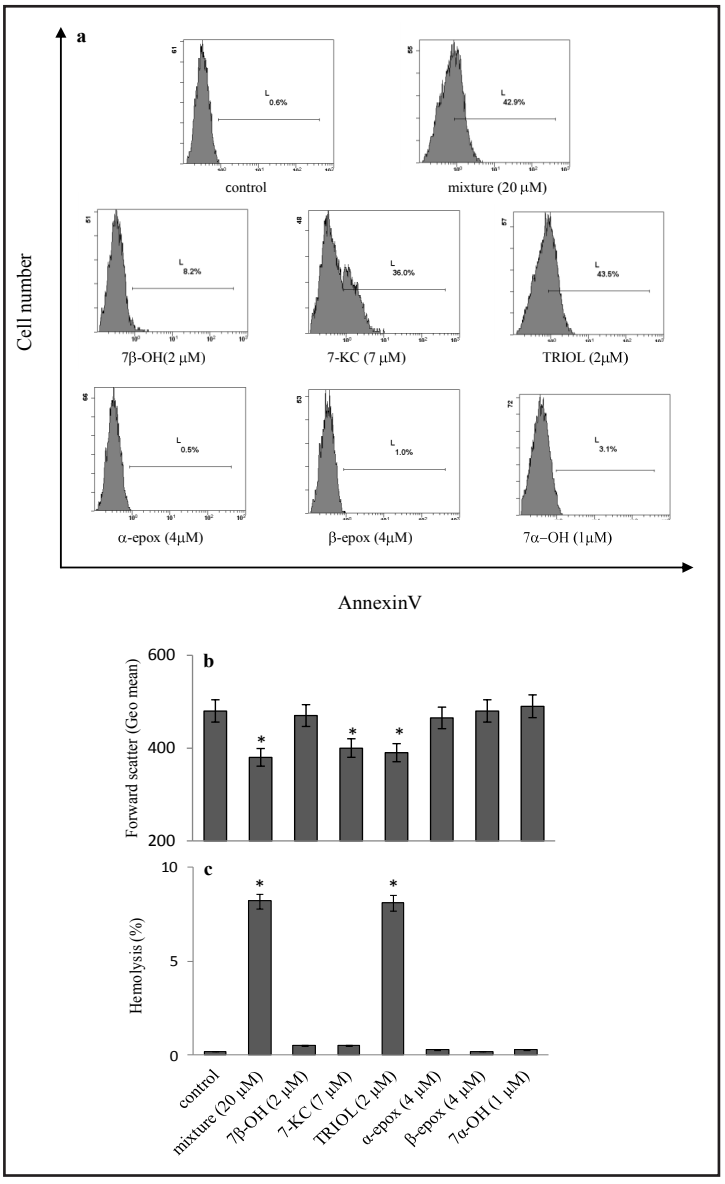

Fig. 1. Eryptosis and hemolysis by oxysterols added individually or in mixture to human RBCs. Histograms of annexin V binding (a), arithmetic means±SD (n=6) of the forward scatter (b), percentage of hemolysis (c), after a 48 h incubation in the absence (control) or in the presence of oxysterols. (a) Image representative of six experiments carried out in triplicate with comparable results. (b) and (c)*Significantly different vs control or other experimental groups (P<0.001; Anova associated with Bonferroni's test).

buffer containing 0.1 mM EGTA and 1% bovine serum albumin, that extracts the NBD-PS probe from the outer plasma- membrane. Cytofluorimetric measurements of residual fluorescence of the sample reveals the amount of NBD-PS localized on the inner leaflet in the plasma-membrane as a result of the APLT activity.

Ex vivo spiking of blood with oxysterols

Blood samples from healthy volunteers (n=5), after an overnight fasting, were individually incubated (37°C, 5% CO_2, 95% humidity, 48 h) either in the absence or in the presence of the oxysterol mixture (20 μM final concentration). RBCs were isolated by centrifugation over Ficoll as described above, washed and re-suspended in PBS to obtain a 0.4% hematocrit. ROS level and PS externalization were cytofluorimetrically measured by DCFDA and annexinV-FITC, respectively, as described above.

Statistics

Results are given as mean±SD of n independent experiments carried out in triplicate. Statistical comparisons were made using one-way ANOVA test, with Bonferroni's correction for multiple comparisons by Instat-3 statistical software (GraphPad Software Inc., San Diego, CA, USA). In all cases, significance was accepted if the null hypothesis was rejected at the P<0.05 level. Comparison between matched-paired samples was by Student's t test.

Results

Eryptosis by oxysterols in mixture or individually

Eryptotic activity of oxysterols was evaluated by cytofluorimetric analysis of FITC-labelled annexin V binding and forward scatter, to measure PS exposure and cell shrinkage, respectively. In comparison with cells incubated in their absence, a 48 h incubation with

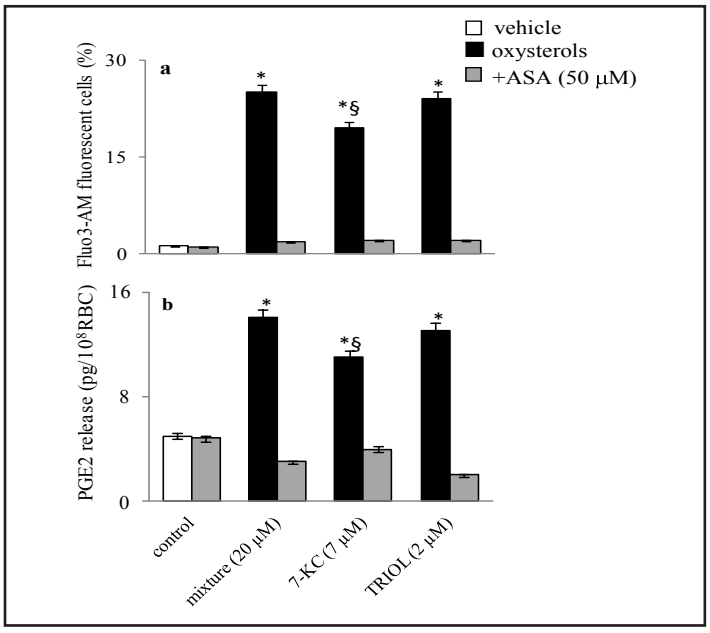

Fig. 2. Ca^{2+} entry (a) and PGE2 release (b), induced by a 48 h treatment with oxysterol mixture or individual 7-KC or TRIOL, in human RBCs and effect of COX-inhibitor ASA. Values are the means±SD of six separated experiments carried out in triplicate. * Significantly different vs control (P<0.0001); § significantly different vs mixture and TRIOL (P<0.05) (Anova associated with Bonferroni's test).

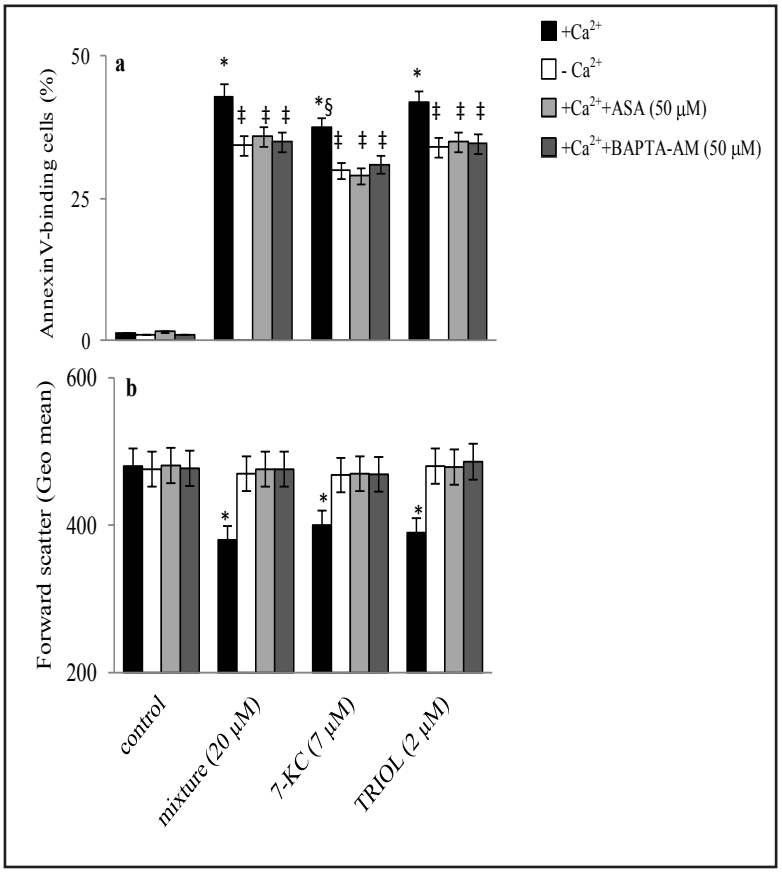

Fig. 3. Effect of Ca^{2+} removal from the medium, or intracellular calcium chelator BAPTA-AM or COX inhibitor ASA, on the oxysterol-induced eryptosis (a) and cell volume (b). Arithmetic means±SD (n=6) of percentage of bounded AnnexinV-FITC (a) and forward scatter (b) of human RBCs after a 48 h incubation in the absence (control) or presence of oxysterols. * Significantly different vs control (P<0.0001); § significantly different vs mixture and TRIOL (P<0.05); ‡ significantly different vs samples incubated in the presence of Ca^{+2} of the relevant group (P<0.05) (one-way Anova associated with Bonferroni's post test).

a mixture including oxysterols at a hyper-cholesterolemia-relevant proportion (20 μM total oxysterols) caused a net increase of annexin V binding RBCs (42±6%, n=6, Fig. 1, a), and a significant decrease of forward scatter (Fig. 1, b). Exposure of erythrocytes to the individual oxysterols provided evidence that only 7-KC and TRIOL were effective in causing PS externalization and reduction of cell volume (Fig. 1, a, b). Hemolytic effects of oxysterols were also investigated. Exposure of RBCs to the oxysterol mixture for 48 h caused increased

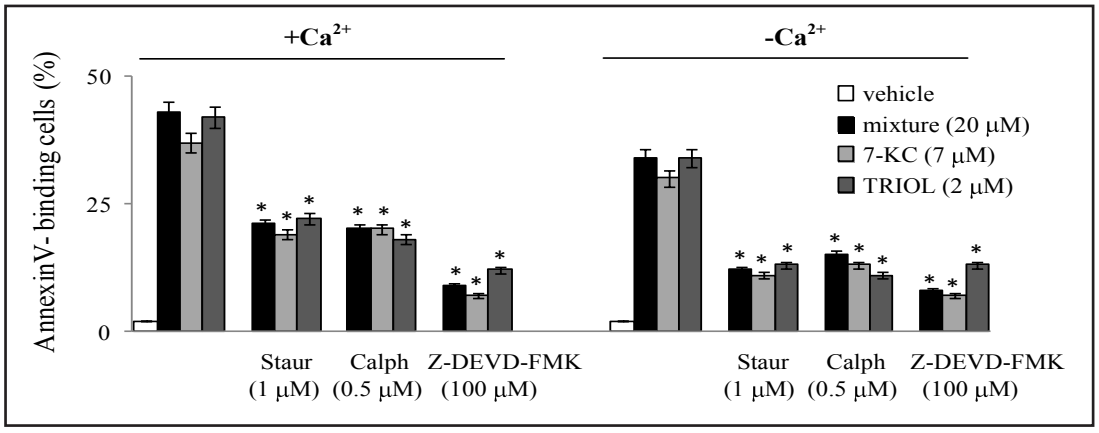

Fig. 4. Effect of pre-treatment with staurosporine (staur), or calphostin C (calph), or Z-DEVD-FMK on PS-externalization in oxysterol-treated human RBCs in the presence or in the absence of Ca^{2+} in the medium. Arithmetic means±SD (n=6) of AnnexinV-FITC-cell fluorescence after a 48 h incubation of RBCs with the oxysterols preceded by 1 h pre-treatment in the presence of inhibitors, or vehicle. *Significantly different vs relevant value measured in the absence of inhibitor (P<0.0001; Student's t-test).

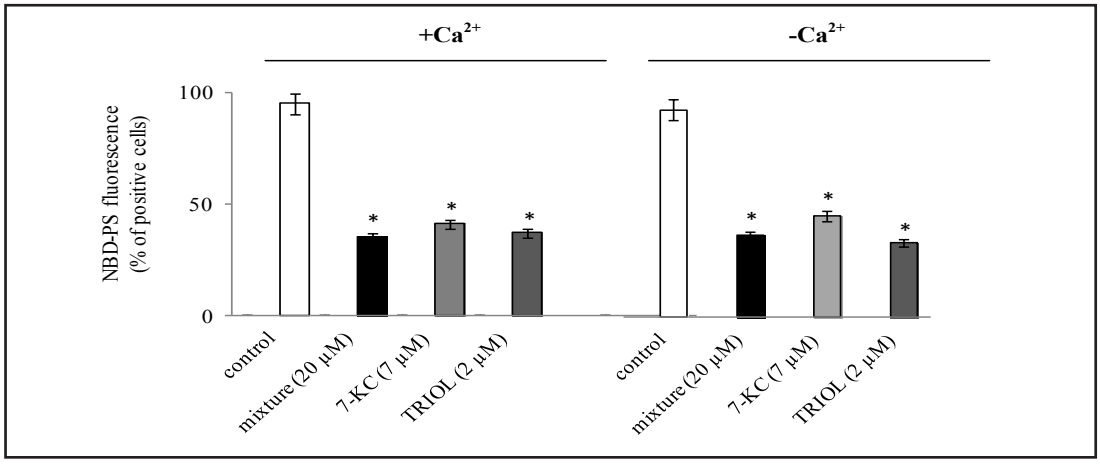

Fig. 5. APL translocase activity in oxysterol-treated human RBCs in the presence or in the absence of Ca^{2+} in the medium. Arithmetic means±SD (n=6) of NBD-PS-associated cell fluorescence after a 48 h incubation of RBCs with the oxysterols. *Significantly different vs control (P<0.0001; Anova associated with Bonferroni's test).

fragility, with release of hemoglobin (Fig. 1, c). The percent amount of lysated cells (8±1%, n=5) was quite lower than the apoptotic ones, showing that oxysterols primarily induced a programmed cell death. Apparently TRIOL only elicited hemolysis. Based on the findings that eryptosis was induced by either the mixture or the individual 7-KC or TRIOL, all other oxysterols were no further assayed individually.

Among several events known to induce eryptosis, an intracellular Ca^{+2} increase is considered a common signaling for PS exposure, associated with a huge number of xenobiotics and a variety of endogenous substances [33, 34]. Cytofluorimetric measurements in the presence of calcium-sensitive Fluo 3-AM provided evidence that a 48 h treatment of RBCs with the oxysterol mixture caused a net increase of the cytosolic calcium level (Fig. 2, a). Both 7-KC and TRIOL were effective, with the effect of TRIOL significantly higher than 7-KC and comparable with that of the mixture. The increase of cytosolic calcium by either the mixture or individual oxysterols was totally prevented by pre-treatment of RBCs with the COX inhibitor ASA (Fig. 2, a), suggesting entry of calcium through the PGE2-activated non-

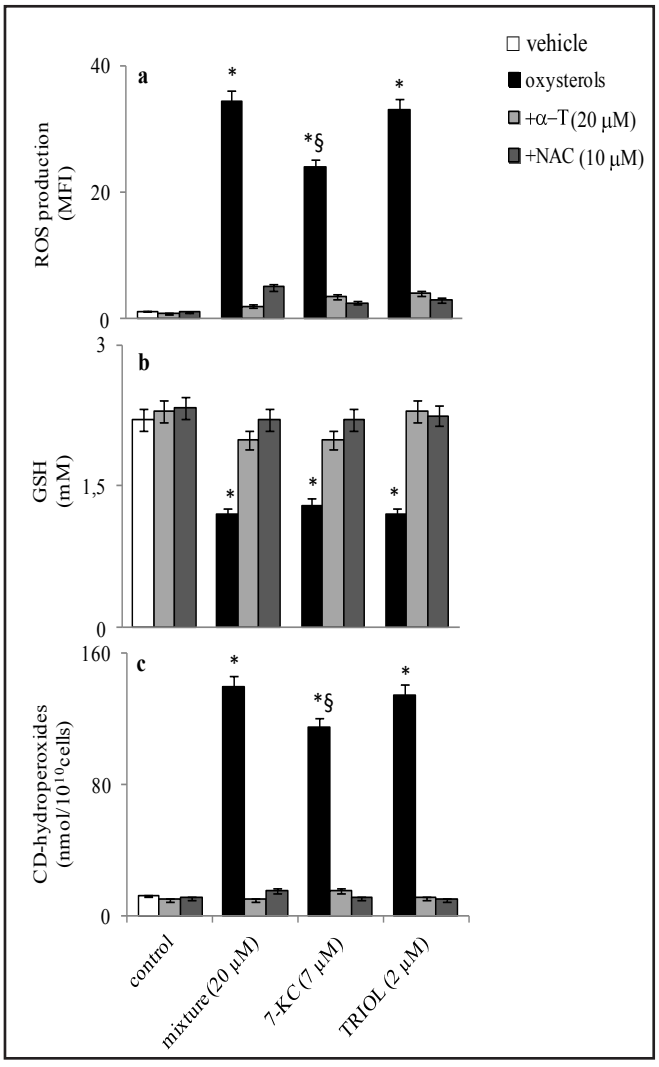

Fig. 6. Oxysterol-induced ROS production (a), GSH depletion (b) and membrane lipid oxidation (c) in human RBCs and effect of antioxidants. Arithmetic means±SD (n=6) of DCFDA-associated MFI (mean fluorescence intensity) (a), GSH levels (b) and CD-hydroperoxides in ghosts (c), after a 48 h incubation of RBCs with the oxysterols preceded by 1 h pre-treatment in the presence of antioxidants or vehicle. * Significantly different vs control (P<0.0001); § significantly different vs mixture and TRIOL (P<0.05; Anova associated with Bonferroni's test).

selective cation channels [35]. Consistent with these results, incubation of the cells with the oxysterols caused a remarkable release of PGE2 into the incubation medium, which was prevented by ASA (Fig. 2, b).

The role of calcium in the eryptotic activity of oxysterols was investigated exposing RBCs to the oxysterols either in the absence of extracellular Ca^{2+}, or in the presence of the cell-permeable calcium-chelating agent BAPTA-AM, or ASA. Either the mixture or the individual 7-KC or TRIOL caused a PS exposure that was ~80% than observed in the presence of Ca^{2+}, under all conditions (Fig. 3, a), whereas the forward scatter was unaltered with respect to control (Fig. 3, b), indicating that the oxysterols induced a substantial calcium-independent membrane scrambling without a modification of the cell volume and shape.

Mechanisms of oxysterol-induced PS externalization

Apart from a calcium-dependent scramblase activity [36], loss of membrane PS asymmetry in activated RBCs may be induced by Ca^{2+}-independent processes, including the activity of protein kinase C [37, 38] and caspase-3 [39, 40]. When RBCs were pre-treated with staurosporin or calphostin C, as PKC inhibitors, or the pan-caspase inhibitor Z-DEV-FMK (Fig. 4), the oxysterol-induced PS externalization appeared strongly inhibited. This occurred both in the presence and in the absence of Ca^{2+} in the medium (Fig. 4). Loss of membrane PS asymmetry may involve inhibition of the membrane APLT or flippase, moving PS from the outer to the inner leaflet of the plasma membrane [41]. The measurement of activity by

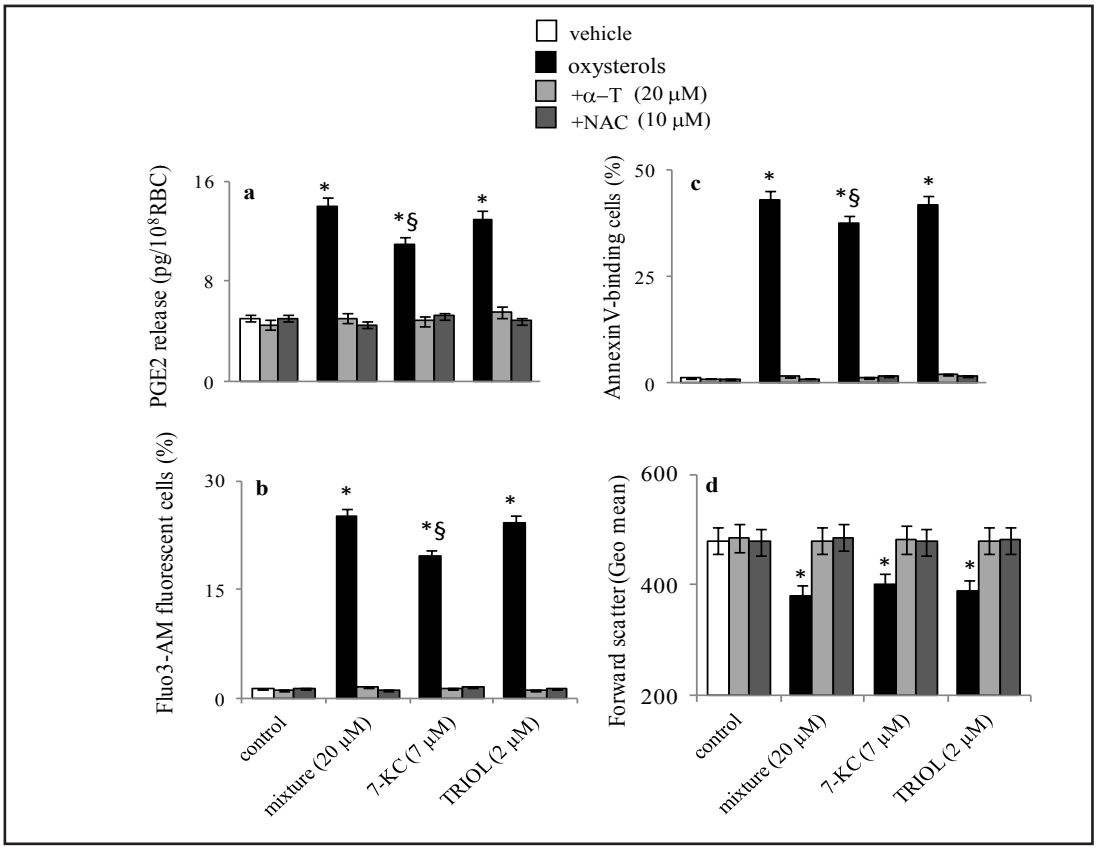

Fig. 7. Effect of antioxidants on the oxysterol-induced PGE2 release (a), Ca^{2+} entry (b), PS externalization (c) and cell shrinkage (d) in human RBCs. Arithmetic means±SD (n=6) of (a), PGE2 released in the medium; (b), fluo3-AM-dependent fluorescence; (c), AnnexinV-FITC associated cell fluorescence; and (d), forward scatter, after a 48 h incubation of RBCs with the oxysterol mixture or individual 7-KC or TRIOL, preceded by 1 h pre-treatment in the presence of antioxidants or vehicle. * Significantly different vs control (P<0.0001); § significantly different vs mixture and TRIOL (P<0.05; Anova associated with Bonferroni's test).

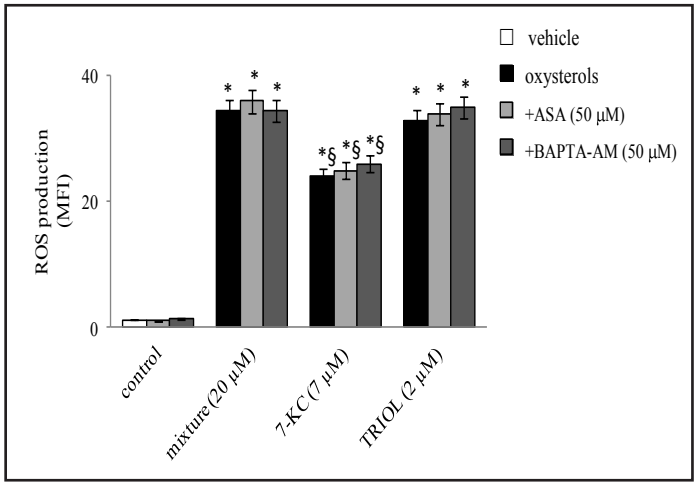

Fig. 8. Oxysterol-induced ROS production in human RBCs in the presence of ASA or BAPTA-AM. Arithmetic means±SD (n=6) of DCFDA-associated MFI (mean fluorescence intensity) after a 48 h incubation of RBCs with the oxysterols preceded by 1 h pre-treatment in the presence of COX inhibitor ASA or cell-permeable Ca^{2+} chelator BAPTA-AM, or vehicle. * Significantly different vs relevant control (P<0.0001); § significantly different vs mixture and TRIOL (P<0.05; Anova associated with Bonferroni's test).

means of fluorescent-labeled phosphatidylserine (NBD-PS) was assessed from the extent of fluorescent phospholipid retained in the plasma membrane after back-extraction with BSA, which extracts exogenously added fluorescent phospholipid from the outer monolayer only. PS-NBD-negative cells therefore are cells in which the flippase is inhibited. Both in

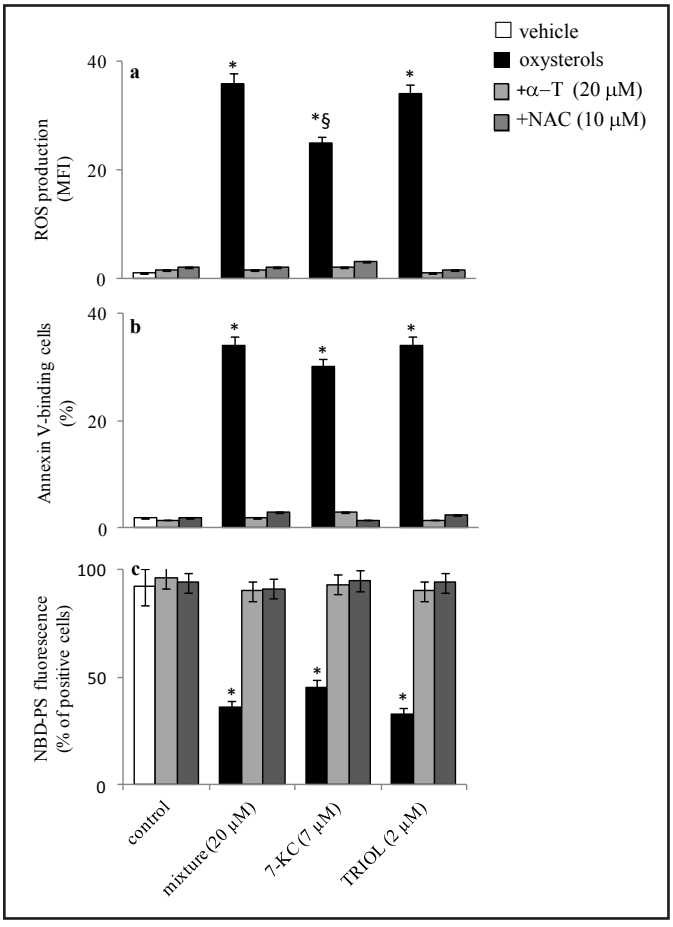

Fig. 9. Effect of antioxidants on ROS production (a), PS externalization (b) and APL translocase activity (c) induced by the oxysterols in human RBCs in Ca^{2+} free medium. Arithmetic means±SD (n=3) of (a), DCFDA-associated MFI (mean fluorescence intensity); (b), AnnexinV-FITC associated cell fluorescence; and (d), NBD-PS-associated cell fluorescence after a 48 h incubation of RBCs with the oxysterol mixture or individual 7-KC or TRIOL in the absence of Ca^{2+}, preceded by 1 h pre-treatment in the presence of antioxidants or vehicle. * Significantly different vs control (P<0.0001); § significantly different vs mixture and TRIOL (P<0.05; Anova associated with Bonferroni's test).

the absence and in the presence of calcium ions, a 48 h treatment with oxysterols resulted in a quite comparable inhibition of the enzyme activity, evident as a loss of residual NBD-PS-associated cell fluorescence (Fig. 5). Overall these findings indicated that apart from calcium-dependent scramblase, oxysterol-induced PS externalization in RBCs involved at least the calcium-independent PKC and caspase-3 activities, as well as inhibition of the APLT-mediated inward transport of PS.

Role of oxidative stress in oxysterol-induced eryptosis

ROS have appeared as mediators of oxysterol-induced apoptosis in a number of cells [42, 43]. Our cytofluorimetric analysis with DCFDA showed that a 48 h treatment with either the oxysterol mixture or the individual 7-KC or TRIOL, induced a remarkable ROS production in RBCs (Fig. 6, a), with a concomitant loss of GSH (Fig. 6, b) and oxidation of membrane lipids evaluated by formation of CD hydroperoxides (Fig. 4, c). The pro-oxidant activity of oxysterols was totally counteracted by pre-treatment with the water-soluble antioxidant NAC, or the lipid antioxidant α-T, which prevented ROS production, GSH depletion and lipid oxidation (Fig. 6). Either NAC or α-T entirely prevented PGE_2 production, calcium entry, externalization of membrane PS, and cell shrinkage (Fig. 7), whereas the oxysterol-induced ROS production was not affected by pre-treatment with ASA or BAPTA-AM (Fig. 8), indicating that ROS generation was upstream production of PGE_2 and calcium entry in the oxysterol-induced signaling axis.

Further experiments explored whether or to what extent oxidative stress influenced the oxysterol-induced PS externalization in the absence of calcium. RBCs were pre-treated with either NAC or α-T before incubating with the oxysterols in the absence of calcium in the medium, for 48 h. Either NAC or α-T prevented ROS production and PS exposure (Fig. 9, a, b), while APLT activity did not appear to be modified with respect to control (Fig. 9, c), showing

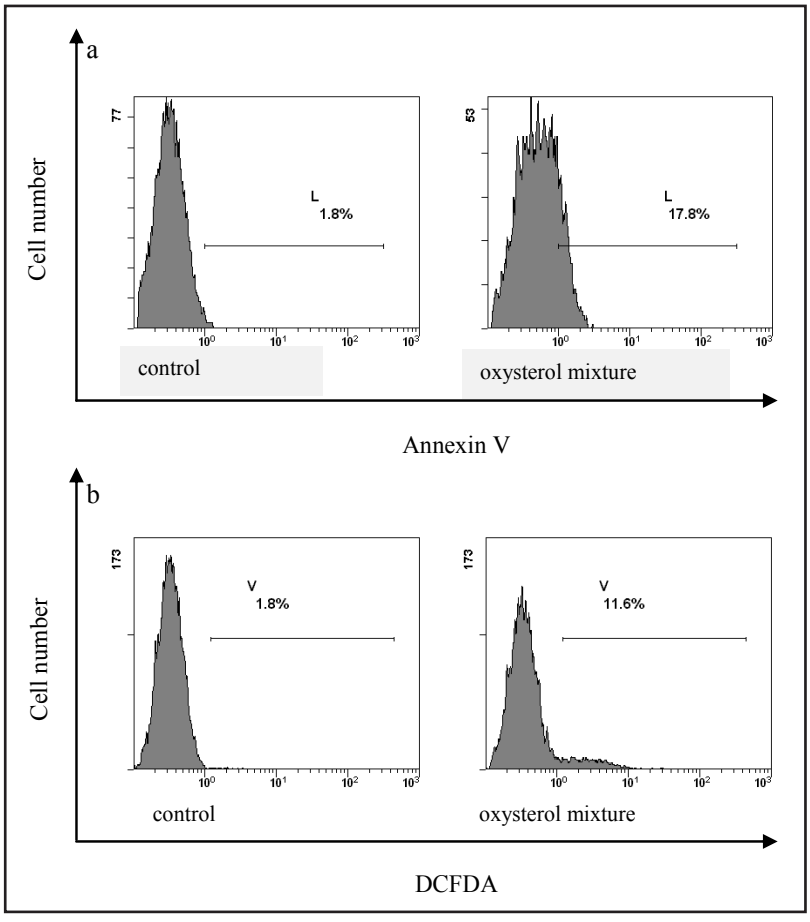

Fig. 10. Externalization of PS (a) and ROS level (b) in RBCs isolated from fresh human blood after a 48 h *ex-vivo* spiking with oxysterol mixture (20 microM). Cells isolated from homologous blood incubated for 48 h with the vehicle only were taken as control. Image representative of separate experiments carried out in duplicate with blood from five volunteers.

that oxidative stress played a pivotal role in the eryptosis triggered by these compounds even in the absence of calcium.

Oxysterol-induced eryptosis ex vivo

A pathophysiological condition of hypercholesterolemia was simulated by e*x vivo* spiking of healthy human blood with the oxysterol mixture. After 48 h incubation, annexin V-binding analysis of isolated RBCs provided evidence of a ten-fold increase of the percent eryptotic erythrocytes with respect to cells from homologous blood incubated in the absence of oxysterols (Fig. 10, a). Parallel measurement of the DCFDA-associated fluorescence showed a remarkable increase of ROS production in cells from the oxysterol-treated *vs* -untreated blood (Fig. 10, b).

Discussion

Toxicity of oxysterols has been reported in various cells [10-17], however activities of these compounds on RBCs are not known. For the first time this study shows that a mixture of oxysterols, qualitatively and quantitatively consistent with the oxysterol pool in plasma of hyper-cholesterolemic subjects, triggered oxidative stress in healthy RBCs, leading to programmed cell death, or eryptosis. The effect was observed either after incubation with isolated cells, or after *ex vivo* spiking of whole blood with the oxysterols. Only 7-KC and TRIOL appeared individually capable of exerting eryptotic activity.

Eryptosis has been associated with a number of events either patho-physiological or caused by external factors, including a huge and ever-increasing number of xenobiotics [33]. These studies provided a very composite picture of this process and individuated many cell

pathways, however various molecular aspects wait to be clarified [34]. In general, a rise of the cytoplasmatic calcium concentration is a main signal for the subsequent series of events leading to the suicidal RBC death, i.e. PS scrambling, activation of K^+ channels, exit of K^+ and Cl^-, followed by loss of water and cell shrinkage [44]. In accordance, a calcium influx into the erythrocytes, with a reduction of cell volume and loss of membrane PS asymmetry were observed as a consequence of the treatment of erythrocytes with the oxysterols.

Main molecular mechanisms through which oxysterols have been shown to influence Ca^{2+} entry into cells include direct biophysical perturbation of plasma-membrane [45], or interaction with protein subunits forming calcium channels leading to modulation of their gating properties [46]. In RBCs, elevation of cytosolic calcium results from opening of plasma-membrane non-selective cation channels of the TRPC6 type activated by PGE2 [47], or eventually of the P-type CaV2.1 [48, 49]. We observed that treatment of RBC with ASA, an inhibitor of COX, fully prevented the oxysterol-induced calcium influx. While ruling out a direct interaction of the oxysterols with these channels [50] these data implied an oxysterol-induced formation of PGE2 in the RBCs, which was also observed. Activation of the arachidonic acid cascade with formation of PGE2 as a part of the apoptotic signaling pathway has been reported in oxysterol-treated macrophages [51], and fibroblast [52], as well as in kidney cell lines [53].

In accordance with the role of calcium in the activity of the Gardos K^+ channels [54], we did not observe cell shrinkage neither in the presence of ASA nor of intracellular Ca^{2+} chelator, nor in the absence of calcium in the incubation medium. These findings exclude that under the applied conditions oxysterols activated K^+ or Cl^- channels independently of Ca^{+2}, as suggested to explain the eryptotic activity of other agents [55].

The increase of the intracellular steady-state levels of ROS has been associated with the cytotoxicity of various oxysterols of patho-physiological interest [51, 56, 57]. Here we observed that oxysterol-induced eryptosis appeared entirely controlled by oxidative stress and such antioxidants as the water-soluble NAC or lipid vitamin E totally prevented ROS generation, calcium entry and PS externalization. In this context, it seems interesting that the oxysterol-induced activation of ROS producing systems in RBCs did not depend on calcium. Our previous observations in human macrophages treated with 7-KC [58] also showed that production of ROS preceded cytoplasmic calcium increase. Consistent with our findings, other reports described anti-eryptotic activity of vitamin C [59] and probucol [60], under conditions of energy depletion and oxidative stress. The molecular mechanisms underlying the effects of all these antioxidants are not entirely clear in our as well as in the reported studies.

Under physiological conditions, the outward gradient of PS is guaranteed by the APL-translocase or flippase [41], counteracting the slow gradient-guided translocation of PS from the inner to the outer leaflet of the bilayer. A rapid and persisting PS exposure occurs following various stimuli, including the apoptotic ones, mainly through a cytosolic calcium increase that activates the phospholipid scramblase activity [61] and inhibits the APL-translocase [62, 63], which finally confines PS at the outer side of the membrane. Nevertheless, in accordance with recent publications [55, 64-66] in our oxysterol-stimulated RBCs Ca^{+2} contributed to, but apparently did not fully account for, PS externalization which was only partially abrogated (20%) by calcium removal. The contribution of calcium-independent mechanisms has been shown by other researchers and considered in this study. The erythrocyte expresses various isoforms of PKC, including calcium-independent [67], the activation of which can cause membrane phospholipid scrambling by a mechanism not requiring Ca^{2+} entry [37, 38]. In addition, the involvement of caspase-3 in the PS externalization has been observed in the absence of calcium in human erythrocytes under oxidative stress [39, 40]. In the absence of calcium we observed ROS production and a PS externalization remarkably blunted in the presence of PKC inhibitors, either calphostin C or staurosporine, or the pan-caspase inhibitor of Z-DEV-FMK, providing evidence of an involvement of these enzymes. It may be mentioned that these inhibitors blunted the oxysterol-induced PS externalization to the same extent either in the absence or presence of calcium ions, suggesting that calcium-

independent isoform of PKC and caspase-3 may play key roles in the eryptotic signaling of these compounds. These considerations cannot rule out the involvement of other PKC isoforms in the presence of calcium. Caspase-3 activation, GSH decrease and membrane lipid oxidation have been associated to impairment of the membrane APL translocase activity in RBCs [40, 68, 69], which has also been observed in the present study. This enzyme is inhibited by calcium ions (62, 63), however oxidative stress has appeared a required event even in the presence of calcium [39, 40, 69, 70]. Our findings that in the absence of calcium either vitamin E or NAC, while preventing ROS production, prevented the oxysterol-induced inactivation of the enzyme, provides evidence of a major role of oxidative stress, over PKC- or caspase- dependent mechanisms, in the APLT inhibition. Generation of ceramide, a factor known to increase RBC sensitivity to inner calcium reported to cause PS translocation in xenobiotic-treated RBCs in the absence of extracellular Ca^{+2} [54, 55], has appeared to be associated with the activity of 7-KC in leukemia cells [71]. However, the evidence that PS scrambling occurred also in the presence of an intracellular calcium chelating agent may suggest, but not prove that ceramide was not involved in the eryptotic activity of oxysterols.

Taken together, these findings suggest that oxysterol-induced and oxidative stress-dependent PS appearance at the RBC surface results from coordinated and complementary processes involving different factors. Signaling pathway/s and molecular mechanism/s leading oxysterol-treated RBCs to activate these processes deserve further studies, now in progress in our laboratory.

In this study, among the components of the mixture, only the individual 7-KC and TRIOL induced oxidative stress, Ca^{2+} influx and eryptosis, showing each compound an activity almost comparable with that of the whole mixture. It is not possible to conclude which one and how much, when combined, TRIOL and 7-KC concur to the eryptosis or if their effects are mutually attenuated or dampened by other component/s of the mixture. As both oxysterols appeared to share a similar pathway of action involving ROS production, it seems reasonable that sensitivity or abundance of the involved RBC system(s) may represent a limiting factor to their cooperation. In this context it may be interesting that TRIOL also showed hemolytic activity suggesting some additional activity.

Finally, the observation that redox unbalance and eryptosis were observed in RBCs isolated after *ex vivo* spiking of whole blood with the oxysterol mixture, suggests a patho-physiological significance of our findings. Oxysterol toxicity at endothelial cells and macrophages has been involved in the pathogenesis of atherosclerosis and cardiovascular diseases [1]. It is interesting to mention that RBCs from hyper-cholesterolemic patients show higher levels of oxidized lipids and lower concentration of membrane protein thiols than healthy RBCs [72], which could be an indication of the oxysterol toxicity and proneness of cholesterolemic RBCs to eryptosis. Since PS-exposing RBCs can adhere to vascular endothelial cells [25, 27] impairing microcirculation, and possess pro-thrombotic and blood clotting activity [23, 24], our study suggests that the eryptotic activity of oxysterols can also contribute to development of atherosclerosis and ischemic problems associated with hyper-cholesterolemia. In this context, the observed release of inflammatory mediator PGE2 from oxysterol-treated RBCs, could further enhance the intravascular inflammatory status.

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