7-KETOCHOLESTEROL INHIBITS Na,K-ATPase ACTIVITY BY DECREASING EXPRESSION OF ITS α1-SUBUNIT AND MEMBRANE FLUIDITY IN HUMAN ENDOTHELIAL CELLS

M. J. DURAN5, S. V. PIERRE6, P. LESNIK2, G. PIERONI3, M. BOURDEAUX4, F. DIGNAT-GEORGES5, J. SAMPOL5, J. M. MAIXENT1

1 INSERM U927, Poitiers, France
2 INSERM U-551, Paris, France.
3 INSERM U-476, Marseille, France.
4 Laboratoire de Biophysique II, Faculté de Pharmacie, Marseille, France.
5 INSERM U603, Marseille, France.
6 Department of Physiology and Pharmacology, University of Toledo, Toledo, Ohio, USA.

Université de Poitiers, IUP Génie Physiologique-Informatique, Faculté des Sciences Fondamentales et Appliquées, 40 Av. du recteur Pineau 86022 Poitiers Cedex France. Tel: +33 5 49 45 36 32, Fax: +33 5 49 45 36 41, E-mail: jean.michel.maixent@univ-poitiers.fr

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Abstract – As cholesterol, oxysterols, can insert the cell membrane and thereby modify the functions of membrane-bound proteins. The Na,K-ATPase is very sensitive to its lipid environment, seems to be involved in important endothelial functions as the regulation of nitric oxide (NO) release. The effects of 7-ketocholesterol, an oxysterol present in oxidized LDL, was investigated on Na,K-ATPase in isolated human endothelial cells. Cells were incubated 24h with lecithin-, cholesterol- or 7-ketocholesterol liposomes (6 µg/ml). K+-stimulated paranitrophenyl phosphatase activity, reflecting Na,K-ATPase activity, was evaluated as well as cell viability and lipoperoxidation. The expression of Na,K-ATPase subunits mRNAs and membrane fluidity were also investigated. As Na,K-ATPase and nitric oxide seem to be related, we determined the production of NO and the expression of endothelial NO synthase mRNAs. Na,K-ATPase activity was strongly decreased by 7-ketocholesterol. This decrease, not related to lipoperoxidation, was correlated with a decreased expression of the Na,K-ATPase α1-subunit messengers and with rigidity of plasma membranes. Cholesterol induced similar effects but was less potent than 7-ketocholesterol. Basal NO production and expression of endothelial NO synthase mRNAs were not modified by 7-ketocholesterol. Our new findings demonstrate that 7-ketocholesterol, used at non toxic doses, was very potent to disrupt the transport of ions by Na,K-ATPase and perturb membrane structure. These data demonstrate that 7-ketocholesterol induces endothelial dysfunction without cell death that may contribute to early events in atherosclerosis.

Key words: Na,K-ATPase, oxysterol, 7-ketocholesterol, endothelium.

INTRODUCTION

Oxysterols, like 7-ketocholesterol, seem to be implicated in the initiation and/or development of atherosclerosis (5,46). High levels of cholesterol oxides, produced in vivo by autooxidation of cholesterol or derived from food (6,38), have been found in human hypercholesterolemic plasma (49) and in atherosclerotic aortas from cholesterol-fed rabbits (36).

Oxysterols have been shown to possess many potent and diverse biological activities in the vascular wall (20). Indeed, numerous studies have reported the cytotoxicity of oxysterols towards endothelial cells (4,33,41), smooth muscle cells (28,20,26) as well as fibroblasts (10). Oxidized derivatives of cholesterol are also known to impair histamine-activated nitric oxide release in endothelial cells (11) and may contribute significantly to the oxidized LDL-induced alteration of vasomotor phenomena (25). In particular, 7-ketocholesterol, one of the major oxysterol present in oxidized low density lipoproteins, is a potent inhibitor of endothelium-dependent relaxation (12). As cholesterol, oxygenated sterols can insert in cell membranes and thereby modify the structure and the function of membrane-associated proteins like the Na,K-
ATPase (27,24). The membrane-bound enzyme Na,K-ATPase or Na pump, present in all the plasma membranes of mammalian cells and specifically inhibited by digitalis, catalyzes the coupled active transport of Na+ and K+ across the plasma membrane (3) and is very sensitive to its lipid environment (16,24). This enzyme plays a fundamental role in cellular function and may be important in endothelial cells as it is implicated in vasomotion of arteries (36) and seems to be involved in the regulation of nitric oxide release (48).

In a previous study, we have shown that cholesterol (24) and oxidized LDL (31) was able to inhibit Na,K-ATPase activity in human endothelial cells. However, the effect of oxysterols on Na,K-ATPase activity is unknown in this model. The role of oxygenated cholesterol derivatives in the atherogenic process was investigated by exposing cultured human umbilical vein endothelial cells (HUVECs) to 7-ketocholesterol. The purpose of this study was to test the effects of this oxysterol on human endothelial Na,K-ATPase.

**MATERIALS AND METHODS**

**Cell culture**

Human umbilical vein endothelial cells (HUVECs) were obtained from fresh umbilical cord veins and cultured according to the method of Jaffe et al. (1973). Cells were grown in RPMI 1640 medium (Gibco BRL, Cergy Pontoise, France) containing 20% fetal calf serum (Gibco), 1% penicillin-streptomycin (Sigma, St. Louis, MO), 1.25% endothelial cell growth supplement (ECGS, Sigma), 2 mM L-glutamine (Gibco) and 1% heparin (Sigma) at 37°C under 95% air (5% CO2). After reaching confluence, cells were subcultured onto T-25 dishes in order to obtain sufficient amount of cellular material. We used only 100% pure cultures as assessed by morphologic and immunologic criteria (von Willebrand’s factor expression).

**Lipid vesicles**

Lipid vesicles were made up according to a modified method from Argobast et al. (1976). 7-ketocholesterol and lecithin were mixed in ethanol. Evaporation of solvent was carried out by gentle warming under reduced pressure and nitrogen atmosphere. Dispersions were performed on physiological saline (0.9% NaCl) by 10 min pulse with a ultrasonic probe (Sonoreactor, Undatim Ultrasonics, Louvain-la-Neuve, Belgium) and the lipid vesicles produced were stored at 4°C under nitrogen atmosphere until use. Pure lecithin liposomes and cholesterol-enriched liposomes were made up in the same conditions in order to define the effect of lecithin and cholesterol on endothelial cells. The size distribution of the vesicles was estimated using the quasi-elastic light scattering method (Granulometer R.T.G., SEMATEch, Nice, France). Before incubation with endothelial cells, dispersions were sterilized by filtration through a 0.45 μm Millipore filter (St Quentin en Yvelines, France).

**Lipid treatments**

At confluence, subcultured cells were incubated with pure lecithin vesicles (6 μg/ml), cholesterol-enriched liposomes, 7-ketocholesterol enriched vesicles or just culture medium serving as control. Cholesterol and 7-ketocholesterol liposomes were also given at the dose of 6 μg lecithin/ml which provided 6 μg/ml of cholesterol or 7-ketocholesterol to the cells. Incubations were performed at 37°C for 24h.

**Cell viability**

Cell viability was assessed with the Cell Proliferation Reagent WST-1 (Boehringer Mannheim, Mannheim, Germany), a colorimetric assay. The quantification of cell viability is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. After incubation, the ready-to-use solution WST-1 was added to the cells cultured in microtiter plates (10 μl per well). Cells were then incubated 2 hours at 37°C (5% CO2) and absorbance of the samples was measured at 450 nm against a background control (culture medium plus WST-1 in the absence of cells).

**K+-stimulated paranitrophenyl phosphatase activity**

Na,K-ATPase activity was measured as K+-stimulated paranitrophenyl phosphatase (pNPPase) activity (31) using a modified method from Wald et al. (1996). Briefly, assays were performed on intact cells cultured in 24-wells plates. Cell monolayer was washed two times with buffered isotonic sucrose (250 mM sucrose, 20 mM imidazole, pH 7.4). pNPPase activity was then assessed using a reaction mixture with or without KCl during 5 min at 37°C. The composition of the reaction medium used was: 6 mM MgCl2, 20 mM imidazole, 250 mM sucrose, 8 mM pNPP (Sigma) pH 7.4 with or without 20 mM KCl. After the reaction, NaOH 1 M was added to permit colour development of the reaction product, paranitrophenyl phosphate (pNP), and reaction medium was kept at 4°C during 5 minutes. Optical density was then measured at 410 nm using a UV/Vis Kontron spectrophotometer. K+-stimulated pNPPase activity was calculated as the difference in paranitrophenyl (pNP) production with or without KCl. Values were expressed as μmole of pNP produced per hour and per milligram of proteins.

**Lipoperoxidation assay**

Lipoperoxidation was assessed using the measurement of the thiobarbituric acid reactive substances (TBARS) as described previously (31). Briefly, after incubation, culture medium was removed and 0.2 ml of 50% (w/v) thichloroacetic acid plus 0.3 ml of 1.3% (w/v) thiobarbituric acid in 0,3% (w/v) NaOH were added. Samples were heated at 60°C in a water bath for 40 minutes and then cooled on ice. Absorbance was measured as the difference at 530 and 600 nm to eliminate light scattering interference. TBARS were quantified using a standard curve of malondialdehyde.

**Nitrite assay**

Nitric oxide production was evaluated by nitrite quantification as described previously (2). After incubation, culture media were removed and nitrates were quantified using the Griess reagent. Briefly, the reagent was added to culture medium (1:1), incubated for 10 minutes at room temperature and absorbance was measured at 540 nm. Nitrite concentration was evaluated with sodium nitrite as a standard.
**Semi-quantitative RT-PCR**

Expression of α1 and β3 subunits of the Na,K-ATPase, endothelial NO synthase and glyceraldehyde 3-phosphate dehydrogenase mRNAs was evaluated by semi-quantitative RT-PCR after extraction of total RNAs. Culture medium was removed, cells were carefully washed twice in phosphate buffered saline and 1 ml of TRI REAGENT® (Molecular Research Center, Cincinnati, OH) was added to culture dishes (2.5 x 106 cells/dish). Isolation of RNA was undertaken according to the guanidium thiocyanate-phenol-chloroform protocol (9). Total RNA was recovered in diethyl pyrocarbonate treated water and A260/A280 ratio was measured. Only RNA preparations whose ratio exceeded 1.7 were further treated (stored at -80°C until use). The RNA mixture was heated for 5 min. at 65°C. The reverse-transcription was then performed with 2 µg of RNA in a final volume of 20 µl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 125 µM of each deoxynucleotides (Eurogentec, Angers, France), 10 mM dithiothreitol, 100 pmoles of hexarandom primers (pd(N)6, Pharmacia Biotech, Orsay, France), 40 units of RNAsin (Promega, Charbonnières, France) and 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Cergy Pontoise, France). After two hours at 37°C, the enzyme was inactivated by boiling (5 min. at 95°C). One µl of the reverse transcribed mixture was added to the PCR incubation mixture containing 67 mM Tris-Cl (pH 8.8), 16 mM (NH4)2SO4, 0.1% Tween 20, 1.5 mM MgCl2, 50 µM of each deoxynucleotides, 20 pmoles of each primers, 2.5 units of Taq DNA polymerase (Eurobio, Les Ulis, France) up to a final volume of 50 µl. The conditions for the amplification reactions are reported in Table 1. Amplification products were resolved by electrophoresis in a 2% agarose gel, stained by ethidium bromide (0.5 µg/ml) and photographed under UV light by a computer assisted camera (Kodak Digital Camera DC 120). Quantification of each band was performed with an image analysis software (MatLab Software, The Mathworks, Natick, MA).

<table>
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<th>Target gene (Accession number)</th>
<th>Sequences (5’ to 3’)</th>
<th>Position</th>
<th>Product size</th>
<th>Annealing temperature</th>
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<td>ATC ATG CTT CAT GAA CAA ATT ACC ATC C (S) CTA ATG GTA GGT TCC CTC TTC CAC CCA (AS)</td>
<td>2865-2899</td>
<td>525 bp</td>
<td>58°C</td>
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<td>Na,K-ATPase 33 (NM_001679)</td>
<td>GAG CAG ATT CCT AGA GCA GGC GAC A (S) AGA GGA CTC TGA ACA CTA TGG TGC C (AS)</td>
<td>220-241</td>
<td>789 bp</td>
<td>59°C</td>
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<tr>
<td>eNOS* (N93738)</td>
<td>CCA GCT AGC CAA AGT CAC CAT (S) GTC TCG GAG CCA TAC AAG ATT (AS)</td>
<td>1276-1296</td>
<td>354 bp</td>
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<tr>
<td>G3PDH* (NM_002046)</td>
<td>GGG AAG CTC TGG CGT GAT G (S) CTG TGG CTG TAG CGG AAT TC (AS)</td>
<td>541-559</td>
<td>380 bp</td>
<td>55°C</td>
</tr>
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</table>


**Membrane fluidity**

Membrane fluidity was assessed on microsomal fractions prepared according to Mayol et al. (1999). Endothelial membranes, suspended in phosphate buffer, were labelled with the lipid-soluble fluorophore 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH, Sigma), which is incorporated at the lipid-water interface of the membrane bilayer (13). Equal volumes of the plasma membrane suspension (100 µg proteins per ml) and 5 µM TMA-DPH suspension were incubated 30 min. at 37°C in the dark with gentle stirring. The TMA-DPH suspension was prepared in phosphate buffer just before use from a 5 mM stock solution in dimethylformamide. Fluorescence measurements were performed at 37°C. Excitation and emission wavelengths were 365 and 428 nm, respectively. Anisotropy measurements were carried out on a model SLM 4800 polarization spectrofluorometer as described previously (7).

**Statistical analysis**

Results are expressed as means ± SE. Statistical analysis was done using one-way analysis of variance (ANOVA) followed with the Tukey’s post-test (GraphPad Prism®, GraphPad Software Inc., San Diego, CA). Values of p<0.05 were considered statistically significant.

**RESULTS**

**Cell viability**

Cell viability was assessed with the WST-1 reagent cleaved by mitochondrial dehydrogenases in viable cells. After the different incubations, no toxic effect on cell viability was observed when cells were treated with either lecithin, cholesterol or 7-ketocholesterol (data not shown).

**K+-stimulated paranitrophenyl phosphatase activity**
The K+-stimulated paranitrophosphoethyl phosphatase (pNPPase) activity, reflecting the Na,K-ATPase activity, measured on intact endothelial cells is shown in Figure 1. Incubation of HUVECs with lecithin liposomes significantly decreased the K+-stimulated pNPPase activity in comparison to control conditions (-21%, p<0.01 vs. Control). When cells were incubated with cholesterol, K+-pNPPase activity was slightly inhibited but not statistically different from the lecithin group. In contrast, 7-ketocholesterol markedly decreased the K+-dependent pNPPase activity (-44%) in comparison with the lecithin-treated group (p<0.001).

Figure 1. K+-stimulated paranitrophosphoethyl phosphatase activity. Na,K-ATPase activity was measured as the K+-stimulated paranitrophosphoethyl phosphatase activity in endothelial cells incubated for 24h with lecithin (6 µg/ml), cholesterol (6 µg/ml), 7-ketocholesterol (6 µg/ml) or culture medium. Values, expressed in µmol pNP/h/mg proteins, are means ± SE of ten experiments. * p<0.01, ** p<0.001 vs. Control, # p<0.001 vs. Lecithin, $ p<0.001 vs. Cholesterol. 7K: 7-ketocholesterol.

Lipoperoxidation

Cell lipid peroxidation was evaluated by the thiobarbituric reactive substances (TBARS) assay after incubation of HUVECs monolayers for 24h with the different types of liposomes. The results in pmoles of malondialdehyde, (means ± SE of cells from six different cords), are respectively for Control 132 ± 9, Lecithin 121 ± 11, Cholesterol 131 ± 2, 7-ketocholesterol 124.8 ± 11.6 and show that exposure of cells with lecithin, cholesterol or 7-ketocholesterol did not modify basal TBARS content.

Expression of α1 and β3 messengers of Na,K-ATPase

The expression of mRNAs was assessed by semi-quantitative RT-PCR. All the results were corrected with the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNAs expression and expressed as percent from control. In a previous study, we have shown that only Na,K-ATPase α1 and β3 subunits messengers are expressed in HUVECs (30). The expression of the Na,K-ATPase α1 subunit mRNAs in the different groups is represented in Figure 2. Cholesterol and 7-ketocholesterol strongly decreased the expression of the α1 subunit mRNAs (p<0.05). No changes were observed when cells were incubated with lecithin.

The expression of the Na,K-ATPase β3 subunit mRNAs was not modified by the different incubations (Figure 3).

Figure 2. RT-PCR analysis of Na,K-ATPase α1 subunit. HUVECs were incubated for 24h with lecithin (6 µg/ml), cholesterol (6 µg/ml), 7-ketocholesterol (6 µg/ml) or culture medium. Electrophoresis analysis of endothelial Na,K-ATPase α1 subunit amplification products is representative of four separated experiments. Amplification products (525 bp) were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gel. Na,K-ATPase α1 subunit / G3PDH mRNAs ratios were defined as 100% in control conditions. Values are means ± SE of four separated experiments. *p<0.05 vs. Control, # p<0.05 vs. Lecithin. 7K: 7-ketocholesterol.

Endothelial membrane fluidity

Anisotropy was determined on purified endothelial plasma membranes at 37°C using the fluorescent probe TMA-DPH usually chosen to measure fluidity changes at the surface of the lipid bilayer. Incubation of HUVECs with lecithin did not induce changes in membrane fluidity (Figure 4). Values of anisotropy were higher in cholesterol and 7-ketocholesterol-treated human endothelial cells, indicating that the membranes from these groups were more rigid than those from the control group (respectively p<0.05 and p<0.001 vs. Control). 7-ketocholesterol was more potent than cholesterol to induce membrane rigidification of HUVECs.

Nitric oxide

Nitric oxide was measured using an indirect
method by the quantification of the nitrites produced in the medium culture. Results, represented in Figure 5, show that lecithin led to an increase in the nitrite content which was not statistically different from the control. In a same way, cholesterol increased nitrite production in a greater extend (1.8-fold when compared to the lecithin group, p<0.001). Nitrite content of 7-ketocholesterol-treated cells was not different from the lecithin ones, indicating that 7-ketocholesterol, at the dose used, has no effect on nitrite production.

Figure 3. RT-PCR analysis of Na,K-ATPase β3 subunit.
HUVECs were incubated for 24h with lecithin (6 µg/ml), cholesterol (6 µg/ml), 7-ketocholesterol (6 µg/ml) or culture medium. Electrophoresis analysis of endothelial Na,K-ATPase β3 subunit amplification products is representative of four separated experiments. Amplification products (798 bp) were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gel. Na,K-ATPase β3 subunit / G3PDH mRNAs ratios were defined as 100% in control conditions. Values are means ± SE of four separated experiments. 7K: 7-ketocholesterol.

Figure 4. Endothelial cell membrane fluidity.
Anisotropy measurements were determined at 37°C using 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene as the fluorescent probe. HUVECs were incubated 24h with lecithin (6 µg/ml), cholesterol (6 µg/ml), 7-ketocholesterol (6 µg/ml) or culture medium. Electrophoresis analysis of endothelial NO synthase expression levels was representative of four separated experiments. Amplification products (354 bp) were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gel. eNOS / G3PDH mRNAs ratios were defined as 100% in control conditions. Values are means ± SE of four separated experiments. ** p<0.001 vs. Control, $ p<0.005 vs. Lecithin, # p<0.05 vs. 7K. 7K: 7-ketocholesterol.

Figure 5. Nitric oxide assay.
Nitric oxide was measured as nitrite production in culture medium of HUVECs incubated 24h with lecithin (6 µg/ml), cholesterol (6 µg/ml), 7-ketocholesterol (6 µg/ml) or culture medium. Values, expressed as percent from control, are means ± SE of six separated experiments. * p<0.001 vs. Control and Lecithin, # p<0.001 vs. Cholesterol. 7K: 7-ketocholesterol.

Expression of eNOS mRNAs

The expression of endothelial nitric oxide synthase mRNAs, evaluated by semi-quantitative RT-PCR, is shown in Figure 6. In HUVECs, nitric oxide production depends only of the endothelial NO synthase activity (17,34). Endothelial NO synthase messengers of lecithin-treated cells were slightly decreased but not statistically different from control ones. More, cholesterol decreased eNOS mRNAs to a higher degree than lecithin (30%, p<0.005 vs. Lecithin). No changes were observed when cells were incubated with 7-ketocholesterol in comparison with the lecithin-treated cells.

Figure 6. RT-PCR analysis of endothelial NO synthase.
HUVECs were incubated 24h with lecithin (6 µg/ml), cholesterol (6 µg/ml), 7-ketocholesterol (6 µg/ml) or culture medium. Electrophoresis analysis of endothelial NO synthase amplification products is representative of four separated experiments. Amplification products (354 bp) were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gel. eNOS / G3PDH mRNAs ratios were defined as 100% in control conditions. Values are means ± SE of four separated experiments. ** p<0.001 vs. Control, $ p<0.005 vs. Lecithin, # p<0.05 vs. 7K. 7K: 7-ketocholesterol.
ketocholesterol.  

**DISCUSSION**

The aim of the study was to test the effects of 7-ketocholesterol, a major oxysterol present in oxidized LDL, on Na,K-ATPase in human umbilical venous endothelial cells.

Our results clearly showed that 7-ketocholesterol is a potent inhibitor of the Na,K-ATPase activity in human endothelial cells. Similar results have been described in smooth muscle cells (27), while an enhanced Na,K-ATPase activity has been reported in endothelial cells after incubation of cells with a cholesterol oxide mixture for only 2h (24). Lecithin and cholesterol also inhibit basal Na,K-ATPase activity but to a lower degree than 7-ketocholesterol.

As Na,K-ATPase is sensitive to oxidation (14,29,31), one would think that the decreased activity obtained with 7-ketocholesterol was due to an oxidative process mediated by this oxysterol. But 7-ketocholesterol, as well as cholesterol and lecithin, did not lead to the production of oxidized lipids in endothelial cells at the dose used. This oxysterol seems to act, in our model, in another manner than the oxidative stress induced by lipoperoxidation. Indeed, 7-ketocholesterol inhibited the expression of the Na,K-ATPase catalytic subunit, i.e. α1, mRNAs and decreased endothelial membrane fluidity. The fact that 7-ketocholesterol, like cholesterol, inhibited the expression of the Na,K-ATPase α1 mRNAs suggest that the promoting region of the human Na,K-ATPase α1 subunit gene contains a sterol regulatory elements down-regulated by cholesterol and its oxidized derivatives as described by Thewke et al. (1998). In our experiments, 7-ketocholesterol and cholesterol, to a minor degree, induced a decrease in membrane fluidity. Previous reports have shown that oxidative compounds of oxidized LDL were able to decrease membrane fluidity (45,32). In this study, Na,K-ATPase activity was inversely related to membrane fluidity (r² =0.9).

Thus, in our experiments, the decrease in Na,K-ATPase activity induced by 7-ketocholesterol seems to be due to a decrease in the expression of the Na,K-ATPase catalytic subunit mRNAs and to a decrease in membrane fluidity. Sterols and their derivatives are known to be potent inhibitors of cholesterol biosynthesis in cells and animals (28,8) by inhibiting the activity and the gene transcription of HMG-CoA reductase (43,44). This may result in depletion of the cholesterol from cell membranes. As cholesterol depletion increased Na,K-ATPase activity (18), impairment of cholesterol biosynthesis seems to be unlikely in our experiments. Inhibition by cholesterol oxides of cellular cholesterol efflux (15) leading to an increase of cholesterol can explain the decreased activity of endothelial Na,K-ATPase activity as cholesterol enrichment inhibited this activity (24). Moreover, modification of the cellular composition of fatty acids by cholesterol oxides (39) may also participate to the impaired Na,K-ATPase activity by modifying endothelial membrane fluidity. The decreased membrane fluidity observed in our study seems not to be specific to Na,K-ATPase since other membrane bound enzymes like 5’-nucleotidase (27) and Ca²⁺-ATPase (50) are affected by oxysterols.

All the changes induced by 7-ketocholesterol in this study were not linked to cellular toxicity as described by Zhou et al. (2000).

Na,K-ATPase plays a fundamental role in cellular function and may be important in endothelial cells (19) as the inhibition of the enzyme lead to an increase of nitric oxide release (48). Inhibition of Na,K-ATPase by cholesterol and lecithin led to an increased nitric oxide production as described by Xie et al. (1993). In these groups, nitric oxide content was inversely related to the expression of eNOS mRNAs. In contrast to cholesterol and lecithin, 7-ketocholesterol failed to modify NO production and the expression of eNOS although oxysterols are known to impair the release of nitric oxide (11).

Our findings demonstrate that 7-ketocholesterol, used at non toxic doses, was very potent to disrupt the transport of ions by Na,K-ATPase and perturb membrane structure. These results help to understand the mechanism by which 7-ketocholesterol led to vascular injury and atherosclerotic lesion development.

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