

# Oxysterols from oxidized LDL are cytotoxic but fail to induce hsp70 expression in endothelial cells

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**Abstract** Oxidized low density lipoprotein (OxLDL) possesses several proatherogenic characteristics, among which a marked cytotoxicity. *In vitro*, cytotoxicity of OxLDL to endothelial cells is associated with an increase in the expression of the inducible form of heat shock protein 70 (hsp70), generally regarded as a cytoprotective protein. Oxidized derivatives of cholesterol which form upon LDL oxidation are cytotoxic. Moreover, most of the OxLDL cytotoxicity is due to its lipid moiety, in particular to oxysterols. In this report we demonstrate that although oxysterols identified in OxLDL are cytotoxic, they cannot trigger the increase in hsp70 expression observed with intact oxidized lipoproteins. We speculate therefore that oxysterols may represent the most toxic form of oxidized lipids in LDL because they cannot activate a rescue mechanism (i.e. the hsp response) and may contribute significantly to cell death within atherosclerotic plaques.

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**Key words:** Stress protein; 7-Hydroxycholesterol; 7-Hydroperoxycholesterol; 7-Ketocholesterol; 5,6-Epoxycholesterol; 25-Hydroxycholesterol

## 1. Introduction

Oxidation of low density lipoprotein (LDL) is believed to play a key role in the pathogenesis of atherosclerosis [1–3]. One of the potentially atherogenic properties of OxLDL is its cytotoxicity to the cells of the artery wall [3,4]. Cytotoxicity of OxLDL could initiate and worsen atherosclerosis by giving rise to cellular dysfunction in the early stages of lesion formation and by causing cell death in advanced lesions, thus leading to the formation of a necrotic core [5,6].

Cells facing toxic stimuli rapidly and preferentially synthesize a family of cytoprotective proteins, the heat shock proteins (hsps) [7], which can prevent damage to other cellular proteins and allow denatured polypeptides to reacquire their native conformation [8]. This stress response represents one of the basic mechanisms of cellular defense. We previously re-

ported that oxidized LDL triggers the expression of the inducible form of hsp70 in cultured endothelial cells, and that hsp70 induction is closely associated with OxLDL cytotoxicity [9]. However, the components in OxLDL responsible for hsp70 induction are still unknown. It was demonstrated that the cytotoxicity of Cu<sup>2+</sup>-oxidized LDL could be mimicked by its lipid extract and that two cytotoxic oxysterols, 7-keto and 7-OH, are present in OxLDL at concentrations sufficient to account for the toxicity of the lipoprotein to smooth muscle cells [10]. Therefore we evaluated whether oxysterols in OxLDL can induce hsp70 expression in human endothelial cells. Our results show that oxysterols in OxLDL fail to do so, although being cytotoxic. This observation stresses the concept that oxysterols might be the most toxic component of oxidized LDL [11,12], by acting through mechanisms that by-pass the induction of hsp expression.

## 2. Materials and methods

### 2.1. Sterols

Cholesterol, 7 $\beta$ -OH, 7-keto, 25-OH and 5,6 $\alpha$ -epoxycholesterol were from Sigma (St. Louis, USA). 7 $\alpha$ -OH was from Steraloids (Wilton, USA). 5 $\alpha$ -hydroperoxycholest-6-en-3 $\beta$ -ol (5 $\alpha$ -OOH) was synthesized according to Nickon and Bagli [13] from 500 mg pure cholesterol (Sigma, Sigma Grade > 99% purity). 7 $\alpha$ -hydroperoxycholest-5-en-3 $\beta$ -ol (7 $\alpha$ -OOH) was obtained by allylic isomerization of 5 $\alpha$ -OOH present in the mixture described above [14].

### 2.2. Lipoproteins

LDL (1.019–1.063 g/ml) were obtained from freshly isolated human plasma by preparative ultracentrifugation [15] and dialyzed in PBS containing 0.01% EDTA. Protein content was determined by the Lowry method [16]. LDL (0.2 mg protein/ml) were oxidized with 20  $\mu$ M CuSO<sub>4</sub> for 24 h at 37°C, as previously described [9].

### 2.3. Lipids

Total lipids from LDL and OxLDL were extracted with chloroform/methanol (2:1, vol:vol), containing 0.01% butylated hydroxytoluene (BHT) [17]. Lipids were separated in neutral and polar fractions using Extract-Clean NH<sub>2</sub> columns (500 mg/2.8 ml) (Alltech, Deerfield, USA) by the method of Hughes et al. [10]. Oxysterols were obtained by fractionation of neutral lipids on Extract-Clean NH<sub>2</sub> column. Purified lipid fractions were dissolved in and added to the culture medium.

### 2.4. Cells

Human umbilical vein endothelial cells (HUVEC) were isolated according to established procedures [18], cultured under standard conditions in medium M-199 containing 20% FCS, heparin (15 U/ml) and ECGF (20  $\mu$ g/ml) (Boehringer Mannheim, Mannheim, Germany) and used within the 4th passage. The EAhy-926 line [19], a hybridoma with the characteristics of human endothelial cells was a kind gift of Dr. Edgell and was cultured under standard conditions in MEM+10% FCS and 1% HAT (5 $\times$ 10<sup>-3</sup> M hypoxanthine, 2 $\times$ 10<sup>-5</sup> M aminopterin, 8 $\times$ 10<sup>-4</sup> M thymidine).

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**Abbreviations:** LDL, low density lipoproteins; OxLDL, oxidized low density lipoproteins; 7 $\beta$ -OH, 7- $\beta$ -hydroxycholesterol; 7-keto, 7-ketocholesterol; 25-OH, 25-hydroxycholesterol; 7 $\alpha$ -OH, 7- $\alpha$ -hydroxycholesterol; 5 $\alpha$ -OOH, 5- $\alpha$ -hydroperoxycholesterol; 7 $\alpha$ -OOH, 7- $\alpha$ -hydroperoxycholesterol; HUVEC, human umbilical vein endothelial cells; ECGF, endothelial cell growth factor; FCS, fetal calf serum; hsp(s), heat shock protein(s); PAGE, polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

### 2.5. Immunocytochemistry

Non-confluent cells (60 000/well) were incubated with modified lipoproteins or the oxysterol fraction for 12 h, then fixed in 3% paraformaldehyde and processed for immunostaining, as described [9]. Mouse monoclonal antibody specific for the inducible form of hsp70 (C92F3A-5, Stressgen, Victoria, Canada) (1:200) was used, followed by biotinylated goat anti-mouse IgG (Amersham, Little Chalfont, UK) (1:250) and fluorescein-conjugated streptavidin (Amersham, 1:100). Coverslips were mounted on microscopy slides with glycerol:PBS (1:1), examined with a Zeiss Axioscope fluorescence microscope and photographed using 400 ASA Diachrome film.

### 2.6. Immunoblotting

Cells were incubated with different concentrations of OxLDL or lipids from OxLDL. Cells were lysed in Tris-glycine buffer (0.25 M Tris, 0.173 M glycine, pH 8.5), containing 3% SDS and 1 mM PMSF and the extracts were subjected to 10% SDS-PAGE [20], after the addition of  $\beta$ -mercaptoethanol (2%), glycerol and bromophenol blue. Electrophoresed proteins were transferred onto a nitrocellulose membrane [21] using a Trans Blot Cell (Hoefer Scientific Instruments, San Francisco, USA). The membrane was incubated with a 1:1500 dilution of anti-hsp70 antibody, then with a 1:2000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma). Immunocomplexes were detected by an enhanced chemiluminescence method (ECL, Amersham), followed by autoradiography and quantified by the Image program (NIH 1.52).

### 2.7. Cell viability

Cell viability was evaluated by the MTT test [22] using non-confluent cells incubated for 24 h with OxLDL, lipids extracted from OxLDL, lipids or purified oxysterols exactly as previously described [23]. Cell viability was expressed as percent ratio over control cells.

### 2.8. Preparation of 4-hydroxynonenal

4-Hydroxynonenal-diethylacetal (HNE-DEA) was kindly provided by Dr. Esterbauer, Institute für Biochemie, Graz, Austria. An aliquot (0.1 ml) of HNE-DEA was dried under nitrogen to remove chloroform; to the residue 1 ml  $10^{-3}$  N HCl was added and the solution was stirred until it became clear. The concentration was determined using a UV-spectrum (300–200 nm).

## 3. Results

In previous experiments we observed that OxLDL induce the expression of hsp70 in endothelial cells [9,24]. To verify the relationship between OxLDL cytotoxicity and hsp70 induction, HUVEC were incubated with increasing concentrations of OxLDL for 24 h showing a decrease of cell viability that was accompanied by an increase of hsp70 expression (data not shown). This suggested that hsp70 expression may be triggered by the same component(s) present in OxLDL that induce toxicity, as indicated by the fact that the toxic products were present in the total lipid extract of OxLDL, which also induced hsp70 expression (Fig. 1). As expected, lipids from native LDL could not induce hsp70 expression and were not cytotoxic (data not shown).

Neutral lipids were obtained from OxLDL by mean of aminopropyl columns, and further fractionated by solvent mixtures with increasing polarity to obtain fractions with different composition, two of which resulted enriched in oxysterols. These fractions when incubated with HUVEC at concentra-

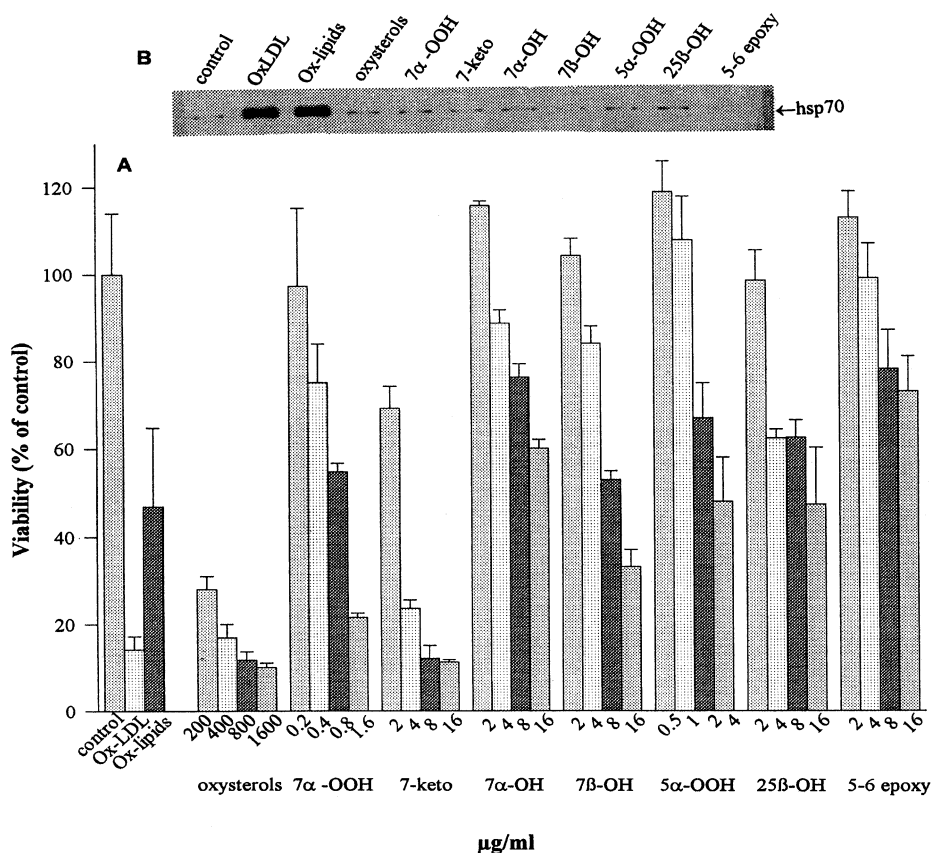


Fig. 1. A: Cytotoxicity of oxysterols from OxLDL to HUVEC. Sparsely grown cells were incubated for 24 h in presence of OxLDL (400  $\mu$ g protein/ml), lipids from OxLDL (800  $\mu$ g/ml) or the indicated concentrations of oxysterols. Cell viability was determined by the MTT test and expressed as percent of the viability of control cells. Values are the mean  $\pm$  S.D. of three determinations from a representative experiment. B: Expression of hsp70. Cells were incubated as described above, then cellular proteins were processed for immunoblotting (see Section 2.6).

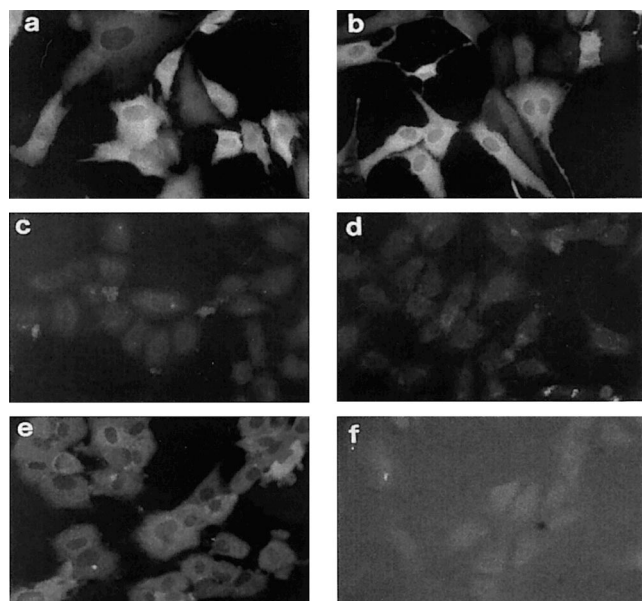


Fig. 2. Analysis of hsp70 induction in HUVEC by immunofluorescence. Sparsely grown cells were incubated for 12 h in M-199 containing: (a) OxLDL (400 µg protein/ml); (b) OxLDL total lipids (1000 µg/ml); (c) neutral lipids from OxLDL (1000 µg/ml); (d) oxysterols from OxLDL (1000 µg/ml); (e) polar lipids from OxLDL (1000 µg/ml); (f) polar lipids from native LDL (1000 µg/ml). Cells were then processed for immunostaining of the inducible form of hsp70 (see Section 2.7). Data are representatives of four different experiments.

tions corresponding to 200–1600 µg OxLDL protein/ml did not induce hsp70 expression, yet toxic to the cells in a concentration-dependent manner (Fig. 1). The other fractions when added to HUVEC at concentrations corresponding to 400 and 800 µg OxLDL protein/ml neither were cytotoxic nor induced hsp70 expression (not shown).

Several oxysterols, including 7-keto, 25-OH, 7 $\alpha$ -OH, 7 $\beta$ -OH, 7 $\alpha$ -OOH, 5 $\alpha$ -OOH and 5,6-epoxycholesterol have been detected in OxLDL [25,26]. Purified oxysterols were individually incubated with non-confluent HUVEC at concentrations corresponding to their estimated content in the OxLDL used in our experiments (from 200 to 1600 µg protein/ml) [25]. All oxysterols, 7 $\alpha$ -OOH above all, were cytotoxic to HUVEC in a dose-dependent manner, but failed to induce hsp70 expression (Fig. 1). This finding was confirmed by immunofluorescence (Fig. 2d). Thus it appears that hsp70 induction by OxLDL is not due to oxysterols.

Oxidation of LDL leads to the formation of a complex array of products, many of which are more polar than oxysterols (highly reactive aldehydes, lysophosphatidylcholine, oxidized fatty acids) [5]. Immunofluorescence results showed that HUVEC incubated with OxLDL, total lipids or polar lipids from OxLDL (Fig. 2a, b, e) displayed cytoplasmic staining for hsp70. Cells incubated with the neutral lipids or the oxysterol-containing fractions showed no hsp70 staining (Fig. 2c, d), in agreement with immunoblotting data. Lyso PC dissolved in ethanol (100 µg/ml) or PLA<sub>2</sub>-treated LDL (containing up to 50% of phospholipids as lyso PC) could not induce hsp70 expression up to 400 µg/ml of LDL protein (data not shown). Another compound that is formed upon LDL oxidation is 4-HNE [27]. We evaluated the effect of 4-HNE in a range from 0.1 µM up to 1000 µM: this compound resulted to

be toxic in a dose-dependent manner, while no induction of hsp70 was observed at all concentrations tested (Fig. 3).

#### 4. Discussion

De novo synthesis of stress proteins is generally regarded as a defense response triggered by a number of toxic stimuli [7,8]. Among stress proteins, hsp70 is highly inducible and is thought to represent an early response of stressed cells to adverse conditions [28]. Hsp70 has been detected in areas of atherosclerotic lesions [29] suggesting that sites of increased hsp expression during plaque evolution represent areas of the arterial wall experiencing cytotoxic stress [30]. Recently, we reported that OxLDL induce the expression of hsp70 in cultured human endothelial cells [9] and smooth muscle cells [23] and hypothesized that this event may represent a cytoprotective response to OxLDL cytotoxicity. In vivo, OxLDL are present in vascular lesions and may play a causal role in atherosclerosis [1]. Therefore, oxidized lipoproteins could contribute to cell damage associated with atherosclerosis. OxLDL cytotoxicity can be mimicked by its lipids [10]. Several lines of evidence point to the cytotoxicity and atherogenicity of oxysterols [31,32], which are also markers for the oxidative modification of lipoproteins [25,26] and have been detected in atherosclerotic plaques [11]. Since oxysterols account for most of OxLDL cytotoxicity [10,11] it is reasonable to speculate that they are responsible for the damage to endothelial cells and for the loss of cells occurring in advanced atherosclerotic lesions. Due to the pathogenic features of oxysterols, and because the inducible form of hsp70 can be viewed as a marker of cells responding to toxic stimuli [28], we speculated that oxysterols, that could represent an oxidative stress for cells, may be responsible for hsp70 expression induced by OxLDL. Oxysterols purified from OxLDL, either tested as a whole mixture or individually, were cytotoxic in a dose-dependent manner but neither as a mixture nor as purified molecules they could induce hsp70 expression in endothelial cells. Oxidized cholesteryl esters were mildly cytotoxic and, like oxysterols, did not induce hsp70 expression (data not shown).

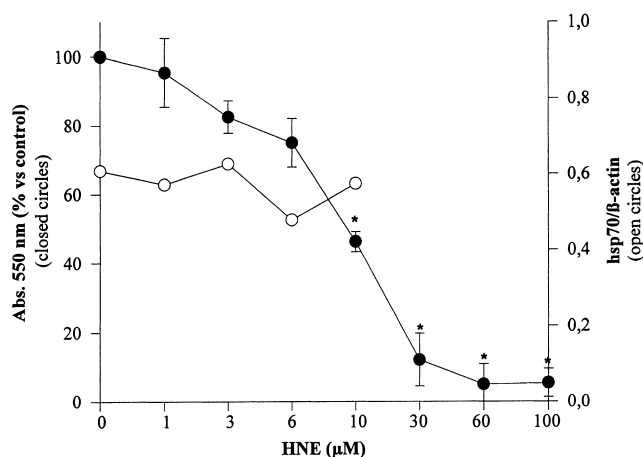


Fig. 3. Cytotoxicity and induction of hsp70 by 4-hydroxynonenal in EAhy-926. Sparsely grown cells were incubated for 18 h with increasing concentrations of 4-HNE (0.1 to 1000 µM). Cell viability was determined by the MTT test and expressed as percent ratio over the viability of control cells. For immunoblotting experiments, the inducible form of hsp70 was investigated with a specific monoclonal antibody.  $P < 0.005$  vs. control.

Thus, although hsp70 induction is associated with OxLDL toxicity, not every toxic component of OxLDL can induce hsp70 expression. These data therefore suggest that the presence of hsp70 in atherosclerotic plaques [33] is not caused by the presence of toxic oxysterols, and that other components of OxLDL must be responsible for this effect.

In a toxic environment, the increase in hsp synthesis is generally linked to the presence of protein denaturants [7,8]. It could be that oxysterols do not trigger a stress response because, rather than being proteotoxic, they are in fact interfering with essential steps of cellular metabolism, as it is known for some oxidized derivatives of cholesterol that block protein synthesis [34,35]. Because oxysterols and other neutral lipids failed to induce hsp70 expression in HUVEC while polar lipids were effective, we tested a major polar product formed upon oxidation of LDL, 4-hydroxynonenal (4-HNE) [27]. This molecule induces hsp70 in cultured hepatoma cells [36]. When 4-HNE was added to endothelial cells we found a dose-dependent cytotoxicity, while no induction of hsp70 was found in this cell system.

In summary, our observations point to the concept that cytotoxicity of oxidized LDL is mediated in part by oxysterols, and that this toxic effect does not induce the heat shock response elicited by OxLDL. We therefore propose that two major classes of oxidized products are present in OxLDL: one responsible for the cytotoxic effect but not able to induce heat shock response (mainly oxysterols); the other, which is less toxic, mediates the induction of hsp70. The balance between these components may result in cell survival versus cells death at sites where OxLDL are present.

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