The role of oxysterols in control of endothelial stiffness

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Abstract Endothelial dysfunction is a key step in atherosclerosis development. Our recent studies suggested that oxLDL-induced increase in endothelial stiffness plays a major role in dyslipidemia-induced endothelial dysfunction. In this study, we identify oxysterols, as the major component of oxLDL, responsible for the increase in endothelial stiffness. Using Atomic Force Microscopy to measure endothelial elastic modulus, we show that endothelial stiffness increases with progressive oxidation of LDL and that the two lipid fractions that contribute to endothelial stiffening are oxysterols and oxidized phosphatidylcholines, with oxysterols having the dominant effect. Furthermore, endothelial elastic modulus increases as a linear function of oxysterol content of oxLDL. Specific oxysterols, however, have differential effects on endothelial stiffness with 7-ketocholesterol and 7α-hydroxycholesterol, the two major oxysterols in oxLDL, having the strongest effects. 27-hydroxycholesterol, found in atherosclerotic lesions, also induces endothelial stiffening. For all oxysterols, endothelial stiffening is reversible by enriching the cells with cholesterol. oxLDL-induced stiffening is accompanied by incorporation of oxysterols into endothelial cells. We find significant accumulation of three oxysterols, 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol, in mouse aortas of dyslipidemic ApoE−/− mice at the early stage of atherosclerosis. Remarkably, these are the same oxysterols we have identified to induce endothelial stiffening.—Shentu, T. P., D. K. Singh, M-J. Oh, S. Sun, L. Sadaat, A. Makino, T. Mazzone, P. V. Subbaiah, M. Cho, and I. Levitan. The role of oxysterols in control of endothelial stiffness. J. Lipid Res. 2012. 53: 1348–1358.

Supplementary key words cholesterol • atomic force microscopy • cell stiffness • oxidized phosphatidylcholine

Multiple studies have shown that oxidative modifications of LDL (oxLDL) are a major factor in the development of atherosclerosis (1–6). The level of oxLDL increases dramatically with hypercholesterolemia in animal models of atherosclerosis (7, 8), and in humans (9, 10), it is found in atherosclerotic lesions (11). It is also well established that dyslipidemia-induced dysfunction of vascular endothelial cells is a critical step in the early stage of atherosclerosis (e.g., Refs. 12–14) and a strong predictor of cardiovascular disease (CVD) development (15–17). The mechanisms, however, of dyslipidemia-induced endothelial dysfunction are still poorly understood. Our recent studies have shown that exposure to oxLDL in vitro or to plasma dyslipidemia in vivo significantly increases the stiffness of aortic endothelial cells, which in turn is associated with an increase in endothelial contractility, enhanced angiogenic potential, and sensitivity to shear stress (18–20). Furthermore, unexpectedly, our studies showed that dyslipidemia-induced endothelial stiffening is caused not by cholesterol loading but by disruption of lipid packing of cholesterol-rich membrane domains in endothelial cells (18, 19, 21). Consistent with these observations, oxLDL-induced endothelial stiffness could be fully reversed by enriching the cells with cholesterol, even though oxLDL had no effect on the cholesterol content of endothelial membranes (19). These studies led us to the hypothesis that oxLDL induces endothelial dysfunction by inserting oxysterols into the plasma membrane, resulting in the disruption of cholesterol-rich membrane domains and endothelial stiffening. The goal of this study, therefore, was to determine the impact of oxysterols on endothelial stiffness.

Abbreviations: ApoE−/−, ApoE-deficient; BAEC, bovine aortic endothelial cell; Chol/CE, cholesterol/cholesteryl ester; mMLDL, minimally oxidized LDL; oxLDL, oxidized LDL; LPC, lysophosphatidylcholine; MBCD, methyl-β-cyclodextrin; MDA, malondialdehyde; PC, phosphatidylcholine; oxPC, oxidized PC; PE, phosphatidylethanolamine; SLO, soybean lipoygenase; SM, sphingomyelin; TBARS, thiobarbituric acid-reactive substance; WT, wild-type.

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OxLDL exists in multiple forms, characterized by different degrees of oxidation and different mixtures of bioactive components (3, 6, 22, 23). Specifically, LDL is progressively oxidized from a minimally oxidized form (mmLDL), which contains mostly oxidized phosphatidylcholines (oxPC), to extensively oxidized LDL, which is strongly enriched in oxysterols, as well as several other bioactive lipids, such as lyposphospholipids and sphingolipids (3, 6, 23). Among the bioactive lipids in oxLDL, oxysterols have been shown to be involved at all stages of atherosclerosis development, and they play an important role in plaque formation (24, 25). In the early stage of atherosclerosis, oxysterols are involved in impairment of several endothelial functions, such as endothelial nitric-oxide synthase (eNOS) activation (26), reactive oxygen species (ROS) production (27), and regulation of the permeability barrier (28, 29). In the late stage of atherosclerosis, an increase in the levels of oxysterols was shown to contribute to apoptosis of vascular cells (29, 30) and degradation of extracellular matrix (31, 32), which led to instability of the plaque. In this study, we demonstrate that oxysterols play a major role in the increase in endothelial stiffness and identify specific oxysterols responsible for this effect.

MATERIALS AND METHODS

Cell culture and reagents

Bovine aortic endothelial cells (BAEC; Cambrex East Rutherford, NJ) were grown between passages 5 and 20 in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO), 10 µg/ml penicillin, streptomycin, and kanamycin sulfate (Invitrogen). Cell cultures were maintained in a humidified incubator at 37°C, with 5% CO2. Cells were split every 3–4 days. Methyl-β-cyclodextrin (MβCD) and cholesterol were purchased from Sigma Chemical, St. Louis, MO. MβCD saturated with cholesterol was prepared as described previously (33). 7-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 25-hydroxycholesterol were purchased from Steraloids, Inc. (Newport, RI). 27-hydroxycholesterol was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Isolation and oxidation of LDL

Normal human plasma prepared in acid citrate dextrose was purchased from the local blood bank (Life Source, Chicago). LDL was isolated by sequential centrifugation in KBr between the density range 1.019 to 1.063 g/ml. The preparation is dialyzed against three changes of 10 mM Tris/HCl buffer, pH 7.4, containing 0.15M NaCl and 1 mM EDTA at 4°C to remove KBr. To proceed to the copper oxidation, further dialysis against three changes of 10 mM Tris/HCl buffer, pH 7.4, containing 0.15M NaCl and 1 mM EDTA at 4°C to remove KBr. To copper oxidation, further dialysis against three changes of 10 mM Tris/HCl buffer, pH 7.4, containing 0.15M NaCl and 1 mM EDTA at 4°C to remove KBr.

Extraction of total lipids in LDL

For sterol analysis, total lipids were extracted from LDL and oxLDL after adding 10 µg 19-hydroxycholesterol (19HC) as an internal standard. Trimethylsilyl ether (TMS) derivatives of the sterols were prepared by incubating with 100 µl mixture of N, O-bis-(trimethylsilyl)-acetamide (BSA): n-decane (1:1) at 60°C for 1 h. The TMS ether derivatives of sterols were analyzed by gas chromatography (Shimadzu GC-17A) using a fused carbon-silica column (30 m × 0.25 mm id) coated with (55% phenyl methylpolysiloxane (DB-5MS; film thickness 1 PM) (J and W Scientific, Folsom, CA) as described previously (39). In brief, samples were injected using a split ratio of 1:10. The temperature was programmed for 80–280°C at a rate of 30°C/min after initial hold of 1 min, and then maintained at 280°C for 15 min. The injector and detector temperatures were 280°C and 290°C, respectively.

Extraction of total lipids in BAECs

After 1 h incubation with LDL or oxLDL (10 µg of protein/ml serum free media), cells were washed three times with PBS and lysed using 50 mM Tris-HCl containing 0.15M NaCl and 0.1% Triton X-100. The total lipids were extracted by the Bligh and Dyer method, and the levels of sterols were determined to the internal standard (19HC, 10µg). For the comparative analysis, values were normalized to LDL-treated cells.

Extraction of total lipids in aorta in ApoE-deficient mice

Aortas were harvested from ApoE-deficient (ApoE−/−) and C57BL/6 wild-type (WT) mice at 10–12 weeks of age. All animals were fed low-fat chow diet. Aortas were dissected and, and samples were put on the filter paper to absorb liquid, and then weighed and ground to a powder before extraction. The total lipids were extracted by the Bligh and Dyer method, and the levels of sterols were determined by gas chromatography as described in the previous section. All investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985). The study was conducted in accordance with the guidelines established by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago.

AFM microindentation

The elastic modulus of individual endothelial cells was measured with a Novascan atomic force microscope (AFM; Novascan Technologies, Ames, IA) as described previously (19, 40). Briefly,
cantilevers with borosilicate glass beads (10 μm diameter, 0.12 N/m) (Novascan Technologies) served as cell indentors. The AFM cantilever probe was positioned above the cell between the nucleus and the cell edge, and each cell was mechanically probed at five different locations, three times at each location, avoiding the perinuclear and edge regions. A total of 50–80 cells were analyzed for each experimental condition (15 force-distance curves acquired from each cell). The force curves were obtained by measuring the cantilever deflection at every vertical z-position of the cantilever as it approached and indented the cell. The cantilever descended toward the cell at a velocity of 2 μm/s until a trigger force of 3 nN was reached, which corresponded to 0.5–1 μm indentation depth, or approximately 10–15% of the total cell height. The force-distance curves were collected and analyzed according to the Hertz model:

\[ F = \frac{4}{3} E \delta^{3/2} \sqrt{R} \]  

(Eq. 1)

where \( F \) is the loading force, \( \delta \) is indentation depth, \( v \) is the cellular Poisson’s ratio (assumed to be 0.5), \( R \) is the radius of the spherical indenter (5 μm), and \( E \) is the local Young’s elastic modulus. The bidomain polynomial model was fit to the experimental force curve using a standard least-squares minimization algorithm. The data are presented as histograms of the elastic moduli for each experimental condition. Statistical significance was calculated using a standard t-test (\( P < 0.05 \)). As there is some variation in elastic modulus measurements between control cell populations from experiment to experiment, to ensure this variability did not contribute to the observed effects, AFM measurements in all experiments were performed on control cells and cells exposed to different experimental treatments on the same days and on the same cell populations.

**Microaspiration**

Micropipette aspiration of attached bovine endothelial cells was performed as described in our earlier studies (41, 42). Briefly, the membranes were visualized with a fluorescent membrane dye, carbocyanide DiIC18 (Molecular Probes, Eugene, OR), and then aspirated using micropipettes with 6–9 μm outer diameter pulled from borosilicate glass capillaries (SG10 glass; Richland Glass, Richland, NJ). Zeiss microscopy (Axiovert 200M) was used for capturing the membrane deformation with 180 s time interval. Negative pressure was applied to a pipette by a pneumatic transducer tester (BioTek Instruments, Winooski, VT).

**Statistical analysis**

Statistical analysis was performed using a standard t-test assuming two-tailed distributions with unequal variance.

**RESULTS**

**Endothelial stiffening and degree of LDL oxidation**

To explore how different oxidation states of LDL affect endothelial stiffness, LDL was oxidized with 25 μM Cu²⁺ for increasing amounts of time (2, 8, 16, and 24 h) to produce mildly or extensively oxidized LDL, as described in previous studies (39, 43, 44). The degree of oxidation was quantified by measuring the levels of TBARS, a common method of determining LDL oxidation status (45). Here we show that progressive LDL oxidation results in continuous increase in cell stiffness (TBARS values ranging between 3 ± 0.5 and 27 ± 1.2 MDA/mg protein) (Fig. 1). An increase in endothelial stiffness is apparent from the right shift in the histograms of the elastic moduli measured in cells exposed to oxLDL compared with cells exposed to nonmodified LDL (Fig. 1A), as well as from the increase in the mean elastic moduli measured for cells exposed to progressively oxidized LDL (Fig. 1B, C). More specifically, a significant increase in endothelial elastic modulus is observed when cells are exposed to oxLDL with a TBARS value of 11 ± 0.5 (8 h of oxidation), which is typically defined as minimally oxidized LDL (46, 47). Moreover, an increase in LDL oxidation due to prolonging the oxidation to 16 h (22 ± 4.8 TBARS) results in further stiffening effect. However, no difference in cell stiffness was observed with further LDL oxidation (24 h, 27 ± 1.2 TBARS). An increase in endothelial stiffness is also observed when cells are exposed to LDL oxidized with SLO (supplementary Fig. 1), another well-established oxidant.

**Fig. 1.** The impact of LDL oxidized status on the endothelial stiffening. A: Histograms of the elastic moduli measured in cells treated with 10 μg/ml LDL or oxLDL (oxidized for 8 h or 16 h). B: Superimposed average elastic moduli (solid square, fitting to black curve) and TBARS values (solid circle, fitting to gray curve) are plotted as a function of the time of LDL oxidation. C: Average elastic moduli shown as a bar graph to highlight the differences between mildly and strongly oxidized LDL. Data are means ± SEM, n = 69–107 cells per experimental condition measured in three independent experiments.
method of LDL oxidation that results in mildly oxidized LDL (34, 35, 47). In the latter case, endothelial stiffness was assessed by measuring progressive membrane deformation into the pipette using microaspiration, an approach that has been extensively used in our previous studies (18, 41). Thus, increase in endothelial stiffness is observed across the full range of LDL oxidation states (Fig. 1B, C).

**Differential effects of oxLDL lipid fractions**

It is known that the lipid extracts of LDL and oxLDL can be separated into several major groups of bioactive components, including LPC, SM, PC, PE, and Chol/CE (48). These components can be separated from lipid extracts of the lipoproteins using TLC analysis, with the individual fractions being indentified by comparing their mobilities to the mobilities of the known standards (49). Therefore, we used this method to determine which fractions induce the stiffening effect. In this series of experiments, we used extensively oxidized LDL (22 ± 4.8 TBARS, 16 h oxidation) because, as described above, maximal stiffening was observed under these experimental conditions.

Fig. 2A shows that the lipid extract of oxLDL is separated to five completely distinct and easily indentifiable fractions, corresponding to Chol/CE, PE, PC, SM, and LPC. The amount of lipids in each fraction was quantified as described in Materials and Methods. To determine the effects of the individual lipid fractions on endothelial stiffness, endothelial elastic modulus was measured in cells exposed to 10 μg/ml lipid for 1 h for each of the fractions separately (Fig. 2B, C). The corresponding fractions of lipid extracts isolated from nonoxidized LDL were used as controls. We show here that the most prominent effect on endothelial stiffness was observed when cells were exposed to the oxidized Chol/CE fraction. This effect was manifested by the appearance of cells with higher elastic moduli, as seen in the right tail of the histogram. In addition, endothelial stiffening was also observed in cells exposed to oxPC. In contrast, exposure of LPC, PE, and SM fractions had no effect on endothelial stiffness (Fig. 2B, C).

**Identification of oxysterols that induce endothelial cell stiffening**

Similar to previous studies (39), five major oxysterols are identified in strongly oxidized LDL (16 h of oxidation) by the GC analysis: 7α-hydroxycholesterol; 7β-hydroxycholesterol; cholesterol 5α,6α-epoxide; cholesterol and 5β,6β-epoxide; and 7-ketocholesterol (Fig. 3A). The most abundant oxysterol is 7β-hydroxycholesterol, followed by 7-ketocholesterol, with 7α-hydroxycholesterol and cholesterol 5β,6β-epoxide representing minor oxysterols (Fig. 3A and supplementary Fig. II). The levels of different oxysterols increase with progressive LDL oxidation with the significant increase observed already in mildly oxidized LDL (8 h oxidation) (supplementary Fig. II). In contrast, formation of oxysterols is accompanied with a decrease in oxLDL cholesterol content. An increase in endothelial stiffness is clearly correlated with the formation of oxysterols (Fig. 3B).

To indentify which oxysterols induce an increase in cell stiffness, cells were exposed to 10 μg/ml of each of the individual oxysterols identified above (Fig. 3C, D). Our results show that oxysterols found in oxLDL complex have differential effects on endothelial stiffness. Specifically, 7-ketocholesterol and 7α-hydroxycholesterol significantly increased endothelial stiffness (Fig. 3C, D), whereas 7β-hydroxycholesterol had a smaller effect that was not statistically significant. No stiffening effects were observed in cells exposed to cholesterol 5α,6α-epoxide and 5β,6β-epoxide isomers (Fig. 3D). To establish whether the difference in the ability of specific oxysterols to induce endothelial stiffening can be associated with the difference in their ability to get incorporated into endothelial cells, we compared the level of incorporation of 7-ketocholesterol, an oxysterol that showed the strongest effect on endothelial stiffness, with 5β,6β-epoxide, an oxysterol that had no effect. Our analysis shows that there was no difference in the levels of the incorporation of the two oxysterols into the cells (supplementary Fig. III).

In addition, we tested the effects of two more oxysterols, 25-hydroxy and 27-hydroxy cholesterol. These two oxysterols do not constitute significant components of oxLDL complex, but they were found in human atherosclerotic lesions (25, 50, 51). 27-hydroxycholesterol is especially...
Fig. 3. Impact of oxysterol components of oxLDL on endothelial stiffness. A: Typical GC chromatographs of oxysterols separated from LDL or oxLDL (22 ± 4.8 TBARS, 16 h of oxidation). 19-hydroxycholesterol (19HC, 10 µg) is used as an internal standard. B: Average elastic moduli plotted as a function of LDL oxidation (TBARS) (the correlation coefficient between the two values is \( R = 0.91 \)). C: Histograms of elastic moduli measured in control (Ctrl) and 7K-treated cells (10 µg/ml). D: Histograms of elastic moduli measured in control and 7A-treated cells (10 µg/ml). Control cells shown in C and D reflect different cell populations that were measured in parallel with 7K- and 7A-treated cells, respectively. E: Average elastic moduli of cells treated with different oxysterols identified above in oxLDL normalized to control cells recorded in the same experiment. The values are means ± SEM, n = 32–80 cells per experimental condition obtained in three independent experiments. * \( P < 0.05 \) vs. control. 5A, cholesterol 5, 6-epoxide; 5B, cholesterol 5, 6-epoxide; 7A, 7α-hydroxycholesterol; 7B, 7β-hydroxycholesterol; 7K, 7-ketocholesterol.

abundant in human atherosclerotic lesions and macrophage-derived foam cells (25, 50, 51). We show here that exposure to 27-hydroxycholesterol results in a significant increase of endothelial stiffness, whereas exposure to 25-hydroxycholesterol has no effect (Fig. 4).

Reversibility of oxysterol-induced endothelial stiffening with MβCD-cholesterol

We have previously demonstrated that oxLDL-induced increase in endothelial stiffness can be fully reversed by exposing the cells to MβCD-cholesterol, a potent cholesterol donor to the cell membranes (19). These observations led us to the hypothesis that exposing the cells to MβCD-cholesterol might cause the exchange between cholesterol and oxysterols incorporated into the membrane and thus rescue the cells from the stiffening effect. In this study, we tested this hypothesis by sequentially exposing the cells to 10 µg/ml of 7-ketocholesterol, an oxysterol that was identified in our study as having a profound effect on endothelial stiffness, and then to 5 mM MβCD-cholesterol. As expected, exposing the cells to 7-ketocholesterol resulted in its incorporation into the cell membranes, and this effect was almost completely reversed by the sequential exposure of the cells to MβCD-cholesterol (Fig. 5A, B). Next, we tested whether treating the cells that were preexposed to 7-ketocholesterol with MβCD-cholesterol also reverses the stiffening effect of the oxysterol. We showed that this is indeed the case: an increase in the elastic modulus in 7-ketocholesterol-treated cells was fully reversed by the subsequent exposure to MβCD-cholesterol (Fig. 5C, D). Endothelial cell stiffening induced by 27-hydroxycholesterol was also fully reversed by MβCD-cholesterol (Fig. 5E, F).
Cholesterol enrichment protects against oxysterol-induced endothelial stiffening

To evaluate further the impact of membrane cholesterol on oxysterol-induced endothelial stiffening, we tested whether cholesterol enrichment prior to the exposure to oxysterols alters the effect of oxysterols on endothelial stiffness. To address this question, cells were first exposed to MβCD-cholesterol, using a standard protocol that results in enriching the cells with cholesterol, and then exposed to 7-ketocholesterol. Consistent with the experiments described above, exposure to 7-ketocholesterol alone resulted in a significant increase in endothelial elastic modulus, demonstrating the robustness and the reproducibility of this effect. However, no stiffening was observed when cells were exposed to 7-ketocholesterol after being cholesterol enriched (Fig. 6). These observations indicate that an increase in membrane cholesterol may have a protective effect against oxysterol-induced endothelial stiffening.

Incorporation of oxysterols into endothelial membranes

Next, we addressed the question of whether significant amount of oxidized lipids is incorporated into the endothelial membranes. This question was addressed by estimating the levels of different oxysterols in total membrane lysates of endothelial cells exposed to oxLDL or to non-modified LDL used as a control. Endothelial cells grown to confluence were exposed to 10 µg/ml of extensively oxidized LDL (16 h oxidation, 22 ± 4.8 TBARS) or to non-modified LDL for 1 h, then cells were washed with PBS to remove oxLDL/LDL particles and lysed for the preparation of lipid extracts. The levels of oxysterols were determined in the whole-cell lipid extracts by the GC analysis. This analysis showed that exposure to oxLDL resulted in a highly significant increase in the level of 7-ketocholesterol in lipid extracts of endothelial cells, as is demonstrated by the normalized ratio of 7-ketocholesterol level in cells treated with oxLDL compared with cells treated with nonmodified LDL (Fig. 7). There was also some increase in the levels of 7α- and 7β-hydroxycholesterol, but these increases were not statistically significant. The difference in the basal levels of the three oxysterols may be the reason that exposure to oxLDL resulted in a significant increase in 7-ketocholesterol but not in 7α- and 7β-hydroxycholesterols. Consistent with our earlier studies (18), we found no change in the level of membrane cholesterol in cells exposed to oxLDL.

Incorporation of oxysterols in aorta tissue extracts of ApoE−/− mice

Finally, we addressed the question whether dyslipidemia in vivo also results in the accumulation of oxysterols in the vascular tissues of mouse aortas at the early stage of atherosclerosis development in ApoE−/− mice, a well-established model for atherosclerosis (6, 52, 53). Earlier studies have shown that ApoE−/− mice fed low-fat diet can be used as a model for the early stage of atherosclerosis because, although these mice are dyslipidemic, development of atherosclerosis is significantly delayed, with lesions containing foam cells forming only after 15 weeks and advanced lesions appearing after 20 weeks (52). In our study, therefore, the levels of oxysterols were measured in aortas harvested from 10- to 12-week-old ApoE−/− mice fed a low-fat diet. Sex- and age-matched WT mice of the same genetic background (C57BL/6) were used as controls. The relative amounts of each of the oxysterols, as well as cholesterol, normalized to the total level of tissue sterols are shown in Fig. 8. Our study shows that in male ApoE−/− mice, there is a significant increase in the two oxysterols that were identified in this study to induce endothelial stiffening: 7-ketocholesterol and 7α-hydroxycholesterol. There was also some increase in 7β-hydroxycholesterol. Interestingly, although the levels of oxysterols increase, the level of cholesterol does not change significantly, and therefore, its relative contribution to total tissue sterols decreases, as shown in Fig. 8A. Note, however, that in contrast to male mice, no change in oxysterol composition was observed in age-matched female mice (Fig. 8B). No difference was observed in the levels of plasma cholesterol between males and females (Fig. 8, inset).

**DISCUSSION**

A growing number of studies suggest that endothelial biomechanical properties play major roles in endothelial function (20, 42, 54–57). The factors that control endothelial biomechanics under normal and pathological conditions, however, are still poorly understood. Our recent studies have shown that endothelial elastic modulus...
components that are critical for an increase in endothelial elastic modulus.

Controversies regarding the mechanisms of oxLDL-induced effects stem from the fact that the degree of LDL oxidation varies significantly under different experimental or pathological conditions, which in turn have major impact on its biological effects (1, 6, 58). Our study shows that initial cell stiffening is observed in response to mildly oxidized LDL, and the effect increases as the oxidation progresses. Mildly oxidized LDL that is defined by its ability to bind to LDL receptor and TBARS values of 2–13 nmol is strongly affected by oxidative modifications of LDL (19), a major proatherogenic lipoprotein associated with the development of atherosclerosis (1–6). However, oxidation of LDL is a complex process that yields an array of bioactive compounds with different biological properties and the individual composition depends on the degree of LDL oxidation. The key question is, What are the critical components that are responsible for oxLDL-induced biological effects? The goals of our current study were to determine the impact of the degree of LDL oxidation on endothelial stiffness and to identify oxLDL components that are critical for an increase in endothelial elastic modulus.

Fig. 5. Cholesterol reverses oxysterol-induced endothelial stiffening. A: Typical GC chromatographs of oxysterols in lipid extracts isolated from control cells (Ctrl), cells exposed to 10 μg/ml 7-ketocholesterol or cells sequentially exposed to 10 μg/ml 7-ketocholesterol and then to 5 mM MBCD-cholesterol. B: The levels of 7-ketocholesterol in the three cell populations normalized to control (n = 3, P < 0.05). C: Histograms of the elastic moduli measured in control cells (Ctrl), 7K-treated cells (10 μg/ml), and cells treated sequentially with 10 μg/ml 7K and 5 mM MBCD saturated with cholesterol complex (7K→Chol). D: Average elastic moduli for the Ctrl, 7K, and 7K→Chol cells (data are shown as means ± SEM, n = 60 cells for each experimental condition obtained from three independent experiments, *P < 0.05 vs. control). E: Histograms of elastic moduli measured in control cells (Ctrl), 27HC-treated cells (10 μg/ml), and cells treated sequentially with 10 μg/ml 27HC and 5 mM MBCD saturated with cholesterol (27HC→Chol). F: Average elastic moduli for the Ctrl, 27HC→Chol-treated cells. Data are means ± SEM (n = 45–60 cells per experimental condition obtained in three independent experiments, *P < 0.05 vs. control).
Mildly oxidized LDL contains oxPC as its major oxidized product (23), strongly oxidized LDL contains both oxPC and oxysterols (39, 43). Furthermore, within the group of oxysterols, the stiffening effect is not universal but differentially induced by specific oxidized species, with the sterols oxidized at position 7 having the most pronounced effects. The same oxysterol species were shown to accumulate in atherosclerotic lesions (39) and impair endothelium-dependent relaxation (63). We also show that endothelial stiffening is induced by 27-hydroxylcholesterol, which was shown to be the most abundant oxysterol in atherosclerotic lesions (25). These observations suggest that oxysterol-induced endothelial stiffening may be an important factor in endothelial dysfunction in proatherogenic environments.

In terms of the mechanism, we showed earlier that exposure to oxLDL results in a decrease in lipid packing of cholesterol-rich ordered membrane domains and an increase in homogeneity of the membrane, suggesting that cholesterol is redistributed between ordered and fluid domains (19). Furthermore, exposure to 7-ketocholesterol was shown to directly interact with caveolin, the major scaffolding protein of caveolae, a subtype of cholesterol-rich membrane domains (64). On the basis of these observations, we proposed that oxLDL-induced endothelial stiffening results not from cholesterol loading but from the incorporation of oxysterols into the endothelial plasma membrane. In the current study, we tested this idea directly by three complementary approaches: 

1. identifying specific oxysterols that induce endothelial stiffening,
2. demonstrating the reversibility of oxysterol-induced endothelial stiffening by exchanging oxysterols with cholesterol, and
3. demonstrating that exposure to oxLDL results in significant incorporation of specific oxysterols, particularly 7-ketocholesterol, into the endothelial membranes.

Our observations show that specific oxysterols have differential effects on endothelial stiffness but that this effect cannot be attributed to the differences in the ability of these oxysterols to be incorporated into the membrane. An alternative possibility is that due to differences in their molecular structure,
oxysterols may have differential effects on the lipid packing of the membrane and therefore differ in their ability to disrupt the structure of lipid-ordered domains. More studies, however, are needed to test this hypothesis. We also show that enriching the cells with cholesterol prior to the exposure to oxysterols may protect endothelial cells against the detrimental effect of oxysterols on endothelial stiffness. Note that enriching endothelial cells with cholesterol by itself has no effect on endothelial stiffness, indicating that neither the reversal of oxysterol-induced stiffening nor the protective effect of cholesterol enrichment can be attributed to a simple mutual cancelation of the two opposite effects. We suggest that the protective effect of cholesterol enrichment can be due to diminishing the incorporation of other sterols into the membrane. Taken together, our observations suggest that, although incorporation of oxysterols into endothelial membranes alters endothelial biomechanics and is expected to be detrimental to endothelial function, increasing the level of membrane cholesterol may be beneficial and protective.

In terms of the presence of oxysterols in vivo, earlier studies have shown that oxysterols are found in vascular tissues of ApoE−/− mice with advanced atherosclerosis, as well as in human atherosclerotic lesions (25, 65). Our studies show that a significant accumulation of oxysterols was found in aortic tissue of male ApoE−/− mice fed a low-cholesterol chow diet at a very early stage of disease progression (10–12 weeks of age) when typically no foam cell lesions are observed (52). Moreover, we show here that, whereas oxysterol accumulation was observed in ApoE−/− male mice, no change in sterol composition was observed in age-matched ApoE−/− female mice. The difference between males and females cannot be attributed to the level of total cholesterol in the plasma because it appears to be the same. A difference in oxysterol accumulation between males and females at the early stages of atherosclerosis might be a very significant finding because earlier studies have shown that males are significantly more susceptible to the development of atherosclerosis than females (66, 67). We propose that accumulation of the oxysterols in the vascular tissue results in stiffening of endothelial cells and that this plays an important role in the initiation of the disease.

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Fig. 8. Oxysterol accumulation in aortic tissue of ApoE−/− mice.Inset: Levels of total cholesterol in plasma of male (n = 10) and female (n = 4) mice. A: Sterol levels in the vascular walls of aortas harvested from male WT and ApoE−/− mice sacrificed at 10–12 weeks. The levels of individual sterols are expressed as a percentage of total sterol content in the same sample. B: Sterol levels in the vascular walls of aortas harvested from age-matched female WT and ApoE−/− mice. N = 4–5 animals in each experimental group. Values are means ± SEM, *P < 0.05 vs. WT.

References

1. Witztum, J. L., and D. Steinberg. 1991. Role of oxidized low density lipoprotein in atherogenesis. J. Clin. Invest. 88: 1785–1792.
2. Witztum, J. L., and D. Steinberg. 2001. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? Trends Cardiovasc. Med. 11: 93–102.
3. Stocker, R., and J. F. Keaney, Jr. 2004. Role of oxidative modifications in atherosclerosis. Physiol. Rev. 84: 1381–1478.
4. Libby, P. 2002. Inflammation in atherosclerosis. Nature. 420: 868–874.
5. Paoletti, R., A. M. Gotto, and D. P. Hajjar. 2004. Inflammation in atherosclerosis and implications for therapy. Circulation. 109: III-20–III-26.
6. Levine, I., S. Volkov, and P. V. Subbaiah. 2010. Oxidized LDL: diversity, patterns of recognition and pathophysiology. Antioxidants & Redox Signaling. 13: 39–75.
7. Hodis, H. N., D. M. Kramsch, P. Avogaro, G. Bitollo-Bon, G. Cazzolato, J. Hwang, H. Peterson, and A. Sevanian. 1994. Biochemical and cytotoxic characteristics of an in vivo circulating oxidized low density lipoprotein (LDL). J. Lipid Res. 35: 669–677.
8. Holvoet, P., G. Theilmeier, B. Shivalkar, W. Flameng, and D. Collen. 1998. LDL hypercholesterolemia is associated with accumulation of oxidized LDL, atherosclerotic plaque growth, and compensatory vessel enlargement in coronary arteries of miniature pigs. Atherosclerosis. Thromb. Vasc. Biol. 18: 415–422.
9. Cazzolato, G., P. Avogaro, and G. Bitollo-Bon. 1991. Characterization of a more electronegatively charged LDL subtraction by ion exchange HPLC. Free Radic. Biol. Med. 11: 247–253.
10. van Tits, L. J., T. M. van Himbergen, H. L. Lemmers, J. de Graaf, and A. F. Stalenhoef. 2006. Proportion of oxidized LDL relative to plasma apolipoprotein b does not change during statin therapy in patients with heterozygous familial hypercholesterolemia. Atherosclerosis. 185: 307–312.
11. Ylä-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witzum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J. Clin. Invest. 84: 1086–1095.
12. Shimokawa, H. 1999. Primary endothelial dysfunction: atherosclerosis. J. Mol. Cell. Cardiol. 31: 23–37.
13. Kinlay, S. P., Libby, and P. Ganz. 2001. Endothelial function and coronary artery disease. Curr. Opin. Lipidol. 12: 383–389.
14. Sima, A. V., C. S. Stancu, and M. Simionescu. 2009. Vascular endothelial dysfunction. Cell Tissue Res. 335: 191–203.
15. Gonzalez, M. A., and A. P. Selwyn. 2003. Endothelial function, inflammation, and prognosis in cardiovascular disease. Am. J. Med. 115(Suppl. 8A): 99S–106S.
16. Heitzer, T., T. Schlimzig, K. Krohn, T. Meintz, and T. Munzel. 2001. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation. 104:2673–2679.
17. Schächinger, V., M. B. Britten, and A. M. Zeiher. 2000. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary disease. Circulation. 101: 1899–1906.
18. Byfield, F. J., S. Tikku, G. H. Rothblat, K. J. Gooch, and I. Levitan. 2006. OxLDL increases endothelial stiffness, force generation and network formation. J. Lipid Res. 47: 715–723.
19. Shentu, T. P., I. Titushkin, D. K. Singh, K. J. Gooch, P. V. Subbaiah, M. Cho, and I. Levitan. 2010. oxLDL-induced decrease in lipid order of membrane domains is inversely correlated with endothelial stiffness and network formation. Am. J. Physiol. Cell Physiol. 299: C218–C229.
20. Levitan, I., and T. P. Shentu. 2011. OxLDL and endothelial biomechanics. J. Biol. Chem. 286: 1344–1357.
21. Lenz, M. L., H. Hughes, J. R. Mitchell, D. P. Via, J. R. Guyton, A. A. Taylor, A. M. Gotto, Jr., and C. V. Smith. 1990. Lipid hydroperoxide and hydroxy derivatives in copper-catalyzed oxidation of low density lipoprotein. J. Lipid Res. 31: 1043–1050.
22. Dziletovic, S., A. Babiker, E. Lor, and U. Diczfalusy. 1995. Time course of oxysterol formation during in vitro oxidation of low density lipoprotein. Chem. Phys. Lipids. 78: 119–128.
23. Schuh, J. F. F. Gordon, and R. H. Haschemey. 1978. Oxygen-mediated heterogeneity of apo-low-density lipoprotein. Proc. Natl. Acad. Sci. USA. 75: 3173–3177.
24. Thorne, S. A., S. E. Abbot, P. G. Winyard, D. R. Blake, and P. G. Mills. 1996. Extent of oxidative modification of low density lipoprotein determines the degree of cytotoxicity to human coronary artery cells. Heart. 75: 11–16.
25. Sigari, F., C. Lee, J. L. Witzum, and P. D. Reaven. 1997. Fibroblasts that overexpress 15-Lipoxygenase generate bioactive and minimally modified LDL. Arterioscler. Thromb. Vasc. Biol. 17: 3639–3645.
26. Bloom, R. J., and J. C. Elwood. 1981. Quantitation of lipid profiles from isolated serum lipoproteins using small volumes of human serum. Clin. Biochem. 14: 119–125.
27. Cuzner, M. L., and A. N. Davison. 1967. Quantitative thin layer chromatography of lipids. J. Chromatogr. 27: 388–397.
28. Björkhem, I., O. Andersson, U. Diczfalusy, B. Sevastik, R. J. Xiu, C. Duan, and E. Lund. 1994. Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human arterial macrophage. Proc. Natl. Acad. Sci. USA. 91: 8592–8596.
29. Grisby, M., J. Nilsson, V. Kostulas, I. Bjökhem, and U. Diczfalusy. 1997. Localization of sterol 27-hydroxylation immuno-reactivity in human atherosclerotic plaques. Biochim. Biophys. Acta. 1344: 278–285.
30. Nakashima, Y., A. S. Plump, E. W. Raines, J. L. Breslow, and R. Ross. 1994. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler. Thromb. 14: 130–140.
31. Breslow, J. L. 1996. Mouse models of atherosclerosis. Science. 272: 685–688.
32. Chen, C. S., M. Mrksich, S. Huang, G. M. Whitesides, and D. E. Ingber. 1997. Geometric control of cell life and death. Science. 276: 1425–1428.
33. Saito, K., N. Nagayama, N. Kataoka, M. Sasaki, and K. Hane. 2000. Local mechanical properties measured by atomic force microscopy for cultured bovine endothelial cells exposed to shear stress. J. Biomech. 33: 127–135.
34. Sieminski, A. L., R. P. Hebbel, and K. J. Gooch. 2004. The relative magnitudes of endothelial force generation and matrix stiffness modulate capillary morphogenesis in vitro. Exp. Cell Res. 297: 574–584.
35. Oberleithner, H., C. Riethmuller, H. Schillers, G. A. MacGregor, H. E. de Wardener, and M. Hausberg. 2007. Plasma sodium stiffness vascular endothelium and reduces nitric oxide release. Proc. Natl. Acad. Sci. USA. 104: 16281–16286.
36. Jalal, L., and S. Devaraj. 1996. The role of oxidized low density lipoprotein in atherogenesis. J. Nutr. 126: 1053S–1057S.
59. Berliner, J. A., M. C. Territo, A. Sevanian, S. Ramin, J. A. Kim, B. Bamshad, M. Esterson, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J. Clin. Invest.* **85**: 1260–1266.

60. Colles, S. M., J. M. Maxson, S. G. Carlson, and G. M. Chisolm. 2001. Oxidized LDL-induced injury and apoptosis in atherosclerosis. Potential roles for oxysterols. *Trends Cardiovasc. Med.* **11**: 131–138.

61. Hakamata, H., A. Miyazaki, M. Sakai, H. Matsuda, H. Suzuki, T. Kodama, and S. Horiuchi. 1998. Isolation of macrophage-like cell mutants resistant to the cytotoxic effect of oxidized low density lipoprotein. *J. Lipid Res.* **39**: 482–494.

62. Miller, Y. I., D. S. Worrall, C. D. Funk, J. R. Feramisco, and J. L. Witztum. 2003. Actin polymerization in macrophages in response to oxidized LDL and apoptotic cells: role of 12/15-lipoxygenase and phosphoinositide 3-kinase. *Mol. Biol. Cell.* **14**: 4196–4206.

63. Wong, W. T., C. H. Ng, S. Y. Tsang, Y. Huang, and Z. Y. Chen. 2011. Relative contribution of individual oxidized components in ox-LDL to inhibition on endothelium-dependent relaxation in rat aorta. *Nutr. Metab. Cardiovasc. Dis.* **21**: 157–164.

64. Sleer, L. S., A. J. Brown, and E. R. Stanley. 2001. Interaction of caveolin with 7-ketocholesterol. *Atherosclerosis.* **159**: 49–55.

65. Vaya, J., M. Aviram, S. Mahmood, T. Hayek, E. Grenadir, A. Hoffman, and S. Milo. 2001. Selective distribution of oxysterols in atherosclerotic lesions and human plasma lipoproteins. *Free Radic. Res.* **34**: 485–497.

66. Pereira, T. M., B. V. Nogueira, L. C. Lima, M. L. Porto, J. A. Arruda, E. C. Vasquez, and S. S. Meyrelles. 2010. Cardiac and vascular changes in elderly atherosclerotic mice: the influence of gender. *Lipids Health Dis.* **9**: 87.

67. Vitale, C., M. E. Mendelsohn, and G. M. Rosano. 2009. Gender differences in the cardiovascular effect of sex hormones. *Nat. Rev. Cardiol.* **6**: 532–542.