Abstract  Endothelial biomechanics is emerging as a key factor in endothelial function. Here, we address the mechanisms of endothelial stiffening induced by oxidized LDL (oxLDL) and investigate the role of oxLDL in lumen formation. We show that oxLDL-induced endothelial stiffening is mediated by CD36-dependent activation of RhoA and its downstream target, Rho kinase (ROCK), via inhibition of myosin light-chain phosphatase (MLCP) and myosin light-chain (MLC)2 phosphorylation. The LC-MS/MS analysis identifies 7-ketocholesterol (7KC) as the major oxysterol in oxLDL. Similarly to oxLDL, 7KC induces RhoA activation, MLCP inhibition, and MLC2 phosphorylation resulting in endothelial stiffening. OxLDL also facilitates formation of endothelial branching networks in 3D collagen gels in vitro and induces increased formation of functional blood vessels in a Matrigel plug assay in vivo. Both effects are RhoA and ROCK dependent. An increase in lumen formation was also observed in response to pre-exposing the cells to 7KC, an oxysterol that induces endothelial stiffening, but not to 5α,6α-epoxide that does not affect endothelial stiffness. Importantly, loading cells with cholesterol prevented oxLDL-induced RhoA activation and the downstream signaling cascade, and reversed oxLDL-induced lumen formation.

In summary, we show that oxLDL-induced endothelial stiffening is mediated by the CD36/RhoA/ROCK/MLCP/MLC2 pathway and is associated with increased endothelial angiogenic activity.—Oh, M-J., C. Zhang, E. LeMaster, C. Adamos, E. Berdyshev, Y. Bogachkov, E. E. Kohler, J. Baruah, Y. Fang, D. E. Schraufnagel, K. K. Wary, and I. Levitan. Oxidized LDL signals through Rho-GTPase to induce endothelial cell stiffening and promote capillary formation. J. Lipid Res. 2016. 57: 791–808.

Supplementary key words  angiogenesis • endothelial stiffness • RhoA

Oxidative modifications of LDL are a major risk factor in the development of vascular disease and are known to induce endothelial dysfunction, one of the earliest manifestations of atherosclerosis (1, 2). Our studies focus on the oxidized LDL (oxLDL)-induced impact on endothelial biomechanics and its role in vascular dysfunction.

Our recent studies showed that the stiffness of aortic endothelial cells (ECs) is significantly increased by exposing the cells to oxLDL in vitro or by dyslipidemia in the diet-induced porcine atherosclerosis model in vivo (3, 4). An increase in endothelial stiffness was accompanied by an increase in endothelial contractile forces generated on the cell-substrate interface and an enhanced ability of ECs to form branching networks in 3D cultures (3, 4), which is considered a prerequisite of angiogenesis (5). Moreover, earlier studies demonstrated a correlation between increased endothelial force and network formation across several endothelial subtypes (6). We proposed, therefore, that oxLDL-induced endothelial stiffening may lead to increased angiogenic activity of ECs during the development of atherosclerotic plaques. This process is expected to be of major clinical importance because neovascularization of the plaques is increasingly recognized as a critical process and a major risk factor for plaque vulnerability (7). The goal of this study is to elucidate the mechanism of oxLDL-induced endothelial stiffening and evaluate a link between this effect and the ability of ECs to form functional capillaries.

Abbreviations: DN, dominant negative; EC, endothelial cell; HAEcs, human aortic endothelial cells; H&E, hematoxylin and eosin; HMVEC, human microvascular endothelial cell; kPa, kilopascals; Lox1, oxidized LDL receptor-1; MLG, myosin light-chain, MLCP, myosin light-chain phosphatase; oxLDL, oxidized LDL; RBC, red blood cell; ROCK, Rho kinase; SCID, severely compromised immunodeficient; vWF, von Willebrand factor.

To whom correspondence should be addressed.

E-mail: levitan@uic.edu

[This online version of this article (available at http://www.jlr.org) contains a supplement.]

Copyright © 2016 by the American Society for Biochemistry and Molecular Biology, Inc.

This article is available online at http://www.jlr.org
Little is currently known about the mechanism of oxLDL-induced endothelial stiffening. Surprisingly, we showed that it could be reproduced by cholesterol depletion rather than cholesterol enrichment (3, 8) and is associated with disruption of the lipid packing of cholesterol-rich membrane domains (4). More recently, we showed that endothelial stiffness increases in cells exposed to specific oxysterols, a major component of oxLDL (9). We have also shown that endothelial stiffening induced by the loss of cellular cholesterol is abrogated by disruption of F-actin, indicating that the integrity of F-actin is essential for the stiffening response (8). However, the signaling pathway responsible for oxLDL-induced endothelial stiffening is not known. Earlier studies showed that oxLDL triggers activation of RhoA and its downstream signaling pathways (11–13). We address the hypothesis, therefore, that activation of RhoA and its downstream signaling pathway plays a central role in oxLDL-induced endothelial stiffening.

The impact of oxLDL on angiogenesis is currently controversial. Earlier studies suggested that oxLDL inhibits endothelial proliferation (14), migration (15), and in vitro tube formation (16), as well as angiogenesis in the hind limb ischemia model (17). Our studies, however, showed that oxLDL may have a positive effect on the formation of endothelial networks in a 3D in vitro model of angiogenesis (3, 18). Consistent with these findings, a similar effect was shown in a 2D system (19). Most recently, it was shown that oxLDL also has a pro-angiogenic effect in vivo (20, 21), possibly in a macrophage-dependent way (20). Our hypothesis is that oxLDL may increase endothelial angiogenic potential directly by altering endothelial biomechanical properties.

In this study, we demonstrate that both oxLDL-induced endothelial stiffening and oxLDL-induced increase in capillary formation in Matrigel plug assay in vivo is mediated by the RhoA/Rho kinase (ROCK) pathway and that both can be prevented by preloading the cells with cholesterol. Furthermore, we show that 7-ketocholesterol (7KC) also induces endothelial stiffening via the same signaling pathway, an effect that is also accompanied by increased capillary formation in Matrigels in vivo. Taken together, these findings provide the first detailed mechanistic insights into oxLDL/7KC-induced endothelial stiffening and suggest a novel link between oxidized lipids, endothelial biomechanics, and capillary formation.

MATERIALS AND METHODS

Cell culture and reagents

Human aortic ECs (HAECs; Lonza, Allendale, NJ) were grown between passages 5 and 10 in Endogro LS complete (Millipore, Billerica, MA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 10 μg/ml penicillin, streptomycin, and kanamycin sulfate (Life Technologies, Grand Island, NY). Cell cultures were maintained in a humidified incubator at 37°C, with 5% CO₂. Cells were split every 3–4 days. The 7KC was purchased from Steraloids (Newport, RI). MβCD saturated with cholesterol was prepared as described previously (8). Cells were transfected using an Amaxa Nucleofector kit (Lonza) for expression of mutant constructs and then subsequently plated onto glass coverslips for microaspiration. DnRhoA<sup>T17N</sup> tagged with cyan fluorescent protein and DnRac1<sup>T17N</sup> tagged with yellow fluorescent protein were generous gifts of Dr. Graham Carnegie and Dr. Dolly Mehta. A polyclonal rabbit antibody to von Willebrand factor (vWF) was obtained from Dako (Carpinteria, CA), and anti-CD31 antiserum was purchased from Becton Dickinson (Franklin Lanes, NJ). Cell counting medium with DAPI was obtained from Vector Laboratories (Burlingame, CA).

LDL isolation and oxidation

LDL was isolated from human plasma obtained from a local blood bank (Lifesource, Chicago, IL) and oxLDL was prepared as described previously (4, 9). The plasma was separated by sequential centrifugation in KBr with a final density of 1.063 g/ml. The preparation was then dialyzed three times in PBS containing 1 mM EDTA at 4°C to remove KBr and then subsequently dialyzed in PBS alone for 2 days to remove EDTA. Copper sulfate was then added to LDL with a final concentration of 25 μM for 16 h at 37°C to oxidize LDL. This reaction was stopped by adding 1 mM EDTA. The content of thiobarbituric acid-reactive substances in LDL and oxLDL was determined by using a thiobarbituric acid-reactive substance assay kit (ZeptoMetrix, Buffalo, NY) as expressed with malondialdehyde equivalents.

Lipid extraction and LC-MS/MS analysis of oxysterols

Oxysterols and cholesterol were analyzed via ESI-LC-MS/MS as acetate derivatives. LDLs of oxLDL (0.05 ml) were extracted using a modified Bligh and Dyer method with the use of 0.9% potassium chloride for phase separation. The set of appropriate deuterated oxysterol and cholesterol internal standards was added during extraction. Internal standards used were 7KC-d7, 5α,6α-epoxycholestanol-d7, 24(R/S)-hydroxycholesterol-d7, 23-hydroxycholesterol-d6, 22(S)-hydroxycholesterol-d7, 7α-hydroxycholesterol-d7, 27-hydroxycholesterol-d6, and cholesterol-d7 (all from Avanti Polar Lipids, Alabaster, AL). The chloroform phase was evaporated and lipids were subjected to acetylation with acetic anhydride/pyridine (1:2, v/v) at 55°C for 30 min. Then solvents were evaporated under a stream of nitrogen and acetylated samples were dissolved in methanol for the ESI-LC-MS/MS analysis.

Sterol and oxysterol acetates were quantified via an ESI-LC-MS/MS approach using AB Sciex 6500 QTRAP mass spectrometer interfaces with an Agilent 1290 LC system. Sterol and sterol acetates were separated on an Ascentis Express C18 column (50 × 2.1 mm, 2.7 μm particle size) using a gradient elution from solvent system A (methanol:water:formic acid 65:35:5, v/v) to solvent system B (methanol:water:formic acid 99:0.5:0.5, v/v) with both systems containing 5 mM ammonium formate. Column temperature was maintained at 55°C. Sterol and oxysterol acetates were analyzed using ESI in positive ion mode as a transition from corresponding ammonium adduct to the most abundant product ion. The employed transitions were as follows: m/z 446.3 > m/z 369.4 (cholesterol acetate), m/z 445.3 > m/z 383.4 (7KC acetate), m/z 462.4 > m/z 367.4 (5α,6α-epoxycholestanol), m/z 504.4 > m/z 367.4 [24(R/S)-hydroxycholesterol, 22(S)-hydroxycholesterol, 25-hydroxycholesterol, and 7α-hydroxycholesterol], m/z 504.4 > m/z 427.4 (27-hydroxycholesterol). Transitions for deuterated internal standards were increased by the appropriate number of Daltons in the molecular and product ions depending on the number of deuterium atoms in the molecule. Declustered ions and collision energies were optimized for each
sterol/oxyysterol acetate. Quantitation of cholesterol and oxysterols was achieved via an isotope dilution approach.

**Microaspiration**

Microcapillary aspiration of ECs was performed as described in our earlier studies (8, 22). Cells were plated onto glass coverslips and allowed to attach overnight. Cells were then serum starved for 1 h prior to exposure to either 10 µg/ml of oxLDL, LDL, or 7KC for 1 h. Cells were then washed and membranes were visualized using a DiIC18 dye (Invitrogen, Carlsbad, CA) and then aspirated using pulled glass micropipettes of 4–8 µm diameter (SG10 glass; Richland Glass, Richland, NJ). A Zeiss microscope (Axiovert 200M) was used to image membrane deformation at 10 s intervals for 180 s using negative pressure (−15 mm Hg) applied by a pneumatic transducer tester (Fluka/Sigma-Aldrich). Ten to fifteen single cells were measured sequentially in each experiment within the window of 1–4 h after the exposure to oxLDL. Time zero on microaspiration graphs designates the time of the application of the negative pressure to each cell. Cells successfully transfected with DnRhoAT138C or DnRac1T17N were identified by the cyan fluorescent protein and yellow fluorescent protein tags.

**AFM microindentation**

EC stiffness was assessed by Young’s elastic modulus using AFM (Novascan Technologies). A 10 µm diameter borosilicate glass bead affixed to the tip of a cantilever indents the EC. Force curves are generated from the laser’s deflection on the cantilever as it approaches and indents the cell (indentation depth of 0.5−1 µm or ~10−15% of the cell’s total height). Young's elastic modulus [measured in kiloPascals (kPa)] is calculated from the force-distance curves and conforms to the Hertz model:

\[ F = \frac{4}{3} \frac{E}{(1−v^2)} \delta^2 \sqrt{R} \]

where F is the force, E is Young’s elastic modulus (stiffness), v is Poisson’s ratio assumed to be 0.5, δ is the indentation depth, and R is the radius of the cantilever’s bead (50 µm). The force-distance curves were analyzed using a least-square minimization algorithm. Specifically, HAEs, seeded on coverslips the day before, were serum starved for 1 h and treated with a concentration range (0–100 µg/ml) of oxLDL for 1 h. Cell stiffness was probed from 15 to 20 individual cells per condition per experiment (n = 3). An additional study used a 1 h treatment of 50 µM blebbistatin (Tocris Bioscience), a myosin II inhibitor, prior to a 50 µg/ml oxLDL treatment and subsequent elastic modulus measurements (n = 3).

**CD36 and Lox1 knockdown**

Two different constructs for CD36 siRNAs (B and D), Lox1 (A and B), and a scrambled control (AllStars negative control siRNA) were ordered from QiAGEN (FlexibleTube siRNA). HAEcs that were 80−90% confluent were transfected in Opti-MEM (Thermo Fisher Scientific) media using lipofectamine (Invitrogen) and a siRNA mix for 4 h. HAEC-enriched cell culture media (Lonza) was then used to replace the Opti-MEM solution and cells were allowed to grow for 48 h before experiments were performed.

**Western blot analysis**

RhoA activation assay. RhoA activity was assessed as was previously described in RhoA pulldown assays (23, 24). Specifically, HAEcs were serum starved for 1 h prior and were then pretreated with cholesterol for cholesterol experiments or were exposed to a concentration range (0–100 µg/ml) of oxLDL for 15 min. Cells were then washed with PBS, scraped, and lysed. A BCA assay (Pierce) was then used to quantitate protein levels per sample, and then 800–1,000 µg of protein was incubated with Rho kinase beads (Cytoskeleton Inc., Denver, CO) for 1 h at 4°C. The Rhokin bead were then spun and washed before being boiled in sample buffer for 2 min. Samples were then run on a SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane before being probed for RhoA (Santa Cruz Biotechnologies).

**Rac1 activation assay and MLC and MLC2 phosphorylation.** Rac1 activity was analyzed with an active Rac1 detection kit from Cell Signaling Technology. Briefly, cells were lysed in lysis buffer. The lysates were then incubated with glutathione resin and GST-PAK1-PBD mixture at 4°C for 1 h with gentle rocking. The resin was washed three times and eluted with 50 µl 2X SDS sample buffer containing 200 mM DTT. Western blot analysis of the cell lysate or the eluted samples from resin was performed using a Rac1 mouse monoclonal antibody. Confluent HAECs were serum starved for 1 h, cells were then treated with corresponding conditions as marked. Cells were lysed in lysis buffer with protease and phosphatase inhibitor cocktail. The phosphorylation level of myosin light-chain phosphatase (MLCP) (MYPT1) and myosin light-chain (MLC)2 were probed with antibody from Cell Signaling Technology.

**RhoA activation ELISA**

RhoA activation was assessed by RhoA G-LISA quantitative assay (Cytoskeleton Inc.) according to the manufacturer’s protocol. Briefly, confluent HAECs were serum starved first and corresponding treatments were applied. After treatments were finished, the medium was immediately aspirated and the cells were washed with ice-cold PBS, lysed in lysis buffer from the kit, and centrifuged at 10,000 g for 1 min at 4°C. The supernatants were collected, snap-frozen in liquid nitrogen, and stored at −80°C until used. The supernatants’ protein concentration was determined by using the Precision Red Advanced Protein assay supplied with the kit. The same amount of protein was used for ELISA. For all experiments, positive (constitutively active RhoA) and negative (lysis buffer) controls were also used. After incubation with the first and second antibody and color development, absorbance was read at 490 nm using a microplate ELISA reader.

**Real-time PCR**

Cells that were 80−90% confluent were treated with corresponding siRNA or scrambled control siRNA for 48 h. Then total cellular RNA was isolated by using Direct-zol™ RNA MiniPrep Plus kit (Zymo Research, Irvine, CA) and reverse transcribed to cDNA by using a high-capacity cDNA reverse transcription system (Applied Biosystems) according to the manufacturer’s protocols. The cDNA synthesis was performed with 0.5 µg of total RNA in a reaction volume of 20 µl containing 2 µl random primer for 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. The primers for CD36 and GAPDH were ordered from Integrated DNA Technologies (predesigned quantitative PCR primers). The cycling conditions were 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 40 s. Relative expression levels were determined by the 2−ΔΔCt method and normalized to GAPDH.

**Preparation of collagen gels and visualization of EC network formation**

ECs were grown in 3D collagen gels as described previously (3, 4, 6). Collagen gels were prepared according to the manufacturer’s instructions to a final collagen concentration of 1.5 mg/ml (Becton Dickinson). HAECs were seeded into gel mixtures and were allowed to polymerize for 20 min at 37°C in 48-well plates. Thereafter,
the gels were mechanically loosened from the sides of the wells and growth media were supplemented with vascular endothelial growth factor, basic fibroblast growth factor, and PMA at concentrations of 50 μg/ml each. Vascular endothelial growth factor was obtained from PeproTech (Rocky Hill, NJ), and basic fibroblast growth factor and PMA were obtained from Sigma-Aldrich. Gels were cultured for 48 h and gel contraction was quantified using MetaVue (Molecular Devices, Union City, CA). To visualize the cells, gels were fixed in 4% paraformaldehyde at 4°C overnight and stained with 0.1% toluidine blue (5 min). Images were obtained at 10× magnification (Nikon, Eclipse TE200-U) to observe EC networks and analyzed using Scion Image, as described previously (3, 4).

Matrigel plug assay and immunohistochemistry

Matrigel plug assay offers an in vivo model of angiogenesis that allows determination of the impacts of various factors on the formation of functional blood vessels in mice. Comparative analysis of the advantages and disadvantages of different angiogenic models, including Matrigel plug, is described in detail in Auerbach et al. (5). Matrigel plug assays were performed as described in earlier studies (25). Briefly, 3-month-old athymic nude mice [severely compromised immunodeficient (SCID)], 25–30 g body weight (Jackson Laboratory), were housed under pathogen-free conditions at the University of Illinois Animal Care Vivarium and treated humanely in accordance with institutional guidelines. Matrigel containing 7 × 10^6 HAECs exposed to a mock control or oxLDL were injected subcutaneously in the midventral abdominal region of nude mice, allowed to solidify, and monitored after 24 and 48 h to assess the wound. After 7 days, the Matrigel plugs were collected and separated from the abdominal muscle, washed with PBS, fixed in 10% formalin, and embedded in paraffin. Fifteen to eighteen serial sections 5 μm thick were prepared from each Matrigel. To visualize the vessels, paraﬃn was initially removed by immersing the slides three times in Xylene for 10 min. Slides were then subsequently immersed in ethanol for 5 min, progressively decreasing the percentage of ethanol (100, 95, and 70%). Slides were then rinsed in cold water before permeabilization with 0.5% Triton X-100. After washing with PBS, the slides were incubated with a citrate buffer solution at 95°C for 30 min. After cooling and rinsing in PBS, peroxidase activity was inhibited by incubating the slides in 3% cold hydrogen peroxide for 20 min. The Matrigel sections were either stained with hematoxylin and eosin (H&E) for histological examination or stained for specific endothelial markers (CD31 and vWF). In order to stain for endothelial markers, the Matrigel sections were first blocked with 5% BSA solution for 1 h before incubating with primary antibodies overnight at 4°C. After washing the slides three times with PBS, sections were incubated with secondary antibodies for 1 h at room temperature. Slides were then washed three times with PBS and mounted using Vectorshield mounting medium. The imaging was performed using a Zeiss Axiovert 40 phase contrast microscope or a Zeiss microscope (Axiovert 200M) for fluorescent imaging. Capillary lumens were counted for two to three histological sections for each Matrigel plug with each section containing three to four fields of view under the given magnification (20× objective). All counting was performed in a double blind protocol, with the samples masked and randomized by two observers blind to the identity of the sample. After the counting, the samples were unmasked to identify the experimental conditions. The number of the capillaries in each experiment was normalized to controls obtained in the same experiment.

Cell viability assay

Cell viability was assessed using a live cell viability kit (Life Technologies). HAECs were serum starved for 1 h prior to exposure to oxLDL in a dose-dependent manner. The samples were treated with calcine AM, a nonfluorescent cell-permeable dye that is converted to fluorescent calcine upon intracellular esterase activity. As a negative control, HAECs that were incubated with 0.1% saponin that killed cells was used. Calcine’s excitation wavelength was 485 nm and the emission wavelength was measured at 530 nm.

Statistics

For statistical analysis, R software was used to perform one-way ANOVA on mean values (±SEM) to determine statistical significance. This was followed up with an unpaired t-test set with a P value <0.05.

RESULTS

oxLDL-induced endothelial stiffening is mediated by RhoA/ROCK/MLCP/MLC cascade

To probe the role of RhoA in oxLDL-induced endothelial stiffening, we first tested the correlation between the dose-response of oxLDL-induced RhoA activation and endothelial elastic modulus. As described earlier (23), exposing HAECs to oxLDL results in a transient activation of RhoA that peaks at 5–15 min, as evaluated using a Rhoa pulldown assay (Fig. 1A, C). OxLDL-induced activation of RhoA was verified using ELISA as an alternative complementary approach (Fig. 1D). OxLDL-induced RhoA activation is dose-dependent, increasing significantly between 10 and 100 μg/ml oxLDL, a typical range used in earlier in vitro studies (4, 19, 23, 24, 26), at a peak exposure time of 15 min (Fig. 1B, E). To determine the dose-response of oxLDL-induced endothelial stiffening, we measured endothelial elastic modulus using atomic force microscopy. Exposure to oxLDL (10–100 μg/ml, 1 h) resulted in a significant right-shift of the histograms of the EC elastic moduli, indicating a gradual increase in cell stiffness with the concentration of oxLDL leveling off at 50 μg/ml (Fig. 1F, G). Notably, there was a strong correlation between oxLDL-induced RhoA activation and an increase in EC elastic modulus (Fig. 1H).

To test whether activation of RhoA was responsible for oxLDL-induced endothelial stiffening, we tested to determine whether this response could be abolished by the inhibition of RhoA using C3 transferase (C3) or a dominant-negative RhoA mutant (DN-RhoAN19). In these experiments, endothelial stiffness was estimated by micropipette aspiration, a technique that measures cellular deformation into a micropipette in response to negative pressure applied to the cell surface (22). Cellular deformation is measured as the length of the protrusion into the pipette normalized by the pipette diameter to account for the differences in the applied force. As was shown in our previous studies (3, 8), cells exposed to oxLDL (10 μg/ml oxLDL for 1 h) were significantly stiffer (shorter membrane protrusions) than cells exposed to the same level of LDL (10 μg/ml). While this level of LDL is significantly below the physiological range, it was used as control to determine the specific effect of LDL oxidation. Here we show that pretreating the cells with C3 (1 μg/ml) prevented oxLDL-induced
constraint on membrane deformability (see typical images of membrane protrusions in Fig. 2A). Microaspiration was performed within the window of 1–4 h after the oxLDL exposure. Notably, while a measurement of a single cell was complete within 3 min, as shown in Fig. 2B, C, D, a time window of several hours was required to perform the measurement of multiple cells. Average time-courses of progressive membrane deformation in control and oxLDL-treated cells with and without pretreating the cells with C3 show that blocking RhoA activation abolishes oxLDL-induced endothelial stiffening (Fig. 2B). Similarly, transfecting the cells with DN-RhoAN19 also abolishes the stiffening effect (Fig. 2C). Previous studies also showed that oxLDL might activate another small GTPase, Rac1 (23, 27). Consistent with these studies, we also showed that oxLDL induces Rac1 activation in HAECs, but transfecting
Fig. 2. oxLDL induces endothelial stiffening via the RhoA and ROCK/MLCP/MLC2 pathways. A: Representative images of membrane deformation of HAECs visualized by DiI<sub>18</sub> red fluorescent membrane dye. The dotted line indicates the position of the glass pipette. B–D: Average time-courses of progressive membrane deformation cells exposed to LDL (10 μg/ml), oxLDL (10 μg/ml), or oxLDL with C3 (B), Y27632 (D), or in cells transfected with DnRhoA N19 (C) (n > 10–15 cells per condition). E, F: Maximal membrane deformation for conditions described in (B–D) (P < 0.05). G, H: Elastic moduli in kPa of HMVECs exposed to LDL, oxLDL, Y27632, or oxLDL pretreated with Y27632 [histograms (G) and average values (H)] (n > 60 cells per condition, three independent experiments). *P < 0.05.
the cells with DN-Rac1N17 had no effect on endothelial stiffness (supplementary Fig. 1). The effect of oxLDL on endothelial stiffening was also abrogated by inhibiting ROCK, the major downstream target of RhoA. Specifically, pretreating the cells with Y27632 (1 µg/ml), an inhibitor of ROCK, fully reversed oxLDL-induced EC stiffening (Fig. 2D). Maximal cellular deformation for all experimental conditions described above are shown in Fig. 2E, F. These data indicate that activation of RhoA/ROCK is essential for oxLDL-induced endothelial stiffening, oxLDL-induced endothelial stiffening was observed not only in aortic ECs but also in microvascular ECs (Fig. 2G, H). Specifically, exposing human microvascular ECs (HMVECs) to 50 µg/ml oxLDL for 1 h resulted in a significant increase in the elastic moduli of these cells and, similarly to HAECS, this effect was abrogated by pretreating the cells with 1 µg/ml Y27632 to block ROCK activation (Fig. 2G, H).

Our next goal was to establish whether oxLDL-induced activation of the RhoA pathway and subsequent stiffening is mediated by endothelial scavenger receptors. We found that two major endothelial scavenger receptors known to interact with oxLDL, CD36 and oxLDL receptor-1 (Lox1), are expressed in HAECS (Fig. 3A, B). Moreover, because previous studies suggested that Lox1 and not CD36 is the major scavenger receptor in ECs of large vessels, we further confirmed CD36 expression in HAECS by real-time PCR (supplementary Fig. 2). Both CD36 and Lox1 were downregulated using the siRNA approach, as compared with scrambled controls (Fig. 3A, B). A significant (50–70%) reduction of the protein expression was achieved for both receptors with two separate siRNAs for each protein (see typical Western blots in Fig. 3A, B and densitometry analysis in Fig. 3C, D). The specificity of the siRNA effects was verified by checking whether anti-CD36 siRNA affected the expression of Lox1 and vice versa (not shown). Our data demonstrate that oxLDL-induced activation of RhoA was significantly inhibited only by knocking down the CD36, but not the Lox1 receptors (Fig. 3E, F). The baseline levels of RhoA activity observed in the absence of oxLDL was unaffected by knocking down either of the receptors. Importantly, oxLDL-induced increase in EC elastic moduli was also practically abolished by knocking down of CD36 receptors, indicating that recognition of oxLDL by CD36 is required to activate this pathway and induce endothelial stiffening (Fig. 3G, H).

To elucidate the downstream events of the signaling pathway of oxLDL-induced endothelial stiffening, we tested the effect of oxLDL on MLCP, a known target of ROCK (28), and on MLC2 phosphorylation that is negatively regulated by MLCP (28). MLCP activity is estimated by phosphorylation of its subunit Mypt1, which renders MLCP inactive (29). We show here that exposure of HAECS to oxLDL results in an increase in Mypt1 phosphorylation, indicating that MLCP is inhibited, and an increase in MLC2 phosphorylation, an expected outcome of MLCP inhibition (Fig. 4A–G). Notably, significant phosphorylation of both Mypt1 and MLC2 is observed after 15 min of oxLDL exposure and maintained for at least 120 min. Furthermore, oxLDL-induced phosphorylation of both Mypt1 and MLC2 is abrogated by inhibiting RhoA or ROCK activation (Fig. 4D–F). Finally, to determine whether oxLDL-induced endothelial stiffening depends on MLC2 phosphorylation, cells were exposed to blebbistatin (50 µM), a high affinity MLC2 inhibitor. Pretreating the cells with blebbistatin prevents a rightward shift in the elastic moduli of ECs, indicating that MLC2 phosphorylation is essential for oxLDL-induced endothelial stiffening (Fig. 4G, H). Exposure to blebbistatin alone, without oxLDL exposure, does not have a significant effect. These data provide comprehensive evidence that oxLDL-induced endothelial stiffening is mediated by the RhoA/ROCK/MLCP/MLC2 signaling pathway.

7KC and oxLDL regulate endothelial stiffness by the same pathway

Previously, we showed that oxysterols play a dominant role in oxLDL-induced endothelial stiffening (9). We further proposed that the impact of oxLDL on endothelial stiffness is mediated by incorporation of oxysterols in the cell membrane. To identify the most abundant oxysterols in the oxLDL complex, we performed a LC-MS/MS-based analysis of the levels of different sterols present in LDL before and after oxidation. The identity of oxysterols was confirmed by their breakdown pattern and by their LC retention time, and quantitation was performed via an isotope-dilution approach with the use of authentic deuterated oxysterol standards. This analysis showed that in native (non-oxidized) LDL, the only major peak corresponded to cholesterol. However, LDL oxidation resulted in formation of several oxysterols, with 7KC being the most abundant (Fig. 5A). The absolute concentrations of all detectable oxysterols calculated based on the appropriate standards are shown as a table in Fig. 5B. These data show that, while an array of oxysterols can be detected both in LDL and oxLDL with a significant increase in oxLDL particles, the level of 7KC in oxLDL is several fold higher than any other sterol. In our further studies, therefore, we focused on determining whether 7KC activates the RhoA/ROCK/MLCP/MLC2 pathway. First, we tested whether 7KC alone could activate RhoA. Our data show that, similarly to oxLDL, exposing HAECS to 10 µg/ml 7KC, a level that was shown previously to induce endothelial stiffening (9), results in activation of RhoA, as determined both by the pulldown and the ELISA assays (Fig. 5C–E). This level of 7KC is higher than what we found in oxLDL (7.2 µg 7KC in 1 mg protein corresponding to 0.7 µg/ml 7KC in 100 µg/ml oxLDL), but similar or lower than the levels typically used to test the impact of 7KC on cellular function (10 to >100 µg/ml 7KC) (30–32). Further studies need to address the effects of lower levels of 7KC.

Next, we tested to determine whether 7KC-induced endothelial stiffening is RhoA/ROCK dependent. First, we show that, similarly to our earlier observations in bovine aortic ECs (9), exposing HAECS to 10 µg/ml 7KC for 1 h results in significant cell stiffening, as demonstrated by measuring progressive membrane deformation (Fig. 6A).
Here we also show that this effect is prevented by pretreating the cells with C3 (Fig. 6A, B). To evaluate whether exposure to 7KC also resulted in stiffening of human microvascular ECs, this experiment was repeated with HMVECs. Similarly to HAECs, HMVECs responded to 7KC by a significant increase in cell stiffness, an effect that was also abrogated by inhibition of the RhoA/ROCK pathway (Fig. 6C, D).

Furthermore, similarly to oxLDL, 7KC also activated the downstream targets of RhoA, resulting in MYPT1 phosphorylation, manifestation of MLCP inhibition, and MLC2 phosphorylation. Both effects were abrogated by inhibiting

---

Fig. 3. oxLDL-induced RhoA activation and endothelial stiffening is CD36 dependent. A, B: Representative Western blots of siRNA knockdown of CD36 (A) and Lox1 (B). C, D: Densitometry analysis of CD36 and Lox1 knockdown when exposed to siRNAs. E, F: RhoA activity as assessed by RhoA activation ELISA kit for HAECs exposed to oxLDL with or without siRNAs to CD36 or Lox1. G, H: Elastic moduli of HAECs exposed to oxLDL with or without the presence of siRNA to CD36 [histograms (G) and average values (H)] (n ≥ 60 cells per condition, three independent experiments). *P < 0.05.
oxLDL and endothelial biomechanics in angiogenesis

RhoA or ROCK (Fig. 7A–C). Taken together, these data indicate that 7KC induces endothelial stiffening by the same mechanism as oxLDL.

The role of the RhoA/ROCK pathway in oxLDL/7KC-induced force generation and network formation

Next, we tested to determine whether the RhoA/ROCK pathway is also responsible for oxLDL-induced increase in force generation and network formation. HAECs were seeded into 3D collagen gels, where they generated intercellular connections that formed distinct EC networks (3, 6). Contractile forces generated by the cells are estimated by the degree of gel contraction. As expected, exposure to oxLDL increased EC contractility, but pretreating the cells with C3 abrogated this effect (Fig. 8A, B). Inhibiting ROCK also abrogated oxLDL-induced increase in endothelial contractility (Fig. 8C). Furthermore, exposure to 7KC also resulted in increased contractility and, similarly to oxLDL, 7KC-induced endothelial contractility is also RhoA dependent (Fig. 8D).

Our earlier studies demonstrated that oxLDL facilitates formation of EC branching networks in 3D collagen gels (3, 4). Here, we tested to determine whether the oxLDL-induced increase in EC network formation correlates with an increase in EC stiffness and contractility and is RhoA dependent. Representative images of branching EC networks in 3D collagen gels are shown in Fig. 8E, demonstrating that preexposure to oxLDL facilitates formation of the network. Branching network formation was analyzed by quantifying cell elongation and connectivity, as described previously (3, 6). Analysis of EC elongation and connectivity showed that inhibition of both RhoA and ROCK reverse the oxLDL effect on EC network formation (Fig. 8F, G). Similarly, exposure to 7KC significantly facilitated branching network formation via a Rho-dependent pathway (Fig. 8H).
oxLDL facilitates angiogenic activity of ECs in vivo via the RhoA/ROCK pathway

Next, we sought to determine whether pretreatment with oxLDL facilitates angiogenesis in a Matrigel plug assay, an in vivo correlate of angiogenesis (5). Accordingly, Matrigels were seeded with HAECs pretreated with oxLDL or with control cells, and then injected into SCID mice. Matrigels were excised from mice after 7 days and the formation of the blood vessels was analyzed histologically. Representative images of 5–6 μm thin sections stained by H&E reveal neovessel formation, both in cross-sectional and longitudinal orientations (Fig. 9A). Multiple lumens of variable diameters, ranging from ~5 to 30 μm, are seen in most sections under all experimental conditions. Most contain red blood cells (RBCs) (arrow) indicating that these are functional perfused neovessels connected to the mouse vasculature. Identification of the lumens was verified using two endothelial markers, vWF and CD31 (Fig. 9B). Moreover, using anti-mouse CD31 antibody that specifically reacts with murine cells or anti-vWF antibody that recognizes both murine and human cells allows discrimination between HAECs seeded into the gels and murine host ECs that are recruited into the gels; human ECs are positive for vWF (red) only, while mouse cells are stained for both vWF (red) and CD31 (green) (Fig. 9B, E), as verified in supplementary Fig. 3. We found that both human and mouse ECs form blood vessels with a portion of lumens comprised mostly of HAECs [Fig. 9B, left panels, a representative image of a vessel positive for vWF, but negative for CD31 (note that RBCs autofluoresce)] and a portion of the lumens comprised mostly of mouse cells (Fig. 9B, right panels, double stained for vWF and CD31).

The number of the vessels formed under different experimental conditions was counted blindly by an observer masked to the identity of the sample. Our results show that pretreatment with oxLDL significantly increased the number of lumens formed in Matrigels (Fig. 9C, D). This result held for both the total number of vessels and the number of perfused vessels, as defined by the presence of RBCs. Also, human ECs were detected in about a third of the vessels under control conditions, with a significant increase when cells were pre-exposed to oxLDL (Fig. 9E). A similar trend was observed for neovessels formed by mouse cells. Furthermore, because it was proposed earlier that oxLDL has a pro-angiogenic effect only at low concentrations and
can be toxic at high concentrations, we extended these studies to reflect a typical range of oxLDL levels used in previous studies (10–100 µg/ml) (19, 21). Our observations are that oxLDL facilitates lumen formation in a dose-dependent way for the whole range (Fig. 9F). Moreover, there is a significant correlation between oxLDL-induced endothelial stiffening and an increase in lumen formation triggered by oxLDL (Fig. 9G). No effect on cell viability was observed for all experimental conditions used in this study (supplementary Fig. 4). Finally, we also show that oxLDL has a similar pro-angiogenic effect on macro- (HAECs) and microvascular ECs (HMVECs) (Fig. 9H).

To determine whether an oxLDL-induced increase in the angiogenic activities of ECs might be a result of increased endothelial stiffness, we used two complementary approaches: First, we tested to determine whether the RhoA pathway that mediates oxLDL-induced EC stiffening is also responsible for an increase in the angiogenic response. HAECs were preincubated with C3 or Y27632, then exposed to oxLDL, and then seeded into the Matrigels prior to injection into SCID mice (representative images are shown in Fig. 10A). As described above, pretreatment of HAECs with oxLDL resulted in a significant increase in the number of lumens. More importantly, the effect of oxLDL was inhibited by both C3 and Y27632, indicating that this effect is also RhoA/ROCK dependent (Fig. 10A, B). In addition, we also compared the effects of two oxysterols that we showed previously to have differential effects on endothelial stiffness (4): 7KC, which was most efficient in inducing endothelial stiffening, and 5α,6α-epoxide, which had no effect on endothelial stiffness. Here, we show that, similarly to oxLDL, pre-exposure of ECs to 7KC results in a significant increase in endothelial lumens formed in Matrigels, whereas preexposure to 5α,6α-epoxide has no effect (Fig. 10 C, D).

**Prevention of oxLDL-induced pro-angiogenic effect by cholesterol**

Previously we have shown that oxLDL-induced EC stiffening can be prevented or reversed by supplying the cells with cholesterol (4, 9). This was surprising and unexpected and here we extend these studies to test whether enriching the cells with cholesterol can also prevent oxLDL-induced angiogenic response. First, we show that preincubation with cholesterol significantly reduces oxLDL-induced activation of RhoA (Fig. 11A, B). Furthermore, consistent with these results, cholesterol also prevents oxLDL-induced inhibition of MLCP, as assessed by Mypt1 phosphorylation, and inhibits phosphorylation of MLC2 (Fig. 11C–E), demonstrating that cells enriched with cholesterol do not respond to oxLDL with the activation of the RhoA/ROCK/MLCP/MLC2 cascade.

Most importantly, we also show here that cholesterol loading may prevent oxLDL-induced pro-angiogenic response. Specifically, loading the cells with cholesterol after the oxLDL treatment led to a significant decrease in...
on the increase in tissue stiffness as a result of changes in the extracellular matrix. In this study, we provide the first mechanistic insights into the signaling pathway responsible for oxLDL-induced endothelial stiffening. We also provide new evidence for a link between oxLDL-induced endothelial stiffening and an increase in the angiogenic activities of ECs, through the activation of the RhoA/ROCK pathway.

Our previous studies established that, in contrast to the original expectations, oxLDL-induced endothelial stiffening cannot be attributed to cholesterol enrichment of the membrane, but instead is associated with the fluidization of cholesterol-rich membrane domains resulting from incorporation of oxysterols, in particular 7KC (4, 9). We also found that oxLDL and cholesterol depletion have similar effects on endothelial contractility, network formation (3, 4), and flow-induced endothelial alignment (35). In this study, we focus on the downstream signaling mechanism that underlies oxLDL-induced endothelial stiffening.

Several possible pathways might be involved in oxLDL-induced endothelial stiffening, such as formation of stress fibers, strengthening of sub-membrane cortical cytoskeleton, or increased adhesion to the substrate. The data presented in this study demonstrate that activation of RhoA plays the dominant role in oxLDL-induced endothelial stiffening and increase in force generation. Because it is known that RhoA is a key factor in stress-fiber formation, these data suggest that the stiffening is primarily a result of stress fiber formation. Indeed, our previous study demonstrated that exposure to oxLDL induces formation of stress fibers (35). In contrast, activation of Rac1, which was previously shown to be responsible for oxLDL-induced dephosphorylation of MLC in macrophages (27), does not contribute significantly to oxLDL-induced regulation of endothelial stiffness. Furthermore, here we extend these studies to demonstrate that oxLDL-induced endothelial stiffening is mediated by the RhoA/ROCK/MLCP/MLC2 pathway. Specifically, oxLDL activates RhoA leading to activation of ROCK, which results in suppression of MLCP and finally an increase in MLC2 phosphorylation, which increases endothelial stiffness.

Moreover, we have shown that oxLDL-induced activation of the RhoA/ROCK pathway and subsequent endothelial stiffening is mediated by one of the major endothelial scavenger receptors, CD36. ECs express several types of scavenger receptors that recognize and internalize oxLDL. Previous studies suggested that lectin-like Lox1 is the principal oxLDL receptor in ECs (36). It was also shown that downregulation of Lox1 inhibits activation of RhoA in ECs (23, 24). On the other hand, CD36 was shown to be critical for oxLDL-induced regulation of macrophage migration and polarity (27, 37). CD36 was also shown to be responsible for oxLDL-induced RhoA/ROCK activation with the subsequent inhibition of MLCP in platelets (38).

We tested the roles of both Lox1 and CD36 and found that it is CD36 and not Lox1 that plays a major role in regulating RhoA activation and endothelial stiffness in the presence of oxLDL. The relative contributions of these pathways to other endothelial responses should be further investigated. It is also important to note that CD36 may

**Fig. 7.** The 7KC induces the RhoA signaling pathway. A: Representative Western blots of phosphorylation of MYPT1 and MLC2 in response to 7KC with and without C3 or Y27632. B, C: Densitometry analysis of phospho-MYPT1 and pMLC2 for the same conditions. *P < 0.05.
OxLDL and endothelial biomechanics in angiogenesis also results in significant endothelial stiffening, as determined by measuring the deformability of freshly isolated ECs harvested from the pigs' aortas (3). These observations suggest that oxLDL-induced endothelial stiffening observed in vitro reflects endothelial stiffening that occurs in vivo in dyslipidemia. We propose that ECs might be exposed to the extra-high oxLDL levels within the plaque environment during the neovascularization process and that increased endothelial stiffness may contribute to abnormal endothelial function in this environment.

It is well-known that oxLDL particles contain multiple bioactive components (1). Our previous study showed that endothelial stiffening is mediated primarily by the oxysterol fraction, whereas other lipid components have a much smaller or no effect (9). We also showed that incorporation of 7KC into ECs can be prevented by preloading the cells with cholesterol (9). Here, we performed a comprehensive analysis of the sterol composition of LDL particles before and after oxidation using MS/MS. This analysis shows that there are virtually no oxysterols present in LDL prior to oxidation and that 7KC is the dominant oxysterol produced during the oxidation, with the level exceeding, by far, all other oxysterols. Here we show that, similarly to oxLDL, 7KC activates the RhoA/ROCK signaling pathway leading to endothelial stiffening. The latter observation is fully consistent with our hypothesis that oxLDL-induced endothelial stiffening is mediated by the incorporation of 7KC and extends this conclusion to show that oxLDL/7KC-induced endothelial stiffening is mediated by the RhoA/ROCK/MLCP/MLC2 pathway.

One of the important questions in the oxLDL field is: What are the physiological levels of oxLDL in vivo under normal and pathological conditions? This question is difficult to address because of the multiple forms of oxLDL and the different degrees of oxidation that may be present in tissues. Thus, while multiple studies demonstrated accumulation of oxLDL in atherosclerotic plaques using immunohistochemistry (39, 40), analytical determination of precise oxLDL levels in vivo remains controversial. Holvoet and colleagues pioneered measuring oxLDL levels in human plasma, demonstrating that it may range from below 10 μg/ml in people with normal levels of cholesterol to ~30 μg/ml in hypercholesterolemic patients (41–43). In contrast, later studies found significantly lower levels of circulating oxLDL, ranging from 0.2 to 0.4 μg/ml, which was attributed to different methodology (44). It is also generally believed that the level of oxLDL within the vascular wall, particularly within an atherosclerotic plaque, might be significantly higher than circulating oxLDL, but further studies are required to provide the quantitative analysis. Similarly to previous studies from our laboratory and from other investigators (19, 23, 24, 26), we use a range of 10–100 μg/ml oxLDL, which has been shown to cause endothelial stiffening (3, 4). Importantly, our earlier studies showed that diet-induced dyslipidemia in a porcine model of atherosclerosis also results in significant endothelial stiffening, as determined by measuring the deformability of freshly isolated ECs harvested from the pigs’ aortas (3). These observations suggest that oxLDL-induced endothelial stiffening observed in vitro reflects endothelial stiffening that occurs in vivo in dyslipidemia. We propose that ECs might be exposed to the extra-high oxLDL levels within the plaque environment during the neovascularization process and that increased endothelial stiffness may contribute to abnormal endothelial function in this environment.

It is well-known that oxLDL particles contain multiple bioactive components (1). Our previous study showed that endothelial stiffening is mediated primarily by the oxysterol fraction, whereas other lipid components have a much smaller or no effect (9). We also showed that incorporation of 7KC into ECs can be prevented by preloading the cells with cholesterol (9). Here, we performed a comprehensive analysis of the sterol composition of LDL particles before and after oxidation using MS/MS. This analysis shows that there are virtually no oxysterols present in LDL prior to oxidation and that 7KC is the dominant oxysterol produced during the oxidation, with the level exceeding, by far, all other oxysterols. Here we show that, similarly to oxLDL, 7KC activates the RhoA/ROCK signaling pathway leading to endothelial stiffening. The latter observation is fully consistent with our hypothesis that oxLDL-induced endothelial stiffening is mediated by the incorporation of 7KC and extends this conclusion to show that oxLDL/7KC-induced endothelial stiffening is mediated by the RhoA/ROCK/MLCP/MLC2 pathway.
Fig. 9. OxLDL facilitates increased angiogenesis in Matrigel plugs in vivo. A: Representative images of cross-sections or longitudinal sections of vessels (H&E stain). RBCs appear as small spherical objects inside the vessels. Scale bar, 10 µm. B: Representative images of lumens as identified by anti-vWF polyclonal antibody and anti-CD31 mouse-specific monoclonal antibody (note that RBCs autofluoresce). C: Representative sections of Matrigels seeded with HAECs pre-exposed to 10 µg/ml oxLDL as compared with control HAECs. D: Quantification of the number of lumens with or without RBCs (total) or lumens containing RBCs (perfused) (n = 5 gels per condition obtained from five mice). E: Number of lumens identified as formed by human or mouse cells (n = 5 gels per condition obtained from five mice). F: Number of blood vessels in gels seeded with HAECs exposed to 0–100 µg/ml oxLDL (n = 4 gels per condition obtained from eight mice). G: Correlation between the numbers of lumens and cell stiffness of HAECs. H: Comparison of HAECs and HMVECs when exposed to 50 µg/ml oxLDL (n = 8 gels per condition obtained from ten mice). *P < 0.05.
What are the functional implications of oxLDL-induced endothelial stiffening? Our studies (3, 4) and those from others (6, 45) implicated an increase in endothelial stiffness as an important factor in determining angiogenic activities of ECs. However, while an increase in matrix stiffness was shown to have a detrimental effect on angiogenesis, an increase in endothelial stiffness appears to promote endothelial angiogenic activity. Here, we provide the first evidence that oxLDL-induced endothelial stiffening mediated by the activation of the RhoA/ROCK pathway may lead to an increase in angiogenic activities of ECs in vivo and promote the formation of new blood vessels. Specifically,

![Figure 10](image)

**Fig. 10.** OxLDL- and 7KC-increased angiogenesis are mediated via the RhoA/ROCK pathway. A, C: Representative images of lumens (black arrow) formed in Matrigels seeded with HAECs exposed to oxLDL with and without preincubation with C3 or Y27632 (A) or 7KC (C). Scale bar, 10 μm. B, D: Number of blood vessels formed under the experimental conditions described above. *P < 0.05.

![Figure 11](image)

**Fig. 11.** OxLDL-induced RhoA signaling is inhibited by cholesterol loading. A, C: Representative Western blots of RhoA activity, p-MYPT1, and p-MLC2 in HAECs exposed to oxLDL with or without preloading the cells with cholesterol. B, D, E: Densitometry analysis of RhoA activity, p-MYPT1, and p-MLC2 (n > 4 gels per condition). *P < 0.05.
we show a strong correlation between oxLDL effects on endothelial elastic modulus and the propensity of ECs to form branching networks in collagen gels in vitro and capillary lumens in Matrigel plug assays performed in nude mice in vivo across an array of experimental conditions: similar dose response to oxLDL, abrogation of both effects by inhibiting the RhoA/ROCK pathway, and reversal of both effects by loading the cells with cholesterol. Furthermore, two oxysterols that we showed previously to have differential effects on cell stiffness (9), also have distinct effects in the angiogenesis assay: 7KC, which causes endothelial stiffening, also promoted lumen formation in Matrigels in vivo; whereas 5α,6α epoxide, which has no effect on endothelial stiffness, had no effect on lumen formation. These observations support our hypothesis that endothelial stiffness is an important factor in determining endothelial angiogenic potential.

There is also significant similarity between the effects of oxLDL on macrovascular and microvascular ECs, both in terms of stiffness and in terms of facilitation of lumen formation: oxLDL induces endothelial stiffening of both HAEcs and HMVECs via the RhoA/ROCK pathway and enhances lumen formation in Matrigels prepopulated with oxLDL-treated HAEcs or HMVECs. These observations suggest that, while it is well-known that it is the microvascular ECs that are responsible for angiogenesis in vivo, macrovascular ECs also have angiogenic properties and may be incorporated into functional capillaries.

In terms of the relationship between oxLDL-induced endothelial stiffening and its pro-angiogenic potential, while we show a strong correlation between the two, it is surprising that a short pre-exposure to oxLDL may result in a significant increase in endothelial branching in vitro and formation of functional capillaries in vivo. One possibility is that, once initiated, cells maintain their stiff and contractile phenotype for a relatively prolonged period of time that is required to form new lumens. Another possibility is that an early stiffening/contraction generates the

---

**Fig. 12.** Prevention of oxLDL-induced angiogenic response by cholesterol loading. A, B: Representative images of lumen formation in gels seeded with HAEcs treated with oxLDL with or without loading the cells with cholesterol [H&E stain (A) and endothelial markers (B), vWF and mouse specific CD31]. C: Number of blood vessels formed under the same experimental conditions. D: Schematic representation of oxLDL-induced increase in capillary lumens and the signaling reversal by cholesterol loading (n = 4 gels per condition obtained from n = 20 mice). *P < 0.05.
initial "push" for the pro-angiogenic phenotype that results in increased lumen formation, even if the stiffening is not maintained. Finally, even though we see a correlation between endothelial stiffening and increased lumen formation over a range of experimental conditions, we cannot fully exclude the possibility that oxLDL-induced activation of the RhoA/ROCK cascade has a pro-angiogenic response independently of an increase in endothelial stiffness.

It is also important to note that the current study examines the role of oxLDL focusing on a Matrigel plug model in nude mice, a model chosen because it allows the introduction of ECs to an in vivo system after being preexposed to oxLDL. Thus, the oxLDL-induced increase in lumen formation demonstrated in our study, can be attributed specifically to the impact of oxLDL on ECs and not to an indirect macrophage-dependent phenomenon, as was proposed in a recent study (20). However, this is also a limitation of the study and more studies are needed to evaluate the roles of oxLDL and endothelial biomechanical properties in other models of angiogenesis, particularly in the context of dyslipidemia.

The study also provides unexpected insights into the relationship between cholesterol loading and oxLDL, suggesting that elevated levels of LDL in the absence of oxidation may actually have a protective effect. Importantly, the balance between oxidized lipids and cholesterol loading of ECs is likely to vary depending on a plethora of specific conditions, including diet, genetic factors, and disease states. We propose that our findings are most relevant to the environment of an atherosclerotic plaque, which is known to be very high in oxidized lipids and to promote angiogenesis. These findings also suggest that the balance between oxLDL and cholesterol is a factor that might provide further important insights into endothelial dysfunction and angiogenic phenotype in different models of genetic and diet-induced hypercholesterolemia, as well as in hypercholesterolemia-dependent disease states.

In summary, our study delineates the mechanism of oxLDL-induced endothelial stiffening, demonstrating that it is mediated by the CD36/RhoA/ROCK/MLCP/MLC2 pathway and provides evidence that the oxLDL-induced pro-angiogenic effect is also mediated by the CD36/RhoA/ROCK pathway and is associated with an increase in endothelial elastic modulus. We propose that oxLDL/7KG-induced endothelial stiffening promotes unwanted angiogenesis within the atherosclerotic plaques that is well-recognized as a major compounding factor in plaque development. Another novel and surprising conclusion is that oxLDL-induced RhoA activation and its pro-angiogenic effect is preventable and reversible by cholesterol loading, a finding that might have significant clinical implications. Several studies have shown that plasma hypercholesterolemia is associated with increased neovascularization of atherosclerotic plaques and that inhibition of angiogenesis is beneficial for plaque stabilization and limiting disease progression (7). Our study suggests that inhibition of the RhoA/ROCK pathway might have a therapeutic effect on plaque stability through the prevention of endothelial stiffening.

The authors are very grateful to Dr. Pappasani Subbaiah for his help and advice about LDL and oxLDL preparations. They are also very grateful to Dr. Michael Cho for his support in providing access to an atomic force microscope.

REFERENCES

1. Levitan, I., S. Volkov, and P. V. Subbaiah. 2010. Oxidized LDL: diversity, patterns of recognition, and pathophysiology. Antioxid. Redox Signal. 13: 39–75.
2. Libby, P. 2012. Inflammation in atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 32: 2045–2051.
3. 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: 713–723.
4. 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.
5. Auerbach, R., R. Lewis, B. Shimners, L. Kubai, and N. Akhtar. 2003. Angiogenesis assays: a critical overview. Clin. Chem. 49: 32–40.
6. 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.
7. Di Stefano, R., F. Felice, and A. Bartolini. 2009. Angiogenesis as a risk factor for plaque vulnerability. Curr. Pharm. Des. 15: 1095–1106.
8. Byfield, F. J., H. Aranda-Espinoza, V. G. Romanenko, G. H. Rothblat, and I. Levitan. 2004. Cholesterol depletion increases membrane stiffness of aortic endothelial cells. Biophys. J. 87: 3336–3343.
9. Shentu, T. P., D. K. Singh, M. J. Oh, S. Sun, L. Sadat, A. Makino, T. Mazzone, P. V. Subbaiah, M. Cho, and I. Levitan. 2012. The role of oxysterols in control of endothelial stiffness. J. Lipid Res. 53: 1348–1358.
10. Hall, A. 2012. Rho family GTPases. Biochem. Soc. Trans. 40: 1382–1382.
11. Kole, T. P., Y. Tseng, L. Huang, J. L. Katz, and D. Wirtz. 2004. Rho kinase regulates the intracellular micromechanical response of adherent cells to rhodamine. Mol. Biol. Cell 15: 5475–5484.
12. Mammoto, A., T. Mammoto, and D. E. Ingber. 2008. Rho signaling and mechanical control of vascular development. Curr. Opin. Hematol. 15: 228–234.
13. Collins, C., C. Guilluy, C. Welch, E. T. O’Brien, K. Hahn, R. Superfine, K. Burridge, and E. Tzima. 2012. Localized tensional forces on PECAM-1 elicit a global mechanotransduction response via the integrin-RhoA pathway. Curr. Biol. 22: 2087–2094.
14. Chen, C. H., J. Cartwright, Jr., Z. Li, S. Lou, H. H. Nguyen, A. M. Goto, Jr., and P. D. Henry. 1997. Inhibitory effects of hypercholesterolemia and oxLDL on angiogenesis-like endothelial growth in rabbit aortic explants. Essential role of basic fibroblast growth factor. Arterioscler. Thromb. Vasc. Biol. 17: 1305–1312.
15. Chavakis, E., E. Derndach, C. Hermann, U. F. Mondzorf, A. M. Zeiher, and S. Dimmeler. 2001. Oxidized LDL inhibits vascular endothelial growth factor-induced endothelial cell migration by an inhibitory effect on the Akt/endothelial nitric oxide synthase pathway. Circulation, 103: 2102–2107.
16. Wang, D. Y., C. V. Yang, and J. K. Chen. 1997. Oxidized LDL inhibits vascular endothelial cell morphogenesis in culture. In Vitro Cell. Dev. Biol. Anim. 33: 248–255.
17. Zhou, B., F. X. Ma, P. X. Liu, Z. H. Fang, S. L. Wang, Z. B. Han, M. C. Poon, and Z. C. Han. 2007. Impaired therapeutic vasculogenesis by transplantation of OxLDL-treated endothelial progenitor cells. J. Lipid Res. 48: 518–527.
18. Song, S. Y., J. K. Fann, and S. G. Cho. 2008. Proteins and their interactions. Nature 455: 210–211.
effects of oxLDL in vitro and in vivo involving HIF-1alpha activation: a novel aspect of angiogenesis in atherosclerosis. J. Cardiovasc. Transl. Res. 6: 558–569.
21. Camaré, C., M. Trayssac, B. Garmy-Susini, E. Mucher, R. Sabbadini, R. Salvayre, and A. Negre-Salvayre. 2015. Oxidized LDL-induced angiogenesis involves sphingosine 1-phosphate: prediction by anti-SIP antibody. Br. J. Pharmacol. 172: 106–118.
22. Oh, M. J., F. Kuhr, F. Byfield, and I. Levitan. 2012. Micropipette aspiration of substrate-attached cells to estimate cell stiffness. J. Vis. Exp. 67: 3886.
23. Sugimoto, K., T. Ishibashi, T. Sawamura, N. Inoue, M. Kamioka, H. Uekita, H. Ohkawara, T. Sakamoto, N. Sakamoto, Y. Okamoto, et al. 2009. LOX-1-MT1-MMP axis is crucial for RhoA and Rac1 activation induced by oxidized low-density lipoprotein in endothelial cells. Cardiovasc. Res. 84: 127–136.
24. Ryoo, S., A. Blumia, F. Chang, A. Shoukas, D. E. Berkowitz, and L. H. Romer. 2011. OxLDL-dependent activation of arginase II is dependent on the LOX-1 receptor and downstream RhoA signaling. Atherosclerosis. 214: 279–287.
25. Kohler, E. E., C. E. Cowan, I. Chatterjee, A. B. Malik, and K. K. Wary. 2011. NANOG induction of fetal liver kinase-1 (FLK1) transcription regulates endothelial cell proliferation and angiogenesis. Blood. 117: 1761–1769.
26. Zhang, Q., J. Liu, J. Liu, W. Huang, L. Tian, J. Quan, Y. Wang, and R. Niu. 2014. oxLDL induces injury and de-differentiation of human liver sinusoidal endothelial cells via LOX-1. J. Mol. Endocrinol. 53: 281–293.
27. Park, Y. M., J. A. Drazba, A. Vasandji, T. Egellhoff, M. Febbraio, and R. L. Silverstein. 2012. Oxidized LDL/Cd36 interaction induces loss of cell polarity and inhibits macrophage locomotion. Mol. Biol. Cell. 23: 3057–3068.
28. Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, et al. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science. 273: 245–248.
29. Birukova, A. A., K. Smurova, K. G. Birukov, P. Usatyy, F. Liu, K. Kaibuchi, A. Ricks-Cord, V. Natarajan, I. Aieva, J. G. Garcia, et al. 2004. Microtubule disassembly induces cytoskeletal remodeling and lung vascular barrier dysfunction: role of Rho-dependent mechanisms. J. Cell. Physiol. 201: 55–70.
30. Lizard, G., S. Monier, C. Cordelet, L. Gesquiere, V. Deckert, S. Gueldry, L. Lagrost, and P. Gambert. 1999. Characterization and comparison of the mode of cell death, apoptosis versus necrosis, induced by 7beta-hydroxycholesterol and 7-ketocholesterol in the cells of the vascular wall. Arterioscler. Thromb. Vasc. Biol. 19: 1190–1209.
31. Amaral, J., J. W. Lee, J. Chou, M. M. Campos, and I. R. Rodriguez. 2013. 7-Ketocholesterol induces inflammation and angiogenesis in vivo: a novel rat model. PLoS One. 8: e60999.
32. Kiss, L., M. Chen, D. Gero, K. Modis, Z. Lacza, and C. Szabo. 2006. Effects of 7-ketocholesterol on the activity of endothelial poly(ADP-ribose) polymerase and on endothelium-dependent relaxant function. Int. J. Mol. Med. 18: 1113–1117.
33. Weber, T., J. Auer, M. F. O’Rourke, E. Kvas, E. Lassnig, R. Berent, and B. Eber. 2004. Arterial stiffness, wave reflections, and the risk of coronary artery disease. Circulation. 109: 184–189.
34. Janmey, P. A., and R. T. Miller. 2011. Mechanisms of mechanical signaling in development and disease. J. Cell Sci. 124: 9–18.
35. Kowalsky, G. B., F. J. Byfield, and I. Levitan. 2008. oxLDL facilitates flow-induced realignment of aortic endothelial cells. Am. J. Physiol. Cell Physiol. 295: C332–C340.
36. Adachi, H., and M. Tsuchimoto. 2006. Endothelial scavenger receptors. Prog. Lipid Res. 45: 379–404.
37. Park, Y. M., M. Febbraio, and R. L. Silverstein. 2009. CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. J. Clin. Invest. 119: 136–145.
38. Wraith, K. S., S. Magwensi, A. Aburima, Y. Wen, D. Leake, and K. M. Naseem. 2013. Oxidized low-density lipoproteins induce rapid platelet activation and shape change through tyrosine kinase and Rho kinase-signaling pathways. Blood. 122: 580–589.
39. Nishi, K., H. Itabe, M. Uno, K. T. Kitazato, H. Horiguchi, K. Shinno, and S. Nagahiro. 2002. Oxidized LDL in carotid plaques and plasma associates with plaque instability. Arterioscler. Thromb. Vasc. Biol. 22: 1649–1654.
40. Yla-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.
41. Holvoet, P., J. Vanhaecke, S. Janssens, F. Van de Werf, and D. Collen. 1998. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. Circulation. 98: 1487–1494.
42. Holvoet, P., A. Mertens, P. Verhaemme, K. Bogaerts, G. Beyens, R. Verhaeghe, D. Collen, E. Muls, and F. Van de Werf. 2001. Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. Arterioscler. Thromb. Vasc. Biol. 21: 844–848.
43. Holvoet, P., T. B. Harris, R. P. Tracy, P. Verhamme, A. B. Newman, S. M. Rubin, E. M. Simonsick, L. H. Colbert, and S. B. Kritchevsky. 2003. Association of high coronary heart disease risk status with circulating oxidized LDL in the well-functioning elderly: findings from the Health, Aging, and Body Composition study. Arterioscler. Thromb. Vasc. Biol. 23: 1444–1448.
44. Itabe, H., and M. Ueda. 2007. Measurement of plasma oxidized low-density lipoprotein and its clinical implications. J. Atheroscler. Thromb. 14: 1–11.
45. Ghosh, K., C. K. Thodeti, A. C. Dudley, A. Mammoto, M. Klagsbrun, and D. E. Ingber. 2008. Tumor-derived endothelial cells exhibit aberrant Rho-mediated mechanosensing and abnormal angiogenesis in vitro. Proc. Natl. Acad. Sci. USA. 105: 11305–11310.