Cardiovascular Research (2015) **107**, 556–567
doi:10.1093/cvr/cvv178

Cross-talk between LOX-1 and PCSK9 in vascular tissues

Zufeng Ding[1,2], Shijie Liu[1,2]*, Xianwei Wang[1], Xiaoyan Deng[2], Yubo Fan[2], Jiwani Shahanawaz[1], Robert J. Shmookler Reis[1], Kattayi I. Varughese[1], Tatsuya Sawamura[3], and Jawahar L. Mehta[1]*

[1]Central Arkansas Veterans Healthcare System and the University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA; [2]Key Laboratory for Biomechanics and Mechanobiology of Ministry of Education, School of Biological Science and Medical Engineering, Beihang University, Beijing 100191, China; and [3]Department of Physiology, Shinshu University School of Medicine, 3-1-1, Asahi, Matsumoto 390-8621, Japan

Received 4 May 2015; revised 28 May 2015; accepted 11 June 2015; online publish-ahead-of-print 19 June 2015

Time for primary review: 18 days

Aims Lectin-like ox-LDL receptor-1 (LOX-1) plays an important role in inflammatory diseases, such as atherosclerosis. Proprotein convertase subtilisin/kexin type 9 (PCSK9) modulates LDL receptor degradation and influences serum LDL levels. The present study was designed to investigate the possible interaction between PCSK9 and LOX-1.

Methods and results In the first set of experiments, human vascular endothelial cells and smooth muscle cells were studied at baseline and after lipopolysaccharide (LPS) treatment (to create an inflammatory state). Both PCSK9 and LOX-1 were strongly induced by LPS treatment. To define the role of PCSK9 in LOX-1 expression, cells were transfected with siRNA against *PCSK9*, which resulted in reduced LOX-1 expression and function. On the other hand, cells exposed to recombinant *hPCSK9* revealed enhanced LOX-1 expression ($P < 0.05$). To determine whether LOX-1 also regulates PCSK9, cultured cells in which *LOX-1* was knocked down by siRNA expressed less PCSK9, whereas those transfected with hLOX-1 cDNA showed increased PCSK9 expression. The second set of experiments was carried out in wild-type (WT) and gene knockout (KO; LOX-1 and PCSK9) mice; *LOX-1* KO mice showed much less PCSK9 ($P < 0.05$ vs. WT mice). *PCSK9*-KO mice showed much less LOX-1 ($P < 0.05$ vs. WT mice). Furthermore, we observed that mitochondrial reactive oxygen species (mtROS) plays an initiating role in the LOX-1/PCSK9 interaction, since mtROS induction enhanced and its inhibition reduced the expression of both PCSK9 and LOX-1. We also found that both LOX-1 and PCSK9 regulate adhesion molecules vascular cell adhesion molecule-1 expression. Finally, oxidized low-density lipoprotein and tumour necrosis factor-α, pro-inflammatory stimuli besides LPS, regulated PCSK9 expression that is mediated by the NF-κB signalling pathway.

Conclusions These observations suggest that LOX-1 and PCSK9 positively influence each other's expression, especially during an inflammatory reaction. mtROS appear to be important initiators of PCSK9/LOX-1 expression.

Keywords LOX-1 • PCSK9 • VCAM-1 • Mitochondrial ROS

1. Introduction

Lectin-like ox-LDL receptor-1 (LOX-1) is one of the major scavenger receptors responsible for binding, internalizing, and degradation of oxidized low-density lipoprotein (ox-LDL).[1,2] LOX-1 is activated in many inflammatory diseases, including atherosclerosis.[3] The importance of LOX-1 in atherogenesis has been proved by deletion and overexpression studies.[3,4] Proprotein convertase subtilisin/kexin type 9 (PCSK9), an enzyme encoded by the *PCSK9* gene in humans, is produced mainly in the liver, kidney, and small intestine.[5,6] This protein plays a regulatory role in cholesterol homeostasis by binding the epidermal growth factor-like repeat A domain of the low-density lipoprotein receptor (LDLR), inducing LDLR degradation.[7] Inhibition of PCSK9 drastically reduces LDL levels in serum,[8] presumably by stimulating its uptake. Thus, PCSK9 inhibition has emerged as a potential novel drug therapy to treat hypercholesterolaemia and associated disease states.[9]

Although the vast majority of prior studies have focused on the effect of PCSK9 on LDLr expression in liver and kidney, an increasing body of

* Corresponding author. Tel: +1 501 526 7417; Fax: +1 501 526 7417, Email: sliu2@uams.edu (S.L.); Tel: +1 501 296 1426; fax: +1 501 686 6180, Email: mehtajl@uams.edu (J.L.M.).

evidence suggests that PCSK9 is also expressed in significant amounts in extra-hepatic and extra-renal tissues.[10] Inflammation induces marked changes in lipid and lipoprotein metabolism, including up-regulation of *PCSK9* expression.[11] Of note, hypercholesterolaemia and related vascular disease states, such as atherosclerosis, are considered inflammatory states.[12]

Since both LOX-1 and PCSK9 are involved in lipoprotein metabolism and inflammation, we posited that there might be a mechanistic link coupling their expression, which could be demonstrated in cultured aortic endothelial cells (ECs) and smooth muscle cells (SMCs) in which either *LOX-1* or *PCSK9* is knocked down by siRNA or overexpressed. Moreover, we have utilized knockout (KO) mice lacking either *LOX-1* or *PCSK9* gene. We show with these multiple approaches that PCSK9 promotes LOX-1 expression and *vice versa*, particularly during an inflammatory state, and that mitochondrial reactive oxygen species (mtROS) are powerful inducing signals for *PCSK9* and *LOX-1* expression.

2. Methods

2.1 Mouse strains

The generation of LOX-1 KO mice in the C57BL/6 background has been described previously;[3] PCSK9-KO mice were purchased from the Jackson Laboratory (Sacramento, CA, USA); and C57BL/6 mice were used as wild-type (WT) controls. All animals were housed in the Animal Care Facility of our institution. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Usage Committee, and conformed to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Only male mice ~10 weeks of age were used in this study.

2.2 *In vivo* inflammation model

To create an inflammatory state, similar weight mice (WT, LOX-1 KO, and PCSK9 KO) were administered lipopolysaccharide (LPS, 1 mg LPS/kg body weight, given i.p.; Sigma, St Louis, MO, USA) or saline (control). Mice were euthanized 24 h later with sodium pentobarbital (80 mg/kg, i.p.), and blood, aorta, and other tissues were collected.

2.3 Cell culture

Human primary aortic ECs and SMCs were purchased from ATCC (Manassas, VA, USA). ECs and SMCs were maintained in vascular cell basal medium (ATCC) augmented with growth supplement containing VEGF or SMC growth factors. ECs or SMCs at a concentration of 1×10^5 cells/cm^2 were seeded in six-well plastic tissue culture plates and culture continued for three passages. The culture medium was renewed every 2 days. To elicit an inflammatory state, ECs and SMCs were incubated for 24 h with LPS (Sigma, 1–1000 ng/mL), ox-LDL (Biomedical Technologies, Inc., 0–60 μg/mL), or tumour necrosis factor-α (TNF-α; Sigma, 0–40 ng/mL).

2.4 ELISA for PCSK9

PCSK9 secretion was measured in mouse sera or cell culture media using a PCSK9 ELISA kit (mouse/human, MBL International, Nagoya, Japan). IL-1β was measured in mouse serum samples, as an indicator of inflammatory state, with an IL-1β ELISA kit (BD Biosciences, CA, USA) as per the manufacturer's instructions.

2.5 Inhibition of LOX-1 and PCSK9 by siRNA transfection

siRNA duplexes corresponding to human *LOX-1*, *PCSK9*, *TNF-α*, and *NF-κB* exonic sequences were purchased from Santa Cruz Biotechnology. To disrupt gene expression, SMCs were transfected for 24 h with 20 nM of each siRNA in siRNA transfection reagent (Santa Cruz). The medium was then replaced with normal culture medium, and cells were treated with 100 ng/mL LPS for a further 24 h. As controls, cells were transfected with the corresponding scrambled-sequence siRNA duplexes. Cell lysates were utilized for ELISA or western blot analysis to verify efficacy of protein knockdown by siRNA.

2.6 LOX-1 overexpression and PCSK9 administration

For LOX-1 overexpression, SMCs at 70% confluence were transfected with PCI-neo plasmid expressing human *LOX-1* cDNA (*hLOX-1*) or PCI-neo plasmid (empty vector) in FuGene 6 transfection reagent (Roche Diagnostics, Mannheim, Germany). Five hours after transfection, the medium was replaced with fresh, serum-free medium, and the cells were starved for 48 h before they were cultured in complete culture medium. To simulate PCSK9 overexpression, SMCs were incubated for 24 h with recombinant human PCSK9 protein (Life Technologies) at 0.5, 2.5, and 5 μg/mL.

2.7 Western blot

Protein from SMCs was purified in the RIPA Lysis Buffer System (Santa Cruz), and loaded onto 12% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad) for electrophoresis. Size-resolved proteins were transferred to Hybond ECL Nitrocellulose Membranes (GE Healthcare). After blocking in 5% BSA buffer for 1 h, membranes were incubated overnight with primary antibody (1 : 1000 dilution) at 4°C. After washing with PBS containing 0.1% Tween-20, membranes were incubated 1 h with biotin-tagged secondary antibody, which was detected with the Pierce ECL Western Blotting Substrate (Thermo Scientific). Band intensities were quantified with the Image J software and normalized to β-actin. Antibodies directed at PCSK9, LOX-1, and vascular cell adhesion molecule-1 (VCAM-1) were purchased from Abcam (San Francisco, CA, USA).

2.8 Functional assessment of LOX-1

As an index of LOX-1 function, we measured ox-LDL uptake by cultured SMCs. Cells were incubated with Dil-ox-LDL (Biomedical Technologies), 5 μg/mL, for 2 h at 37°C. Cells were then gently washed three times in PBS and digested with trypsin-EDTA. Dil-ox-LDL uptake was measured by flow cytometry (FACS Vantage SE, Becton Dickinson). As another measure of LOX-1 function, VCAM-1 expression was assessed.

2.9 Real-time quantitative PCR analysis

At 24 h after LPS administration, tissues (kidney, brain, liver, and small intestine) were homogenized and total RNA was isolated using RNAzol B (Tel-Test) according to the manufacturer's instructions. Total RNA was reverse-transcribed at 42°C with SuperScript II (Life Technologies). Gene expression was measured using SYBR Green PCR core reagents (Applied Biosystems). Relative mRNA levels for each sample were quantified based on C_t (the amplification cycle threshold) normalized to *GAPDH* as an endogenous mRNA standard, and expressed relative to the control (WT without LPS), set to a value of 1. Primers used were as follows: *PCSK9* forward primer, 5′-TTGCAGCAGCTGGGAACTT-3′; *PCSK9* reverse primer, 5′-CCGACTGATGACCTCTGGA-3′; TNF-α and NF-κB gene expression levels were analysed by the BioRad PrimePCR SYBR Green Assay predesigned primer pair (BioRad): *GAPDH* forward primer, 5′-GGGTCTTTGCAGTCGT ATGG-3′; *GAPDH* reverse primer, 5′-ACCTCCTG TTTCTGGGGACT-3′.

2.10 Mitochondrial ROS measurement

MitoSOX™ Red (Invitrogen), a mitochondrial superoxide indicator, was used to assess mitochondrial ROS levels. mtROS-mediated fluorescence was measured by flow cytometry (FACS Vantage SE, Becton Dickinson), as described previously.[3] To confirm the role of mitochondrial ROS, SMCs were treated for 24 h with three different mtROS stimulants: rotenone (10 μM), thenoyltrifluoroacetone (TTFA; 10 μM), or antimycin (40 μg/mL), and then stained with MitoSOX™. Rotenone, TTFA, and

antimycin were purchased from Sigma. Data were recorded as the 'M2 percentage' fluorescence variation, which indicates the fraction of cells with enhanced production of mtROS.

2.11 Collection of arterial tissues from humans

Human artery blocks were obtained from the Department of Pathology archives of the University of Arkansas for Medical Sciences. The patients were ≈70 years old ($n = 3$) and had severe atherosclerosis interspersed with normal non-atherosclerotic regions. The protocol for collection of the archived tissue sections was approved on an exempt basis by the Institutional Review Board. The human study conformed to the Helsinki declaration.

For immunohistochemical and immunofluorescent analyses, 5 μm thick sections of the artery were stained with different antibodies and analysed using the Mouse/Rabbit-Specific HRP/3,3′-diaminobenzidine detection immunohistochemistry kit (Abcam). Image J. v 1.46 (NIH, Bethesda, MD, USA) was used to semi-quantitatively assess the expression of PCSK9 and LOX-1.

2.12 Statistical analysis

Data from five independent experiments were combined for statistical analysis, with results expressed as mean ± SD. Groups were compared pairwise by Fisher's *t* tests, or by ANOVA with Tukey's *post hoc* correction when more than two groups were contrasted. *P*-values of <0.05 were considered nominally significant, without adjustment for multiple measures, to avoid inflating type II errors.

3. Results

3.1 LPS increases the expression of PCSK9 and LOX-1 in ECs and SMCs

Basal PCSK9 expression (assessed by western blotting) and secretion (assessed by ELISA) were modest in both ECs and SMCs. After incubation with LPS, however, PCSK9 expression/secretion increased several fold in both ECs and SMCs, in an LPS concentration-dependent fashion. The increase in PCSK9 expression/secretion in response to LPS was only modest for ECs, although significant, but it was quite pronounced in SMCs (*Figure 1A* and *B*).

LOX-1 expression was also strongly and significantly induced in ECs and SMCs by LPS treatment, with the increase roughly linearly dependent on LPS concentration. Expression of LOX-1 was consistently greater in SMCs than in ECs, both at baseline or at any LPS input (*Figure 1C* and *D*). Consequently, subsequent *in vitro* experiments were carried out only in SMCs. Furthermore, since LPS (100 ng/mL) treatment resulted in robust expression of PCSK9 and LOX-1, all subsequent experiments utilized LPS-treated cells. This

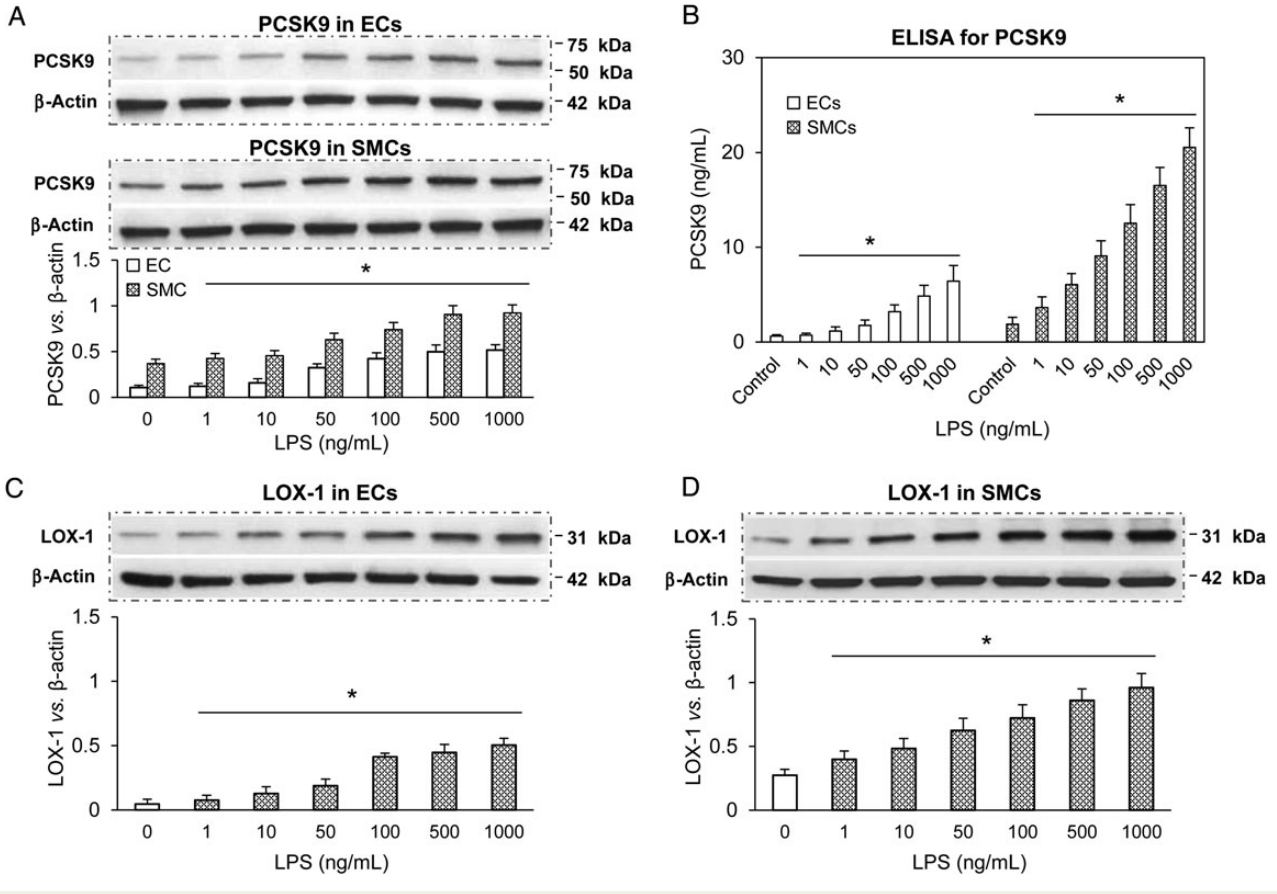

Figure 1 LPS induces secretion of PCSK9 and expression of LOX-1. (*A* and *B*) LPS induces secretion of PCSK9 by SMCs in a dose-dependent fashion (measured by western blot and ELISA). (*C* and *D*) LPS induces LOX-1 protein expression in ECs and SMCs. Bar graphs represent data compiled from five independent experiments, as mean ± SD. *$P < 0.05$ vs. control.

concentration did not induce cell injury (apoptosis or death) in SMCs (data not shown).

3.2 PCSK9 inhibition reduces the expression of LOX-1 and VCAM-1

To assess the link between PCSK9 and LOX-1 expression, PCSK9 was knocked down by siRNA transfection. siRNA targeting PCSK9 reduced its expression more than four-fold by western blot (*Figure 2A*) and more than six-fold by q-PCR (*Figure 2B*), validating the efficacy of siRNA transfection and of knockdown via RNA interference. Importantly, PCSK9 knockdown reduced LOX-1 expression about five-fold (*Figure 2C*). Scrambled siRNAs had no effect on expression of *PCSK9* or *LOX-1* mRNAs (*Figure 2B* and *C*).

Next, we studied the effect of *PCSK9* knockdown on DiI-ox-LDL uptake and VCAM-1 expression, as indices of LOX-1 function. VCAM-1, a cell surface glycoprotein, is expressed in cytokine-activated ECs and SMCs. VCAM-1 mediates the attachment of circulating monocytes and lymphocytes, a critical process in the development of inflammation and atherosclerosis.[13] As shown in *Figure 2D*, *PCSK9* knockdown decreased the fraction of cells strongly positive for DiI-ox-LDL uptake by ~50% (similar to the decrease in modal fluorescence), indicating a marked reduction in ox-LDL internalization. Furthermore, *PCSK9* knockdown significantly reduced VCAM-1 expression, by nearly two-fold (*Figure 2E*). Control treatment with scrambled siRNA had no effect on DiI-ox-LDL uptake or VCAM-1 expression.

To assess whether PCSK9 inhibition regulates LOX-1 expression *in vivo*, we compared WT and *PCSK9*-KO mice after administering LPS. As expected, LPS elicited an approximately eight-fold increase in serum IL-1β levels in WT mice (*Figure 2F*), indicating a systemic pro-inflammatory state, while increasing the LOX-1 level ~70% in aortic tissue (*Figure 2G*). In contrast, the inflammatory response was markedly attenuated in the PCSK9-KO mice—as reflected by a serum IL-1β level only half of that WT mice after LPS treatment ($P < 0.05$ vs. WT mice; *Figure 2F*). *PCSK9*-KO mice also expressed ~40% less LOX-1 and >60% less VCAM-1 in aortic tissue (each $P < 0.05$ vs. WT mice; *Figure 2G* and *H*).

3.3 Exposure to exogenous PCSK9 activates expression of LOX-1 and VCAM-1

Given that PCSK9 knockdown or KO attenuates LOX-1 expression, we inquired whether PCSK9 also positively induces LOX-1. We tested

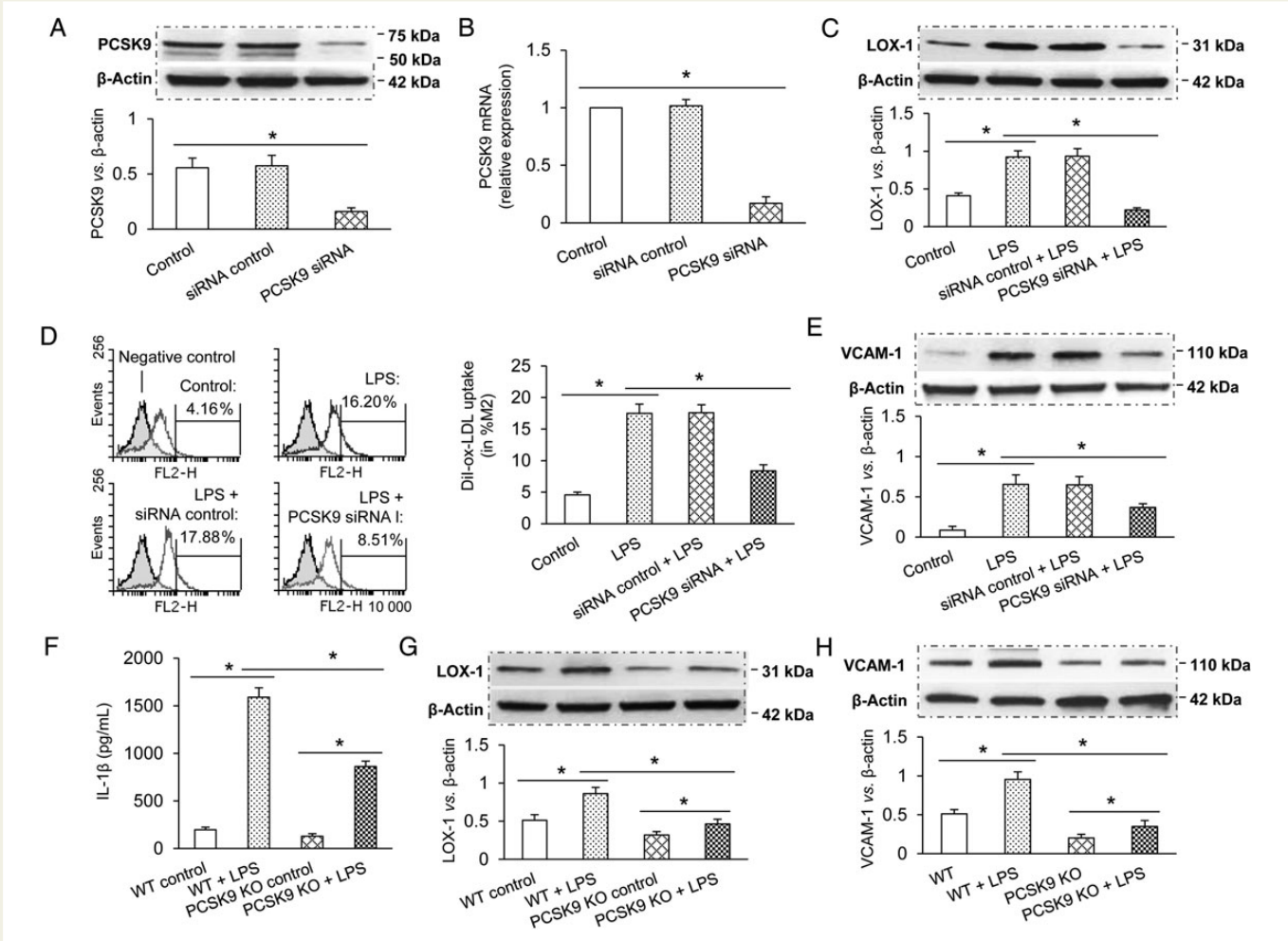

Figure 2 PCSK9 inhibition reduces LOX-1 expression, DiI-ox-LDL uptake, and VCAM-1 expression in SMCs. Transfection of cells with siRNA decreases PCSK9 expression by western blot (*A*) and q-PCR (*B*), and reduces LOX-1 expression in SMCs monolayer (*C*), DiI-ox-LDL uptake (*D*), and VCAM-1 expression (*E*). PCSK9-KO mice show lower serum levels of IL-1β (*F*) and reduced expression of LOX-1 (*G*) and VCAM-1 (*H*). Bar graphs represent data compiled from five independent experiments, as mean ± SD. *$P < 0.05$ vs. appropriate control.

this possibility in SMCs, mimicking their autocrine or paracrine stimulation with PCSK9 by exposure to recombinant hPCSK9. Addition of hPCSK9 evoked a near-linear dose-dependent increase in LOX-1 protein expression (*Figure 3A*). Concurrent with this up-regulation of LOX-1, DiI-ox-LDL uptake (measured by flow cytometry) also increased in response to hPCSK9 (*Figure 3B*). Treatment with hPCSK9 further increased the expression of LOX-1 and VCAM-1 in SMCs already strongly induced by LPS exposure (*Figure 3C* and *D*).

3.4 LOX-1 depletion reduces expression of PCSK9, VCAM-1, and IL-1β

Having demonstrated both *in vitro* and *in vivo* that PCSK9 regulates LOX-1 expression, we wondered whether the converse (regulation of PCSK9 by LOX-1) might also be true, creating a positive-feedback loop. Remarkably, this reciprocal control could be confirmed in four distinct assays. First, SMCs were transfected with siRNA against the *LOX-1* gene. As expected, *LOX-1* siRNA inhibited LOX-1 protein expression about eight-fold (*Figure 4A*), but it also reduced PCSK9 expression by ~60% ($P < 0.05$; *Figure 4B*), consistent with our premise. In keeping with previous studies, which had shown that LOX-1 is a major determinant of adhesion molecule expression,[2] also we found that *LOX-1* knockdown inhibited VCAM-1 expression by >50% (*Figure 4C*), whereas scrambled siRNA had no effect on expression of PCSK9 or VCAM-1.

Secondly, to confirm that LOX-1 inhibition attenuates PCSK9 secretion *in vivo*, we compared WT and *LOX-1*-KO mice. The more than seven-fold rise in IL-1β serum levels elicited by LPS in WT mice, indicating a robust inflammatory response, was severely blunted in *LOX-1*-KO mice (*Figure 4D*). Serum levels of PCSK9, measured by ELISA, increased more than three-fold in LPS-treated WT mice, but attained less than half that level in LPS-injected *LOX-1*-KO mice (*Figure 4E*).

Thirdly, *PCSK9* gene expression was measured by q-PCR in the kidney, brain, liver, and small intestine of LPS-treated mice—tissues known to express significant amounts of PCSK9.[10] As shown in *Figure 4F*, *PCSK9* mRNA levels were much lower in all tissues from *LOX-1*-KO mice than in the same tissues from WT mice (each $P < 0.05$). LPS administration significantly enhanced PCSK9 expression (assessed by western blotting) in aortic tissue of WT mice (*Figure 4G*). However, PCSK9 expression was less than half as great in *LOX-1*-KO mice receiving the same dose of LPS ($P < 0.05$ vs. WT mice). Western blotting results were consistent with serum protein levels for PCSK9 (*Figure 4E*) and *PCSK9* mRNA levels in diverse tissues (*Figure 4F*). As expected, *LOX-1* gene deletion markedly inhibited VCAM-1 expression in the aorta (*Figure 4H*).

3.5 LOX-1 overexpression induces both PCSK9 and VCAM-1

The fourth assay to study regulation of PCSK9 by LOX-1 involved determination of the effect of LOX-1 overexpression on PCSK9 expression. SMCs were transfected with *hLOX-1* cDNA. As expected,

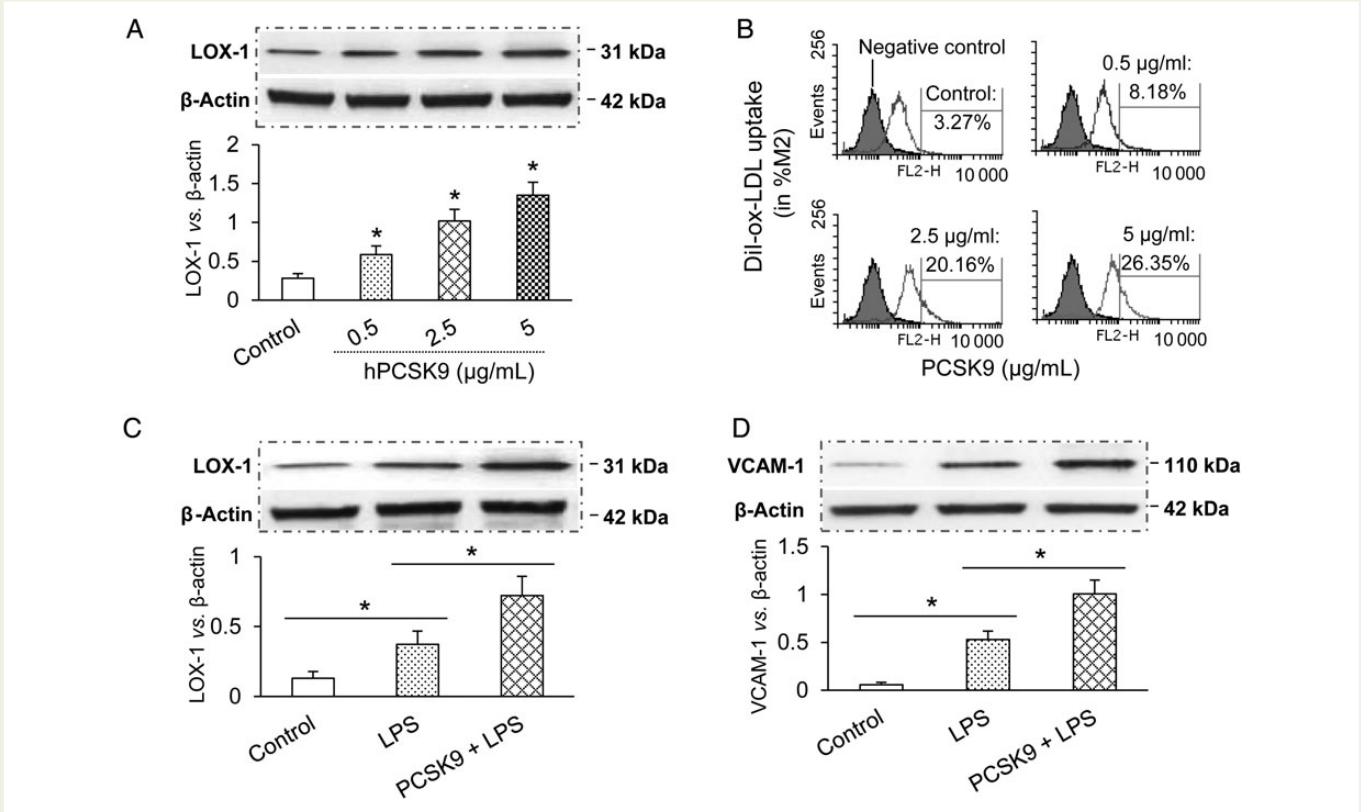

Figure 3 PCSK9 administration induces LOX-1 and VCAM-1 expression in SMCs. (*A* and *B*) PCSK9 treatment (with recombinant hPCSK9) induces LOX-1 expression and DiI-ox-LDL uptake in a dose-dependent manner. (*C* and *D*) In the presence of LPS, PCSK9 administration further enhanced expression of LOX-1 and VCAM-1. Bar graphs represent data compiled from five independent experiments, as mean ± SD. *$P < 0.05$ vs. appropriate control.

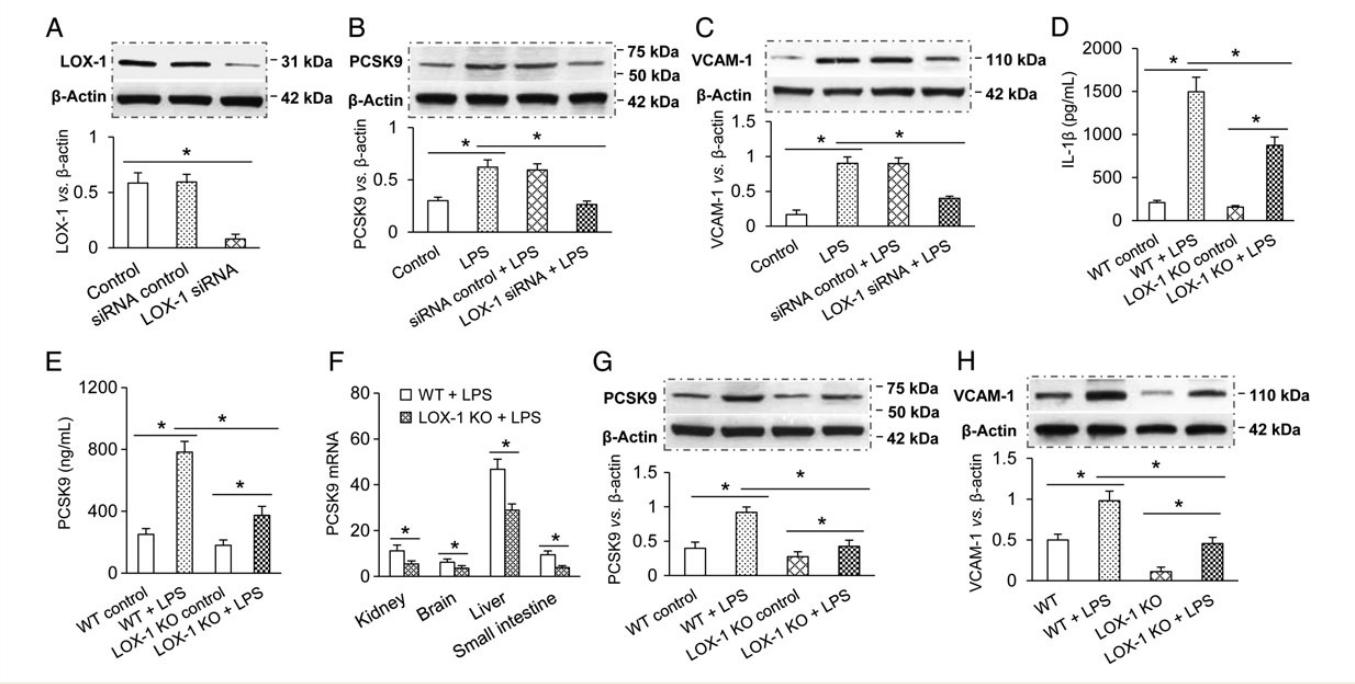

Figure 4 LOX-1 knockdown/deletion decreases PCSK9 and VCAM-1 expression in SMCs. (*A*) LOX-1 expression is inhibited by siRNA transfection. (*B* and *C*) LOX-1 knockdown markedly reduces PCSK9 and VCAM-1 expression in SMCs. (*D* and *E*) Compared with WT mice, LOX-1 KO mice show much lower levels of IL-1β and PCSK9 secretion in plasma (ELISA analysis). (*F*) LOX-1 KO mice show much lower levels of PCSK9 mRNA (measurement by q-PCR) in kidney, brain, liver, and small intestine compared with WT mice. Expression levels were calculated with a real-time quantitative PCR $\Delta\Delta C_t$ method. (*G* and *H*) Expression of PCSK9 and VCAM-1 is lower in the aortas of LOX-1 KO mice than in WT mice. Bar graphs represent data compiled from five independent experiments, as mean ± SD. *$P < 0.05$ vs. appropriate control.

the transfection markedly enhanced LOX-1 protein expression (*Figure 5A*). Consistent with our postulate of bidirectional positive feedback between LOX-1 and PCSK9, LOX-1 overexpression also enhanced the expression of PCSK9 (*Figure 5B*). Treatment with LPS further increased the expression of PCSK9 in cells overexpressing LOX-1 (*Figure 5C*). Not unexpectedly, LOX-1 overexpression enhanced VCAM-1 expression in the presence or absence of LPS (*Figure 5D*).

3.6 Mitochondrial ROS trigger LOX-1—PCSK9 cross-talk

ROS are a major endogenous source of oxidative damage, trigger inflammatory responses, and also serve a signalling function to initiate antioxidant defences.[14] Previous studies had demonstrated a positive bidirectional interaction between ROS and LOX-1.[3,15] Because the main source of cellular ROS is mitochondria, we reasoned that mtROS may induce LOX-1 and thus its reciprocal interplay with PCSK9. Mitochondrial ROS production was induced by blocking two key steps in the mtROS respiratory chain. Complexes I and III are the main sites of mitochondrial electron leakage, resulting in superoxide production. Consistent with a previous report,[16] mtROS were markedly elevated after addition of the complex I inhibitor rotenone, or the complex III inhibitor antimycin A (*Figure 6A*). In contrast, the complex II inhibitor TTFA had only a minor, insignificant effect on mtROS generation (*Figure 6A*). Concomitant with mtROS generation, protein levels of both PCSK9 (assayed by western blot and ELISA) and LOX-1 (by western blot) were elevated significantly after SMCs were exposed to rotenone or antimycin A, but not to TTFA (*Figure 6B–D*).

Based on the above results, we thought that mtROS may play a bridging role in the regulation of expression of LOX-1 and PCSK9. To examine this postulate, we used two different ROS inhibitors—a relatively specific mtROS inhibitor YCG063 and a generalized NADPH oxidase inhibitor apocynin. As expected, both YCG063 and apocynin inhibited LPS-induced mtROS generation (*Figure 7A*), and expression of LOX-1 (*Figure 7B*) and PCSK9 (*Figure 7C* and *D*).

As anticipated based on prior evidence of a reciprocal relationship between ROS and LOX-1,[15] modulation was also observed in the opposite direction. That is, *LOX-1* knockdown in SMCs reduced mtROS production by 55%, and *PCSK9* knockdown decreased mtROS by 45% (*Figure 7A*). Moreover, SMC mtROS rose 53% when *LOX-1* was overexpressed through cDNA transfection, and increased 37% upon stimulation with exogenous hPCSK9 (*Figure 8B*).

3.7 Relationship of non-LPS mediators of inflammation ox-LDL and TNF-α with PCSK9

LOX-1 is a major vascular receptor for ox-LDL, and is thought to play a pro-inflammatory role.[2] As one of the inflammatory cytokines, TNF-α has also been implicated in atherogenesis as well as in inflammatory responses.[17] Therefore, we also investigated the effect of ox-LDL and TNF-α on PCSK9 expression. Similar to LPS, both ox-LDL (20–60 μg/mL) and TNF-α (10–40 ng/mL) induced PCSK9 expression in a dose-dependent manner (*Figure 9A* and *B*). Subsequently, we studied the effect of LOX-1 and TNF-α inhibition on PCSK9 expression. As shown in *Figure 9C*, q-PCR analysis confirmed that TNF-α expression

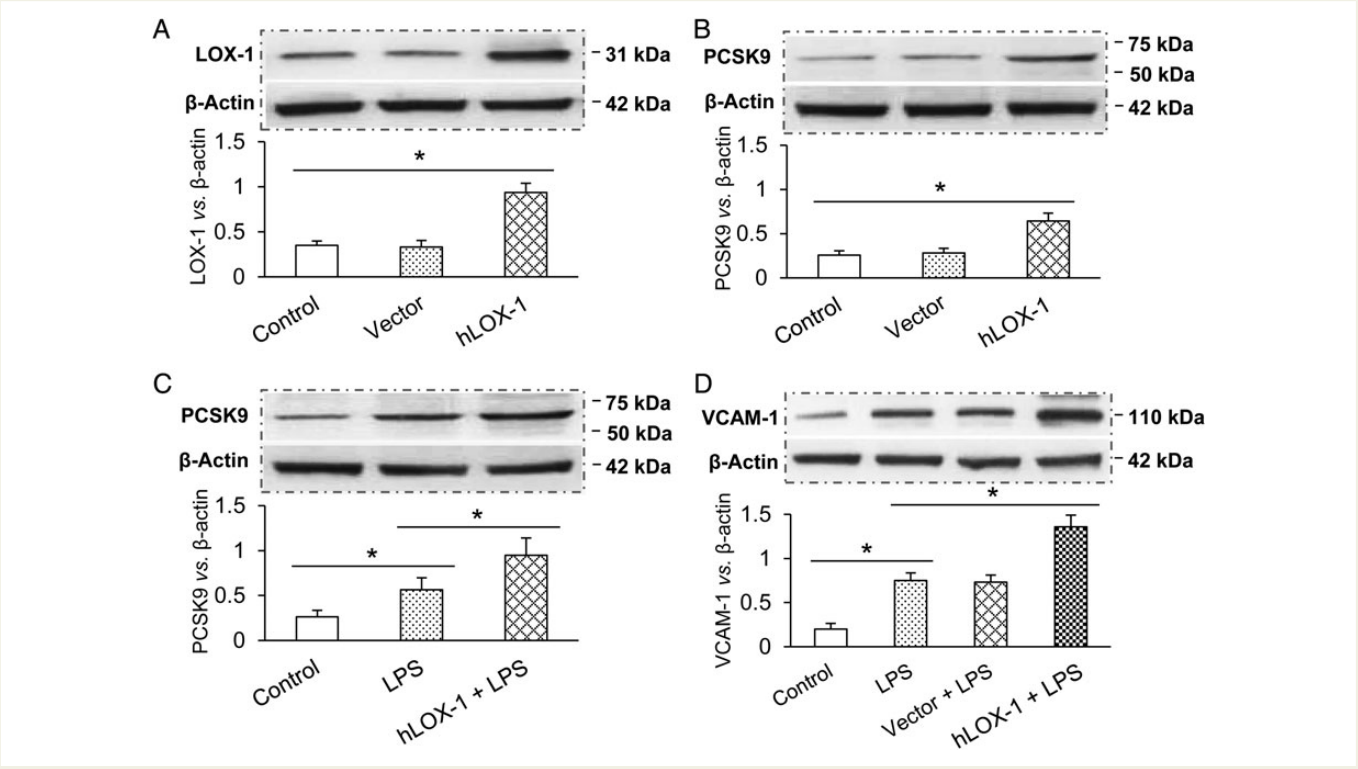

Figure 5 LOX-1 overexpression induces PCSK9 and VCAM-1 expression in SMCs. (*A*) LOX-1 overexpression (by cDNA transfection) is confirmed by western blot. (*B* and *C*) LOX-1 overexpression induces PCSK9 in the absence or presence of LPS. (*D*) In the presence of LPS, LOX-1 overexpression further enhances VCAM-1 expression. Bar graphs represent data compiled from five independent experiments, as mean ± SD. *$P < 0.05$ vs. appropriate control.

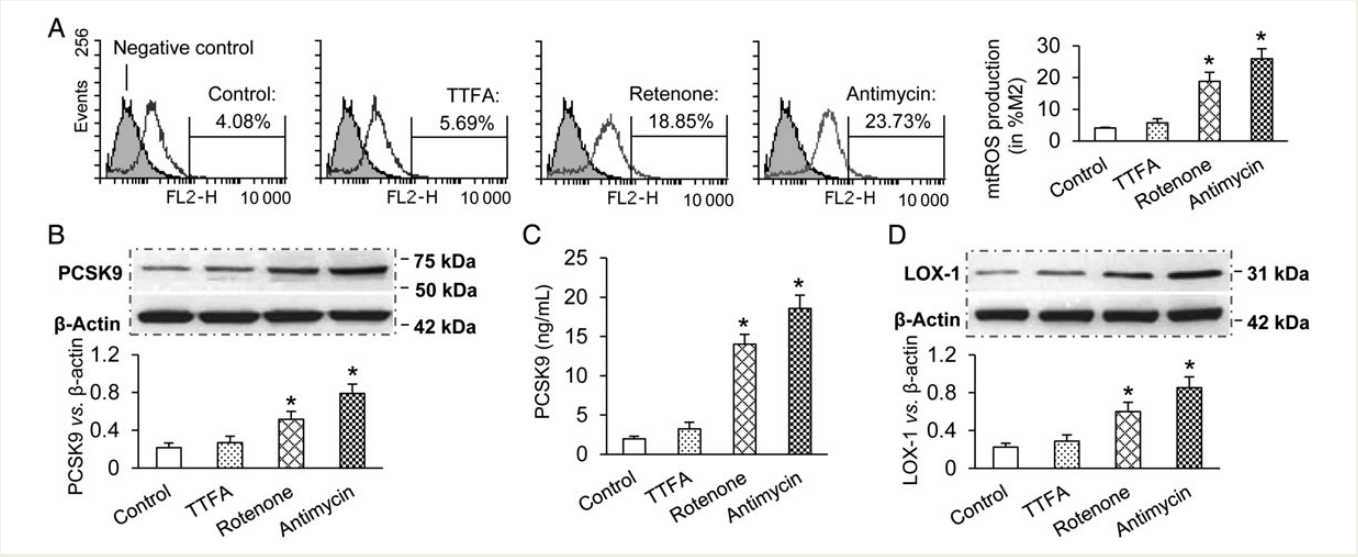

Figure 6 Effect of mtROS inducers on the expression of PCSK9 and LOX-1. (*A*) Rotenone and antimycin, but not TTFA, induce mtROS. (*B*–*D*) Rotenone and antimycin, but not TTFA, induce expression of PCSK9 and LOX-1 proteins. SMCs were treated with 10 μM rotenone, 10 μM TTFA, or 40 μg/mL of antimycin for 24 h. Bar graphs represent data compiled from five independent experiments, as mean ± SD. *$P < 0.05$ vs. appropriate control.

was reduced by its siRNA transfection. As expected, inhibition of LOX-1 and TNF-α by siRNA transfection obviously reduced ox-LDL- and TNF-α-induced PCSK9 expression, respectively (*Figure 9D* and *E*).

Previously, we reported that NF-κB activation plays a bridging role in ox-LDL- and LPS-induced LOX-1 expression.[2,18] Others have also reported that TNF-α induces cell surface expression of LOX-1 via

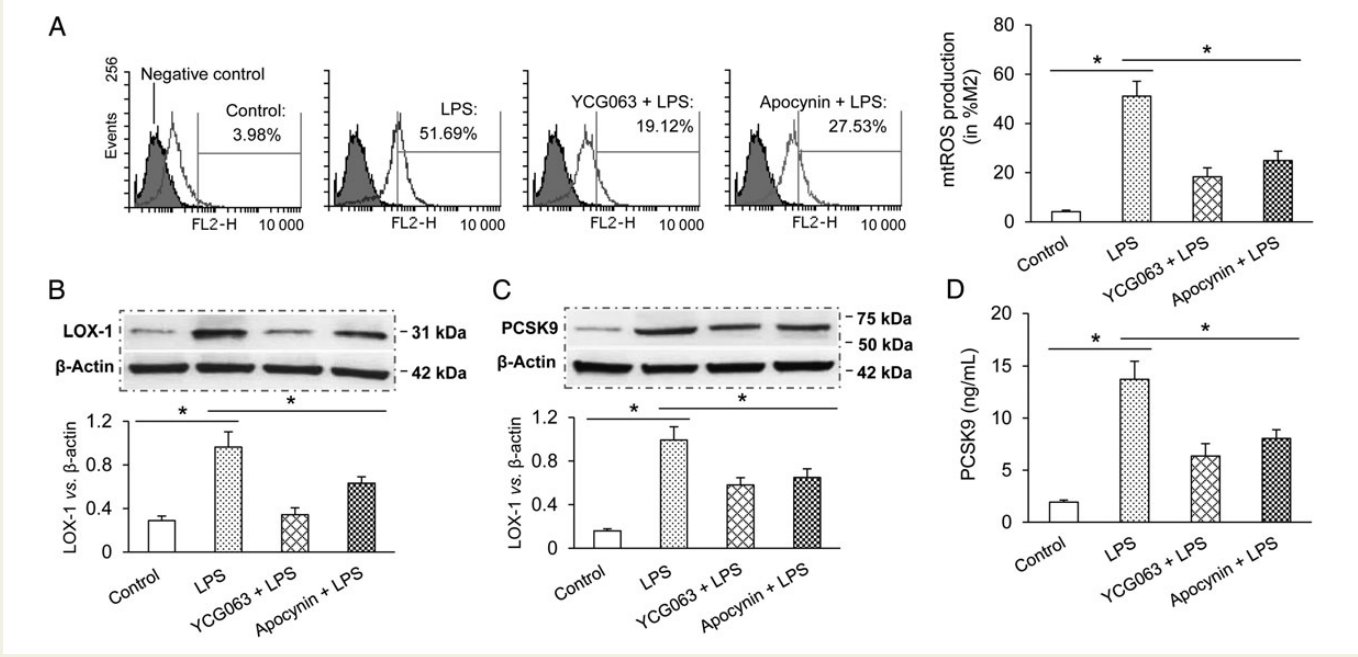

Figure 7 Effect of mtROS inhibition on expression of PCSK9 and LOX-1 in SMCs. Pretreatment with ROS inhibitors apocynin (10 μM) and YCG063 (10 μM) markedly inhibited mtROS production (*A*—flow cytomery), LOX-1 expression (*B*—western blot), and PCSK9 expression (*C*—western blot and *D*—ELISA) in SMCs. Bar graphs represent data compiled from five independent experiments, as mean ± SD. *$P < 0.05$ vs. appropriate control.

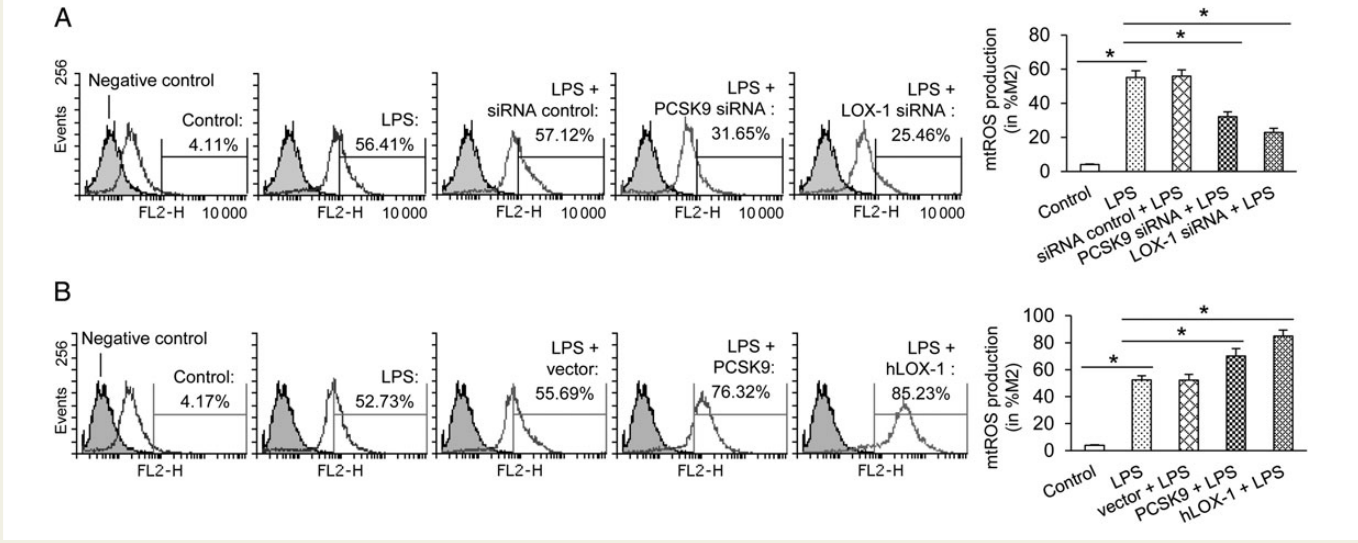

Figure 8 mtROS and PCSK9/LOX-1 expression in SMCs. (*A*) MitoSOX™ Red staining by (flow cytometry analysis) indicates that knockdown of PCSK9 or LOX-1 decreases mtROS production. (*B*) PCSK9 treatment (0.5 μg/mL) or LOX-1 overexpression induces mtROS production. Bar graphs represent data compiled from five independent experiments, as mean ± SD. *$P < 0.05$ vs. appropriate control.

NF-κB.[17] We therefore posited that NF-κB may play an important role in PCSK9 expression via LOX-1, and studied the role of NF-κB in LPS-, ox-LDL-, and TNF-α-induced PCSK9 expression with the use of siRNA. Q-PCR analysis showed that NF-κB expression was inhibited by its siRNA transfection. Furthermore, NF-κB knockdown also inhibited LPS-, ox-LDL-, and TNF-α-induced PCSK9 expression (*Figure 9G–I*), indicating that NF-κB plays a bridging role in LPS-, ox-LDL-, and TNF-α-induced expression of sLOX-1 and PCSK9.

3.8 LOX-1 and PCSK9 in human atherosclerosis

To determine whether human atherosclerotic tissues also express PCSK9 and LOX-1, arterial sections from normal and atherosclerotic arteries were examined by immunostaining and IF. *Figure 10* (upper panel) shows extensive expression of LOX-1 in the intima and media, extending almost to the adventitia of atherosclerotic tissues, similar to that observed by Mehta *et al.* in *ApoE*-KO mice fed a high-fat diet.[2]

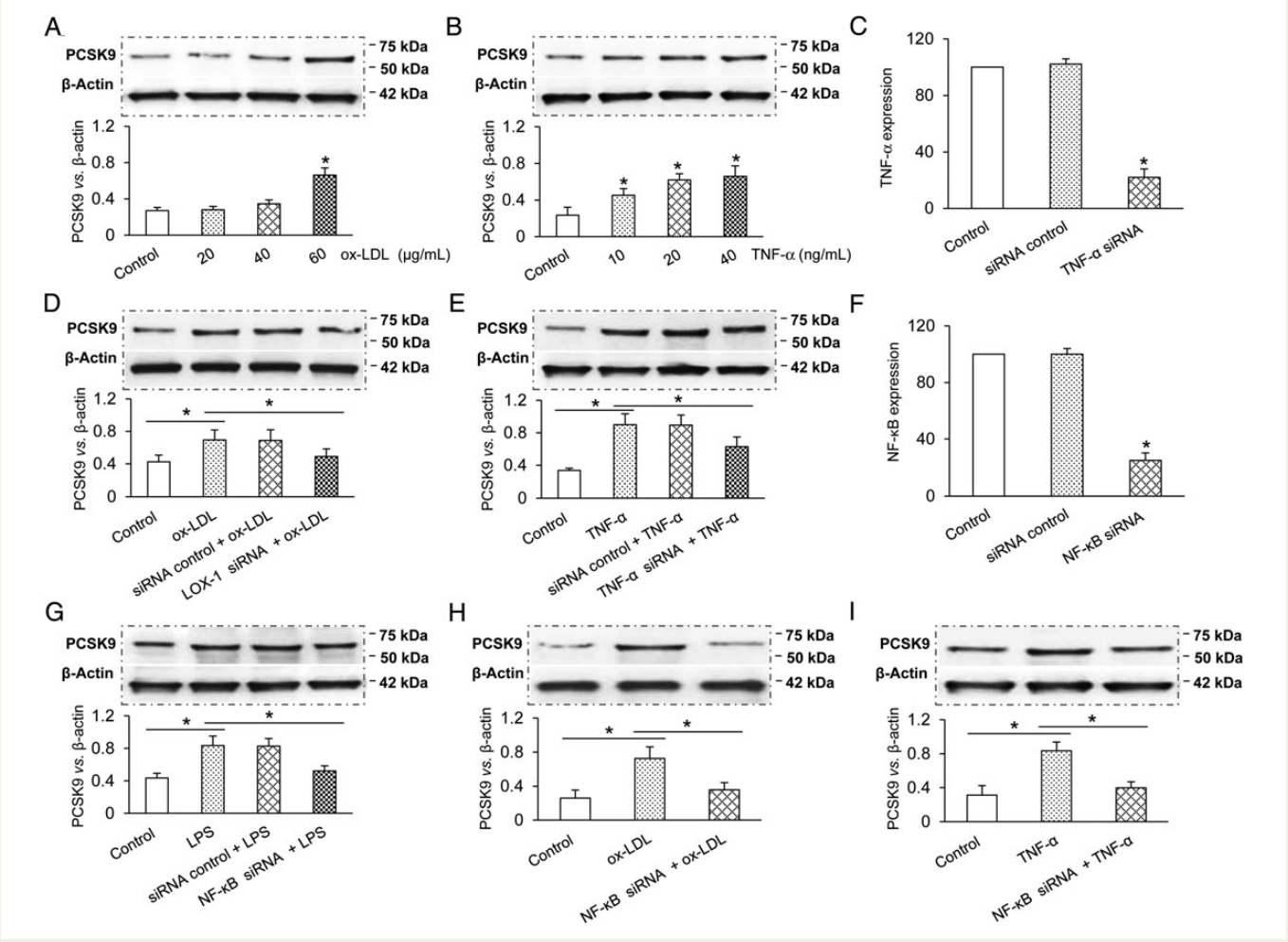

Figure 9 Role of NF-κB in LPS-, ox-LDL-, and TNF-α-induced PCSK9 expression in SMCs. (*A* and *B*) Both ox-LDL- and TNF-α-induced PCSK9 expression in a dose-dependent manner. (*C*) TNF-α was inhibited by its siRNA transfection (q-PCR). (*D*) LOX-1 knockdown inhibited ox-LDL-induced PCSK9 expression. (*E*) TNF-α knockdown inhibited PCSK9 expression. (*F*) NF-κB was by its siRNA (q-PCR). (*G*–*I*) NF-κB knockdown inhibited LPS-, ox-LDL-, and TNF-α-induced PCSK9 expression. Bar graphs represent data compiled from five independent experiments, as mean ± SD. *$P < 0.05$ vs. appropriate control.

Importantly, we noted a diffuse distribution of PCSK9 in atherosclerotic regions. IF (*Figure 10*, lower panels) confirmed the presence of both PCSK9 and LOX-1 in atherosclerotic regions. While the distribution of PCSK9 and LOX-1 was diffuse in the atherosclerotic plaque, it appeared that SMCs were the chief source of intense expression of both PCSK9 and LOX-1. A higher power view of merged images (rightmost panel) confirms co-expression of PCSK9 and LOX-1 in SMCs. These observations were consistent, based on examination of several sections from each of three normal and three atherosclerotic arterial specimens.

4. Discussion

The experiments conducted with cultured aortic ECs and SMCs establish the existence of a positive-feedback loop between LOX-1 and PCSK9, such that induction of either will be stabilized by reciprocal induction of the other. Results from *LOX-1*-KO and *PCSK9*-KO mice lend further support to this interaction, and indicate that it also occurs *in vivo*. Both PCSK9 and LOX-1 were strongly and proportionately induced by LPS in cultured ECs and SMCs, although to a substantially greater extent in SMCs than in ECs (both basally and after LPS exposure). These observations have important implications in atherogenesis, which is considered to be an inflammatory disease.

The interplay between PCSK9 and LOX-1 was shown by both knockdown (or KO) and up-regulation/induction experiments. The functional significance of this cross-talk was corroborated by experiments on ox-LDL uptake and VCAM-1 expression in SMCs. PCSK9 knockdown almost completely blocked, while its increase enhanced, Dil-ox-LDL uptake and VCAM-1 expression.

LOX-1 activation has been implicated in atherosclerosis and related disorders. It is the major ox-LDL receptor on ECs, but is also present in substantial amounts on arterial SMCs. LOX-1 expression in SMCs may become particularly important in disease states characterized by loss of EC continuity during haemodynamic stress, or following EC disruption as may occur during angioplasty. LOX-1 activation induces ox-LDL uptake, increased production of cell surface adhesion molecules, and a state of oxidative stress and inflammation—key steps in atherogenesis.[17] A pro-inflammatory state further enhances LOX-1 expression, resulting in a self-perpetuating pro-atherosclerotic milieu.[5] It is noteworthy that LOX-1 inhibition reduces the state of oxidative

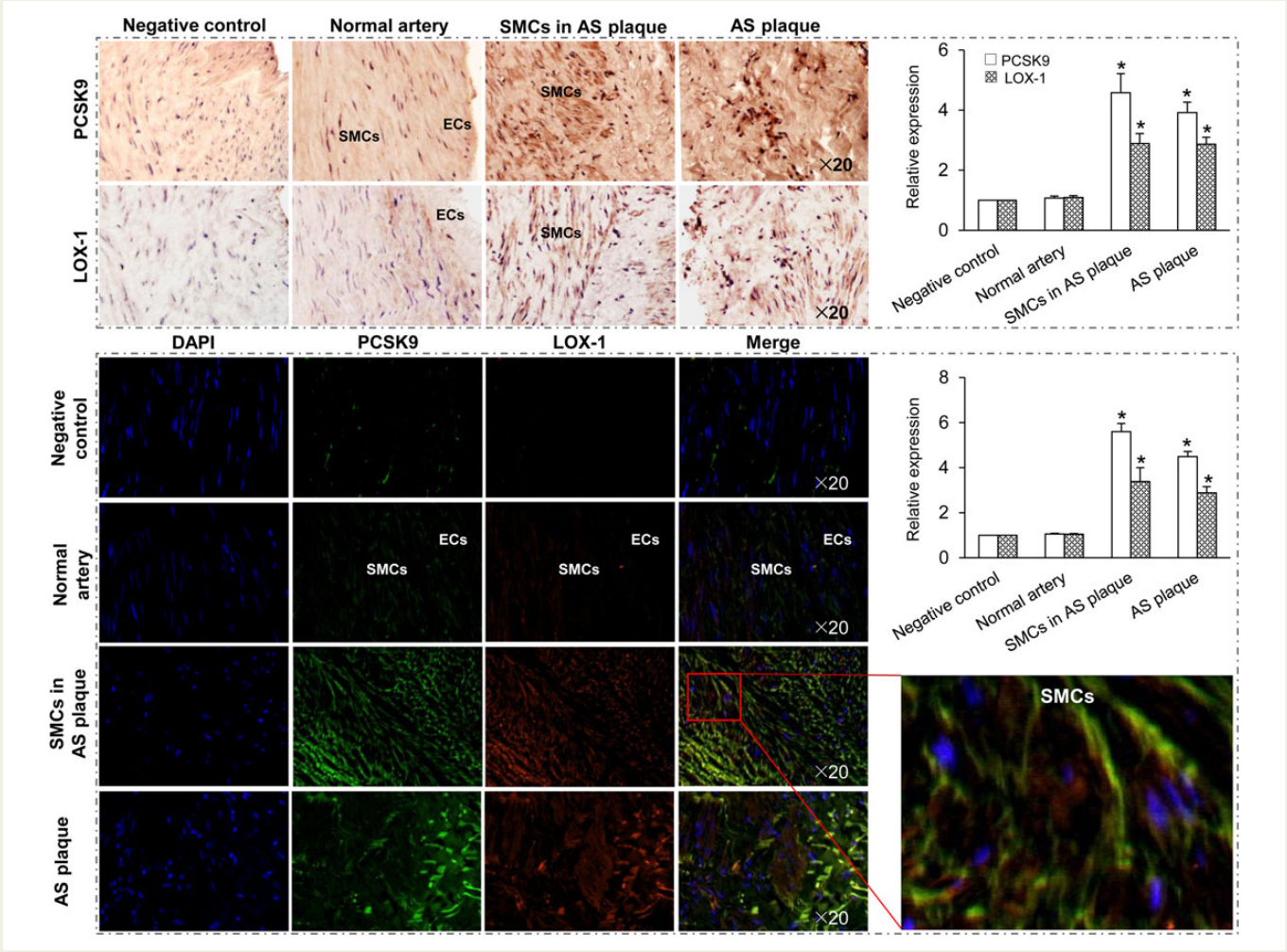

Figure 10 Expression of PCSK9 and LOX-1 in human aorta with atherosclerosis. Representative images of immunohistochemical (upper panel) and IF (lower panel) staining for PCSK9 and LOX-1 expression. The bar graphs on the upper right are data based on observations from all tissue blocks show that while PCSK9 and LOX-1 are increased in the atherosclerotic (AS) plaque, the increase is particularly pronounced in SMCs. Similar data were obtained on immunohistochemistry (upper panel) and IF staining (lower panel). The inset shows high power magnification. Data shown as mean ± SD. $^{*}P < 0.05$ vs. normal regions.

stress, mitochondrial DNA damage, and NLRP3 inflammasome activation in macrophages,[2,19] all of which are markers of atherosclerosis.

The relationship between LOX-1 and lipids has been examined by several investigators. Ding *et al.*[18] showed that ox-LDL decreases LDL receptor expression by ECs, an effect mediated through LOX-1. Pirillo *et al.*[20] showed that 15-lipoxygenase-modified HDL induces LOX-1 expression. Statin drugs have also been shown to reduce LOX-1 expression by ECs.[21] Thus, multiple lines of evidence link LOX-1 and LDLr to the regulation of cellular lipid levels.

PCSK9 secretion in large amounts by SMCs, but only in small amounts by ECs, was recently documented.[22] The present studies, showing two- to three-fold greater secretion of PCSK9 by SMCs than by ECs, provide strong support for those observations while also demonstrating that the inflammatory stimulus LPS further increases PCSK9 expression by SMCs and ECs. Ferri *et al.*[22] showed that SMC-derived PCSK9 reduced the expression of LDLr and lowered LDL uptake by human macrophages and by the J774 macrophage cell line.

The present studies, showing positive feedback between LOX-1 and PCSK9, may have important implications for atherogenesis. PCSK9 overexpression, which occurs particularly during inflammatory conditions, enhances LOX-1 expression. Conversely, PCSK9 expression is itself induced by LOX-1, which is abundantly expressed by diverse cell types in atherosclerotic plaque, including SMCs. A plausible synthesis of these interactions is presented in *Figure 9*, although we acknowledge that other scenarios are also possible. In this portrayal, an inflammatory stimulus such as LPS induces ECs, and especially SMCs, to secrete PCSK9, which in turn induces mtROS production and LOX-1 synthesis. The initiating trigger could also be an oxidative or mitochondrial stress, elevating ROS production. LOX-1 is then activated, and ox-LDL is taken up by cells leading to propagation of the inflammatory response and eventually to atherosclerosis. Atherogenesis in *LDLr*-KO mice can be attenuated by *LOX-1* deletion as noted in previous studies,[23] and may involve a reduction in PCSK9. These studies also showed reduced oxidative stress in atherosclerotic regions. In keeping with the near-total inhibition of LOX-1 expression upon PCSK9 knockdown or KO, it is quite likely that PCSK9 inhibitor strategies may inhibit LOX-1 expression.

Wu *et al.*[24] noted that *PCSK9* siRNA reduced ox-LDL-induced apoptosis of human ECs, implying that PCSK9 can impair EC survival. Like

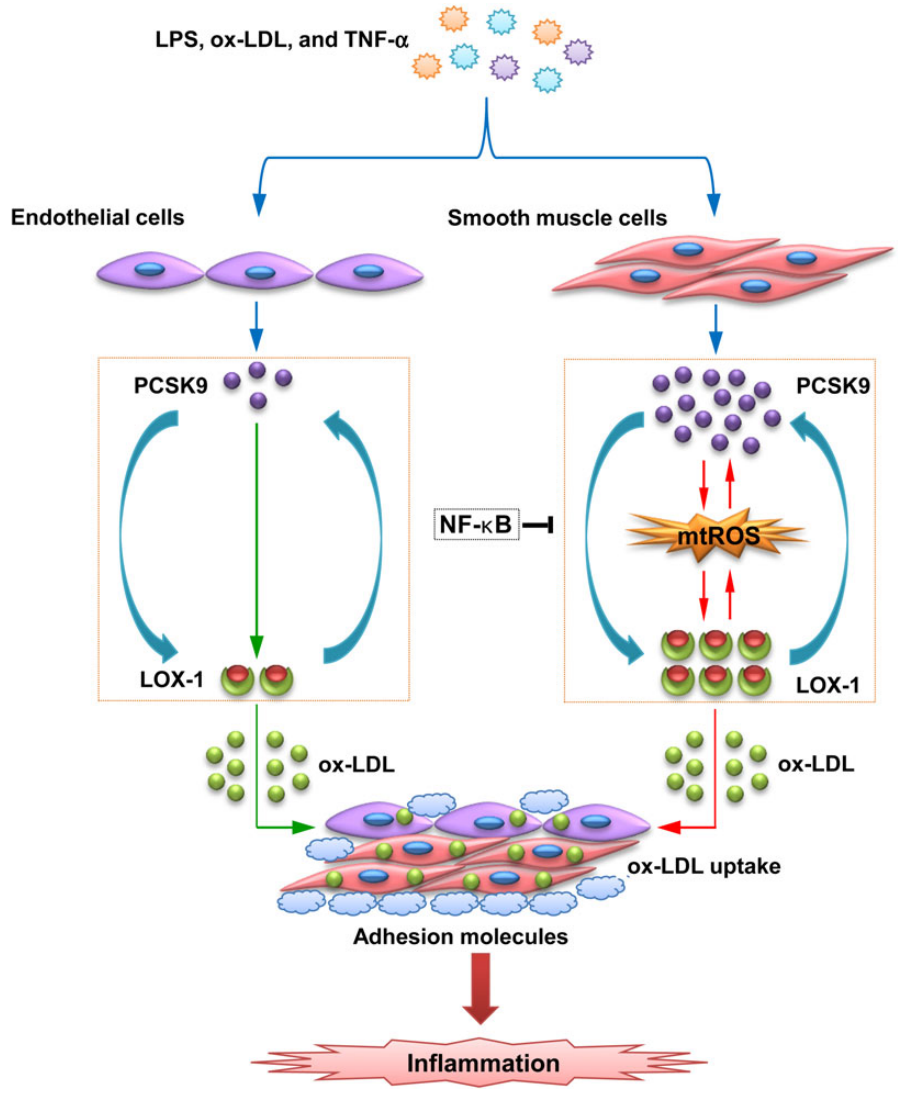

Figure 11 Proposed signalling pathway linking PCSK9, LOX-1, and mtROS. There appears to be a bidirectional link between LOX-1 and PCSK9 especially during inflammatory states. Compared with ECs, LPS induces more PCSK9 expression in smooth muscle cells. Both PCSK9 treatment and LOX-1 overexpression induce mtROS generation, whereas inhibition of PCSK9 and LOX-1 reduces mtROS generation, suggesting a bridging role for mtROS in PCSK9–LOX-1 cross-talk. Both LOX-1 activation and PCSK9 administration enhance ox-LDL uptake and VCAM-1 expression in smooth muscle cells, which is a key event in the development of inflammation.

Ferri *et al.*,[22] we saw only modest expression of PCSK9 by cultured ECs, except in response to LOX-1 overexpression; however, these changes in ECs were small compared with those in vascular SMCs. It is possible that PCSK9 released by SMCs permeates through the interstitial fluid and induces ECs to express LOX-1, accompanied by adverse effects such as apoptosis and up-regulation of adhesion molecules.[24–28]

To investigate the role of other pre-inflammatory factors in PCSK9 expression, we used ox-LDL and TNF-α. We found that both ox-LDL and TNF-α markedly induced PCSK9 expression, indicating that perhaps all pro-inflammatory stimuli play an initial role in PCSK9 activation. NF-κB activation has been confirmed to play a key role in LPS-, ox-LDL-, and TNF-α-induced LOX-1 expression;[2,17–19] we therefore posited that NF-κB may play an important role in LOX-1-mediated PCSK9 expression. NF-κB was knocked down by its siRNA, and this phenomenon inhibited LPS-, ox-LDL-, and TNF-α-induced PCSK9 expression, indicating that NF-κB plays an important signalling role in inflammatory stimulus-mediated PCSK9 expression.

Another novel observation in this study is that LOX-1 and PCSK9 overexpression induced increases in mtROS generation, whereas knockdown of LOX-1 or PCSK9 was associated with reduced mtROS. Although we have depicted mtROS as a bridge between PCSK9 and LOX-1, it is at least equally likely (and consistent with our data) that the primary interaction is between mtROS and LOX-1, with a slightly weaker PCSK9 response arising from its coupling (mutual induction and suppression) to LOX-1. Another line of evidence, implying a primary role of mtROS in PCSK9/LOX-1 induction, is our observation that rotenone and antimycin A (which increase mtROS generation by three- to four-fold), simultaneously induce the expression of PCSK9 and LOX-1 by five- to 10-fold.

Immunohistochemistry of human atherosclerotic tissues reveals co-expression of LOX-1 and PCSK9 by the same cells. Moreover, compared with normal non-atherosclerotic arterial sections, atherosclerotic regions showed diffuse LOX-1 and PCSK9 expression, with the majority of immunoreactivity occurring in SMCs that express both LOX-1 and PCSK9. The identification of PCSK9 in atherosclerotic human tissues and its localization in SMCs in this study are proof of concept and need further confirmation.

In summary, our observations suggest a self-reinforcing cross-talk between PCSK9 and LOX-1 in vascular tissues, which can be initiated by an inflammatory stimulus and/or activation of mtROS. These concepts are summarized in *Figure 11*. PCSK9 inhibitors may thus induce an anti-atherosclerotic state by inhibiting LOX-1 expression.

4.1 Perspectives

Our studies provide evidence for positive feedback between PCSK9 and LOX-1 in arterial tissues and cultured ECS and SMCs, wherein PCSK9 stimulates LOX-1, and LOX-1 stimulates PCSK9. Mitochondrial ROS generation seems to play a critical role in the induction of PCSK9 and/or LOX-1, and possibly in their cross-talk which is exacerbated in the inflammatory state. LOX-1 has been shown to be a potent mediator of atherogenesis, raising the possibility that PCSK9 inhibitors may inhibit atherogenesis in hypercholesterolaemic states by disrupting LOX-1 expression.

4.2 Study limitations

There are some limitations of the present study which need to be addressed. First, although we studied PCSK9 signalling in *LOX-1* gene KO mice, further experiments in *LOX-1* transgenic aorta should be considered in the future. Secondly, we focused our attention on ECs and SMCs, other cells in the aorta, such as macrophages and fibroblasts, which express LOX-1, may also secrete PCSK9, and this phenomenon should be examined.

Conflict of interest: none declared.

Funding

This study was supported by funds from the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development, Washington, DC. Additional support was provided by grants-in-aid from the National Natural Science Foundation of China (nos 11332003 and 61190123). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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