pregnant women with preeclampsia and chronic hypertension: lack of correlation with lipid peroxides. *Hypertens Pregnancy*. 2001;20:177–183.
22. Jain M, Sawhney H, Aggarwal N, Vashistha K, Majumdhar S. Auto antibodies against oxidized low density lipoprotein in severe preeclampsia. *J Obstet Gynaecol Res*. 2004;30:188–192.
23. Raijmakers MT, van Tits BJ, Hak-Lemmers HL, Roes EM, Steegers EA, Peters WH. Low plasma levels of oxidized low density lipoprotein in preeclampsia. *Acta Obstet Gynecol Scand*. 2004;83:1173–1177.
24. Qiu C, Phung TT, Vadachkoria S, Muy-Rivera M, Sanchez SE, Williams MA. Oxidized low-density lipoprotein (oxidized LDL) and the risk of preeclampsia. *Physiol Res*. 2006;55:491–500.
25. Sanchez SE, Williams MA, Muy-Rivera M, Qiu C, Vadachkoria S, Bazul V. A case-control study of oxidized low density lipoproteins and preeclampsia risk. *Gynecol Endocrinol*. 2005;21:193–199.
26. Yamada Y, Doi T, Hamakubo T, Kodama T. Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nervous system. *Cell Mol Life Sci*. 1998;54:628–640.
27. Chen M, Masaki T, Sawamura T. LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. *Pharmacol Ther*. 2002;95:89–100.
28. Oka K, Sawamura T, Kikuta K, Itokawa S, Kume N, Kita T, Masaki T. Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. *Proc Natl Acad Sci U S A*. 1998;95:9535–9540.
29. Sacks GP, Studena K, Sargent K, Redman CW. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. *Am J Obstet Gynecol*. 1998;179:80–86.
30. Hubel CA, Kagan VE, Kisin ER, McLaughlin MK, Roberts JM. Increased ascorbate radical formation and ascorbate depletion in plasma from women with preeclampsia: implications for oxidative stress. *Free Radic Biol Med*. 1997;23:597–609.
31. Llurba E, Gratacos E, Martin-Gallan P, Cabero L, Dominguez C. A comprehensive study of oxidative stress and antioxidant status in preeclampsia and normal pregnancy. *Free Radic Biol Med*. 2004;37:557–570.
32. Wang YP, Walsh SW, Guo JD, Zhang JY. The imbalance between thromboxane and prostacyclin in preeclampsia is associated with an imbalance between lipid peroxides and vitamin E in maternal blood. *Am J Obstet Gynecol*. 1991;165:1695–1700.
33. Rumbold AR, Crowther CA, Haslam RR, Dekker GA, Robinson JS. Vitamins C and E and the risks of preeclampsia and perinatal complications. *N Engl J Med*. 2006;354:1796–1806.
34. Poston L, Briley AL, Seed PT, Kelly FJ, Shennan AH. Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. *Lancet*. 2006;367:1145–1154.

Cardiovascular Research (2009) 82, 493–502
doi:10.1093/cvr/cvp089

Impaired vascular function in small resistance arteries of LOX-1 overexpressing mice on high-fat diet

Birgit Eichhorn[1]*, Gregor Muller[2], Anja Leuner[2], Tatsuya Sawamura[3], Ursula Ravens[1], and Henning Morawietz[2]

[1]*Department of Pharmacology and Toxicology, Medical Faculty Carl Gustav Carus, University of Technology, Fetscherstr. 74, D-01307 Dresden, Germany;* [2]*Department of Vascular Endothelium and Microcirculation, Medical Faculty Carl Gustav Carus, University of Technology, Dresden, Germany; and* [3]*Department of Vascular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka, Japan*

Received 1 August 2008; revised 6 February 2009; accepted 11 March 2009; online publish-ahead-of-print 15 March 2009

Time for primary review: 28 days

KEYWORDS
Endothelial function;
K^+-channel;
Lipoproteins;
Smooth muscle;
Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1)

Aims LOX-1 is a major vascular receptor for oxidized low-density lipoprotein (oxLDL). In this study, we analysed the impact of LOX-1 overexpression and high dietary fat intake on vascular function in small resistance arteries.

Methods and results Relaxation of mesenteric arteries was measured using a wire myograph. Compared with the control group, mice overexpressing LOX-1 on a high-fat diet (FD) had preserved vascular smooth muscle relaxation, but impaired endothelium-dependent relaxation via NO. Vascular NO availability was decreased by exaggerated formation of reactive oxygen species and decreased endothelial NO synthase expression. Endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation via cytochrome P450 metabolites was increased in LOX-1 + FD animals, but did not completely compensate for the loss of NO. Currents of calcium-activated potassium channels with large conductance (BK_{Ca} channels) were measured by the voltage-clamp method. The BK_{Ca} current amplitudes were not altered in endothelial cells, but highly increased in vascular smooth muscle cells from resistance arteries of LOX-1-overexpressing mice on FD. BK_{Ca} currents were activated by low-dose H_2O_2 and cytochrome P450 metabolites 11,12-EET and 14,15-EET as EDHF in control mice.

Conclusion LOX-1 overexpression and FD caused functional changes in endothelial and vascular smooth muscle cells of small resistance arteries.

1. Introduction

Atherosclerosis, with its clinical manifestation in cardiovascular diseases, is the major cause of death in industrialized countries. Functional changes in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) contribute to the initiation and early progression of cardiovascular diseases like atherosclerosis.[1–3] Resistance arteries do not show morphological alterations in response to high-fat diet (FD) or oxLDL, but rather develop functional impairment.[4] Several changes in the early phase of endothelial dysfunction are associated with high plasma levels of lipoproteins. Circulating low-density lipoproteins (LDL) can be modified to oxidized LDL (oxLDL). The major receptor of oxLDL is the lectin-like oxLDL receptor-1 (LOX-1) in the vessel wall.[5–7] LOX-1 mediates endocytosis of oxLDL in ECs,[8] VSMCs, and monocytes.[9] Basal LOX-1 expression is low, but several pathophysiological conditions like hypertension, diabetes mellitus, and hyperlipidaemia and the development of atherosclerotic lesions have been linked with an increased vascular LOX-1 expression.[10] The G501C mutation in the lectin-like oxidized LDL receptor gene (LOX-1/OLR1) has been associated with the risk of myocardial infarction,[11] but not with the risk for stroke.[12] LOX-1 expression is increased in human atherosclerotic lesions (in early lesions mainly in ECs, in advanced lesions also in VSMCs and macrophages).[13] Moreover plaque formation is enhanced in coronary arteries of mice overexpressing LOX-1 against a genetic background of apolipoprotein E deficiency.[14] Cell-culture studies have shown that the endothelial generation of reactive oxygen species (ROS) by NAD(P)H oxidase complexes in response to oxLDL is mediated by LOX-1.[15] Furthermore, *in vitro* studies indicate that activation of LOX-1 also initiates a reduction in NO release.[15] However, little is known about the contribution of LOX-1 to vascular homeostasis and endothelial dysfunction in small vessels. The intact endothelium plays an important role in vascular function by synthesizing and releasing vasodilating factors.[16] Major vasodilating factors in arteries are the endothelium-derived hyperpolarizing factor (EDHF), nitric oxide (NO),

* Corresponding author. Tel: +49 351 458 6261; fax: +49 351 458 6315.
E-mail address: birgit.eichhorn@tu-dresden.de

and prostacyclin. The reduced NO availability in atherosclerosis can be mediated via decreased expression of the endothelial NO synthase (eNOS) or via inactivation of NO by ROS.[2] In addition to eNOS expression, there is also evidence of reduced eNOS activity by lack of cofactors (e.g. BH_4) and increased formation of endogenous inhibitors.[17] The impaired NO-mediated relaxation in different vessels of hypercholesterolaemic and atherosclerotic animal models or patients[18,19] can be compensated by EDHF. Several components for EDHF signalling have been proposed including electrical coupling through gap junctions, certain ROS as for instance H_2O_2, cytochrome P450 metabolites, and vascular Ca^{2+}-activated K^+ channels (K_{Ca})[20] in particular those with large conductance (BK_{Ca} channels).[21–23] Activation of BK_{Ca} channels facilitates relaxation by cell membrane hyperpolarization. Nevertheless their role in endothelial and smooth muscle dysfunction is not completely understood. Binding of oxLDL to LOX-1 was shown to modulate BK_{Ca}-channels in ECs *in vitro*.[15]

In this study, we have used mice overexpressing bovine LOX-1 that were fed an FD to examine a potential functional impairment of small resistance arteries (mesenteric arteries). Based on our experimental findings, we provide evidence that LOX-1 receptors can cause vascular dysfunction in resistance vessels.

2. Methods

2.1 Animals

Male wild-type mice (C57BL/6, WT) and mice overexpressing bovine LOX-1 in a C57BL/6 background under control of the murine preproendothelin-1 promoter (LOX-1 mice[14]), aged 8 weeks, were fed standard chow EF R/M CD88137 or FD EF R/M TD88137 (Ssniff Spezialitäten GmbH, Soest, Germany) for 10 weeks. LOX-1 mice were kindly provided by T.S., Department of Vascular Physiology, National Cardiovascular Center Research Institute Fujishirodai, Suita, Osaka, Japan. The generation and characterization of the mice has been recently described by Inoue *et al.*[14] The LOX-1 mice carry 24 copies of the transgene, resulting in an approximately eight-fold higher mRNA-expression and a marked upregulation of endothelial LOX-1 protein expression. A similar upregulation of the LOX-1 protein has been described in ECs of human carotid arteries, covering early atherosclerotic lesions.[13] Overexpression of the bovine LOX-1 was verified by PCR in mesenteric arteries (see Supplementary material online, *Figure S1*). All performed experiments are in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal research Ethics Committee of the Dresden University of Technology and the Regierungspräsidium Dresden approved the animal facilities and the experiments according to institutional guidelines and German animal welfare regulations (AZ: 24-9168.24-1-2003-13, 24D-9168.24-1/2006-16).

2.2 Serum lipid measurements

Serum lipids were measured at the Institute of Clinical Chemistry and Laboratory Medicine (University of Technology Dresden) using kits for triglycerides, cholesterol, HDLs and LDLs (Roche Diagnostics GmbH, Mannheim, Germany).

2.3 Preparation of mesenteric arteries for *in vitro* studies

Arteries (third-order branch) were dissected and maintained in Ca^{2+}-free physiological salt solution [PSS; mmol/L: NaCl 119.0; KCl 4.7; $MgSO_4$ 1.17; $NaHCO_3$ 25.0; KH_2PO_4 1.18; glucose 5.5, and ethylenediaminetetraacetic acid (EDTA) 0.027; pH 7.4].

2.4 Superoxide anions

Dissected vessels were incubated in Krebs–Henseleit buffer (mmol/L: NaCl 115.0; $NaHCO_3$ 25.0; KCl 4.0; KH_2PO_4 0.9; $MgSO_4 \times 7H_2O$ 1.1; $CaCl_2$ 2.6; glucose 5.5; pH: 7.4) for 30 min at 37°C. Lucigenin (5 μmol/L – a concentration below the threshold of redox cycling[24]) and NADPH (100 μmol/L) were dissolved in Krebs–Henseleit buffer for determination of ROS. Lucigenin solution containing additional 200 U/mL superoxide dismutase (SOD) and 380 U/mL catalase was used to examine superoxide anions and the resulting H_2O_2 formation.[25] Solution without tissue served as control. Photoemission was detected every second for 30 min in a Fluorescence Microplate Reader Fluorimeter FLUOstar OPTIMA (BMG LABTECH, Jena, Germany). The length of the blood vessels was measured with an eye piece scale (ZEISS, Jena, Germany) and used for data normalization. The increase in ROS production in animals fed with an FD was normalized as 100% of the corresponding control.

2.5 Measurement of contractile function

Small sections of mesenteric arteries (length 2 mm) were mounted in microvascular myographs for isometric tension recordings as described previously[26] and maintained in oxygenated PSS (5% CO_2 in 95% O_2; 1.6 mmol/L $CaCl_2$) at 37°C. During equilibration of the vessels, tension (T) corresponding to a pressure (P) of 70 mm Hg according to the equation $P = T2\pi U^{-1}$ (U = inner circumference) was adjusted. In all experiments cyclooxygenase-mediated relaxation was blocked with cyclooxygenase inhibitor diclofenac (0.1 mmol/L; Sigma, Taufkirchen, Germany). The vessel rings were contracted with cumulatively increasing concentrations of phenylephrine (PE). Relaxation was measured by increasing concentrations of acetylcholine (ACh) or sodium nitroprusside (SNP) in PE-precontracted (10 μmol/L) vessels. Relaxing effects of ACh were studied in the absence and presence of NO synthase inhibitor nitro-L-arginine-methylester (L-NAME; 30 μmol/L), BK_{Ca} inhibitor paxilline (1 μmol/L), cytochrome P450 inhibitor N,N-diethylaminoethyl-2,2-diphenylvalerate (proadifen; 50 μmol/L), and epoxygenase inhibitor 6-(2-propargyloxyphenyl)hexanoic acid (PPOH; 30 μmol/L). The effects of ACh or SNP are expressed in percent of the response to PE (=100%).

2.6 Isolation of vascular cells

Mesenteric arteries were stored in low Ca^{2+}-containing PSS (0.16 mmol/L Ca^{2+}) at 4°C. Enzymatic dissociation was carried out in two steps. The first solution (1 mL PPS) contained 0.7 mg papain; 1.5 mg dithioerythritol (Roth, Karlsruhe, Germany), bubbled with O_2, 20 min, 37°C. The second solution (1 mL PPS) contained 1.2 mg collagenase type F, 1.5 mg trypsin inhibitor, 0.5 mg elastase (Serva, Heidelberg, Germany), and 1.0 mg bovine albumin fraction V (Serva, Heidelberg, Germany) gassed with O_2, 12 min, 37°C. Single VSMCs and ECs were obtained by trituration in PSS.

2.7 Electrophysiological experiments

Potassium outward current through the BK_{Ca} channels ($I_{BK,Ca}$) was measured using a HEKA-EPC8 amplifier (HEKA Elektronik, Lambrecht, Germany) in voltage-clamp mode. Bath superfusion buffer (mmol/L): NaCl 127.0; KCl 5.9; $CaCl_2$ 2.4; $MgCl_2$ 1.2; glucose 11.0; HEPES 10.0, pH 7.4. Pipette solution (mmol/L): KCl 134.0; NaCl 6.0; $MgCl_2$ 1.2; $CaCl_2$ 4.2, EGTA 5.0; glucose 11.0; Mg-ATP 3.0; HEPES 10.0; pH 7.4. Pipette tip resistance was 3.0–4.0 MΩ. Experiments were carried out at 21°C. Effects of the following compounds were studied: 1,3-Dihydro-1-[2-hydroxy-5- (trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619; 30 μmol/L), paxilline (1 μmol/L), H_2O_2 (1 μmol/L), 11,12-Epoxy-(5Z,8Z,14Z)-

eicosatrienoic acid (11,12-EET; 300 nmol/L), and 14(R),15(S)-Epoxy-(5Z,8Z,11Z)-eicosatrienoic acid (14,15-EET; 300 nmol/L). The cell capacity was used for data normalization. Unless stated otherwise, all substances were purchased from Sigma, Taufkirchen, Germany.

2.8 RNA isolation and real-time PCR

Total RNA of mesenteric arteries was isolated using the EZNA Total RNA-Kit (Peqlab, Erlangen, Germany). For quantification of mRNA expression real-time PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) in a thermal cycler (Corbett Research, Mortlake, Australia). Primers: bovine LOX-1 (sense: 5′-CCAGGAGAACTGCTTGTCTT-3′, antisense: 5′-GTGC TCTCAATAGATTCGCC-3′), eNOS (sense: 5′-TTCCGGCTGCCACCTGATC CTAA-3′, antisense: 5′-AACATATGTCCTTGCTCAAGGCA-3′), KCNMA (sense: 5′-CGATAAGCTGTGGTTCTGGC-3′, antisense: 5′-AAGAAGACC ATGAAGAGGCGTC-3′), and KCNMB1 (sense: 5′-AGAAGCGCGGAGAGA CACGA-3′, antisense: 5′-CAGCTCTTCCTGGTCCTTGATA-3′). Quantification by one-step RT real-time PCR included 50°C for 30 min, 95°C for 15 min, subsequent cycles of 94°C for 30 s, 60°C for 30 s, and finally 72°C for 50 s. Internal RNA standards were produced as previously described.[27]

2.9 Data analysis and statistics

Potency of agonists was determined as $-\log EC_{50}$ [mmol/L]. All results are expressed as mean ± standard error of the mean (SEM). Unless stated otherwise, number of experiments is given as arteries or cells from *n* mice. Student's *t*-test (unpaired) was used for statistical analysis, differences with $P < 0.05$ were considered significant. Multiple comparisons were done by one-way ANOVA followed by Bonferroni *post hoc* test.

3. Results

3.1 Body weight and serum lipids

Table 1 summarizes body weight and serum lipid concentrations in the four investigated animal groups. LOX-1 mice on normal diet were heavier than WT mice, but did not differ in serum lipid concentrations. FD significantly increased body weight in WT and in transgenic mice when compared with control diet. While serum concentrations of triglycerides were not different between the groups, free cholesterol, LDL, and HDL concentrations were significantly higher in WT and LOX-1 animals on high-fat compared with standard diet. The ratio of LDL:HDL increased during FD from 1:7 to 1:4 in WT mice and from 1:8 to 1:4 in LOX-1 mice. Although FD caused significantly larger weight gain in LOX-1 mice compared with WT animals, no statistically significant differences were found in serum lipid parameters.

3.2 Vascular function

Basal tone of mesenteric arteries was unaffected by FD (compare pre-PE control values in Supplementary material online, *Figure S2A*).

Endothelium-dependent and -independent relaxation in mesenteric arteries was studied in PE-preconstricted vessels. In order to determine the optimum concentration for precontraction, we measured contractile responses to cumulatively increasing PE concentrations. All vessels contracted in the same concentration range [average $-\log EC_{50}$ (mol/L) values between 5.5 and 5.7]. However, the maximum contractile response to PE was significantly lower in the LOX-1 + FD mice than in the other groups (LOX-1 + FD: 1.4 ± 0.1 mN/mm vs. WT: 1.7 ± 0.3 mN/mm; WT + FD: 1.8 ± 0.1 mN/mm; LOX-1: 2.0 ± 0.2 mN/mm; $P < 0.05$; see Supplementary material online, *Figure S2A*). Subsequently, arteries in all further experiments were precontracted with 10 μmol/L PE. Values of maximum contraction produced by 80 mmol/L KCl were similar in the four groups (see Supplementary material online, *Figure S2C*). Absolute values of the constriction induced by 100 μM PE and 80 mmol/L KCl in WT and LOX-1 mice are consistent with previously published results.[28,29]

Endothelium-dependent relaxation was studied by exposing the arteries to increasing concentrations of ACh. The resulting concentration–response curves (CRC; *Figure 1A* and *B*) revealed incomplete relaxation by ACh with significantly reduced relaxation in LOX-1 + FD animals (maximum relaxation, Eff_{max}: 67.8 ± 3.4%; $P < 0.01$) compared with WT, WT + FD, and LOX-1 (Eff_{max}: 89.3 ± 5.0%; 88.1 ± 4.7%; and 88.1 ± 3.6%; *Figure 1C*). Interestingly, potencies were similar in all groups [average $-\log EC_{50}$ (mol/L) values between 6.8 and 7.0]. The relaxing response to ACh was reduced in the presence of the NO synthase (NOS) blocker L-NAME (30 μmol/L). Relaxation was reduced in WT (42.2 ± 5.6%), WT + FD (39.1 ± 7.5%), and LOX-1 mice (40.1 ± 6.1%), but not in LOX-1 + FD arteries (5.5 ± 5.2%). These results suggest that NO-mediated relaxation was significantly impaired in LOX-1 + FD animals compared with the other groups (*Figure 1D*). In addition the basal tone during L-NAME incubation increased only in arteries from WT, WT + FD, and LOX-1 animals, but not in LOX-1 + FD mice. In the presence of L-NAME, the

Table 1 Characterization of body weight and serum parameters

	WT		LOX-1	
	Control	+FD	Control	+FD
Body weight, g (*n*)	27.3 ± 0.8 (8)	33.1 ± 1.1** (22)	30.7 ± 1.2# (7)	36.3 ± 1.5*# (18)
Triglycerides, mmol/L (*n*)	1.1 ± 0.1 (7)	1.3 ± 0.1 (24)	1.1 ± 0.03 (7)	1.3 ± 0.1 (18)
Cholesterol, mmol/L, (*n*)	3.4 ± 0.2 (7)	5.6 ± 0.3*** (24)	3.3 ± 0.1 (7)	5.9 ± 0.3*** (18)
HDL, mmol/L, (*n*)	2.7 ± 0.2 (7)	4.5 ± 0.2*** (24)	2.4 ± 0.1 (7)	4.8 ± 0.3*** (18)
LDL, mmol/L, (*n*)	0.3 ± 0.1 (7)	1.1 ± 0.1*** (24)	0.3 ± 0.04 (7)	1.1 ± 0.1*** (18)

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$ standard diet vs. high-fat diet.
#$P < 0.05$ WT vs. LOX-1.

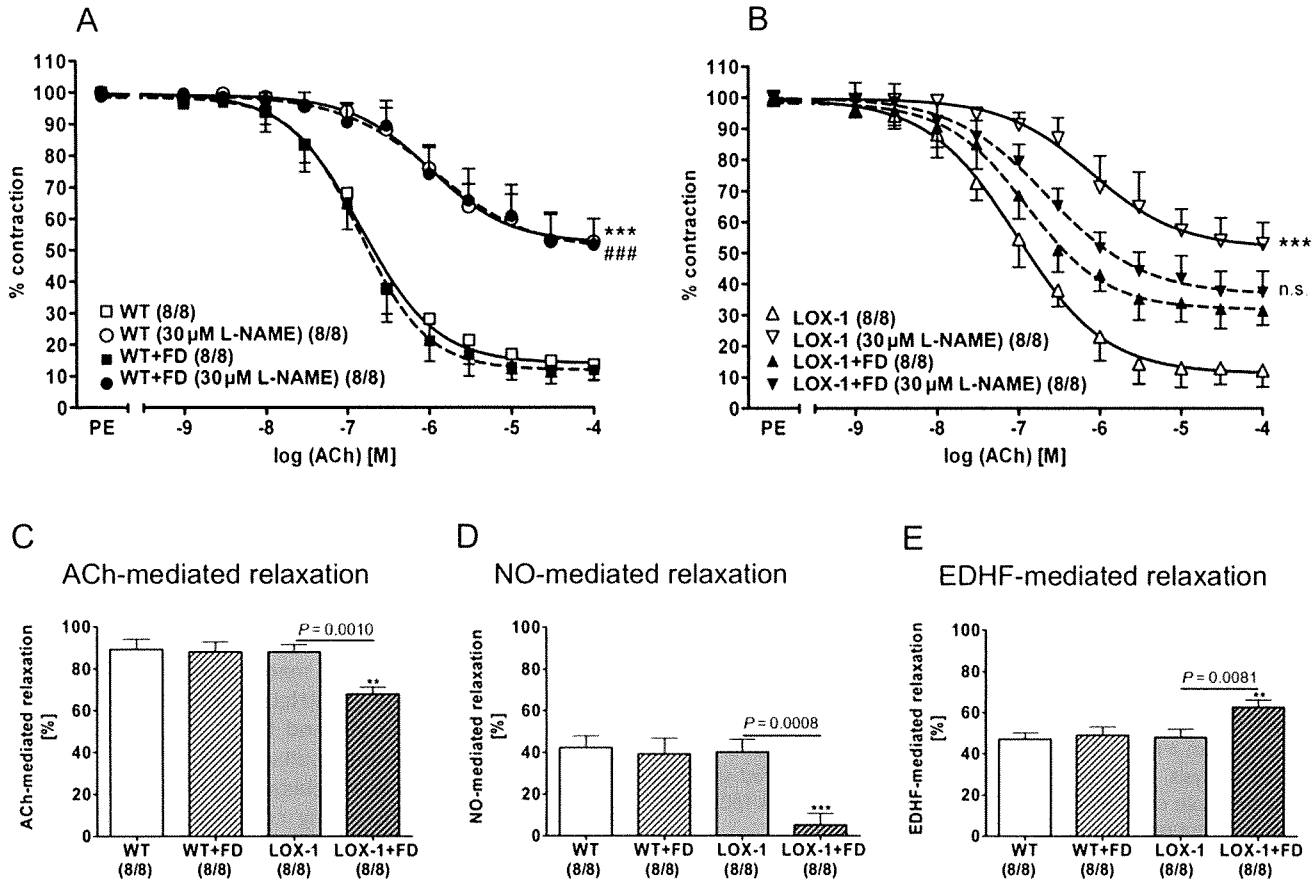

Figure 1 Effects of acetylcholine in mesenteric arteries of WT and LOX-1 mice. (*A*) Concentration-response curves (CRCs) for acetylcholine in arteries of WT mice on standard and high-fat diet without and with L-NAME. (*B*) CRCs for acetylcholine in arteries of LOX-1 mice on standard and high-fat diet without and with L-NAME. Maximum effects of acetylcholine-induced (*C*), NO-mediated relaxation (*D*), and EDHF-mediated relaxations (*E*) of arteries from WT and LOX-1 mice on standard and high-fat diet. $^{***}P < 0.001$ control vs. L-NAME of mice on standard diet. $^{\#\#\#}P < 0.001$ control vs. L-NAME of mice on high-fat diet.

differences in PE-induced contractions between LOX-1 + FD mice and the other three animal groups persisted (see Supplementary material online, *Figure S2D* and *E*).

ACh-induced relaxation in the presence of NOS and cyclooxygenase inhibitors is mediated by EDHF. The EDHF-mediated fraction of relaxation was largest in LOX-1 + FD mice (62.6 ± 3.5%) compared with the other groups (WT: 47.0 ± 3.2%; WT + FD: 49.0 ± 4.0%; LOX-1: 48.0 ± 4.1%; $P < 0.01$; *Figure 1E*). Cytochrome P450 enzymes are a substantial source of EDHF, they are responsible for the transformation of arachidonic acid into epoxyeicosatrienoic acids (EETs).[30,31] The role of these enzymes in ACh-induced relaxation was examined with proadifen, a non-specific blocker of cytochrome P450 isoenzymes and PPOH, a specific epoxygenase blocker. Proadifen and PPOH did not significantly block EDHF-mediated relaxation in WT, WT + FD, and LOX-1 animals, but significantly reduced EDHF-mediated relaxation in LOX-1 + FD (proadifen 41.3 ± 6.3%; $P < 0.01$ and PPOH 28.7 ± 6.3%; $P < 0.05$; *Figure 2A–D*). Both compounds changed the efficacy of ACh to a larger extent in LOX-1 + FD mice compared with the other groups, leading to similar levels of relaxation in all four groups.

The contribution of BK_{Ca} channels as potential EDHF targets in LOX-1 + FD was tested by blocking the channels with the specific BK_{Ca} blocker paxilline (*Figure 2E–H*). Paxilline reduced ACh-mediated relaxation only in LOX-1 + FD (18.42 ± 3.6%; $P = 0.0026$), but not in WT (0.1 ± 3.3%), WT + FD (1.6 ± 2.5%), and LOX-1 animals (1.1 ± 3.9%), indicating that BK_{Ca} channels were activated only in LOX-1 + FD mice. Even in the presence of L-NAME, paxilline was able to inhibit relaxation, indicating a BK_{Ca} channel involvement in the EDHF-mediated relaxation. During paxilline incubation the basal tone significantly elevated in arteries from LOX-1 + FD mice compared with WT, WT + FD, and LOX-1 animals (see Supplementary material online, *Figure S2F*). PE-induced contraction in the presence of paxilline was similar in all four groups (see Supplementary material online, *Figure S2G*).

Endothelium-independent relaxation was measured using the NO-donor SNP. Potency and efficacy of SNP were calculated from cumulative CRCs (see Supplementary material online, *Figure S3*) and were found to be similar in all groups [$-\log EC_{50}$ (mol/L): WT: 7.4 ± 0.2; WT + FD: 7.4 ± 0.1; LOX-1: 7.2 ± 0.2; LOX-1 + FD: 7.2 ± 0.1; Eff_{max}: WT: 0.6 ± 2.5%; WT + FD: 1.2 ± 2.6%; LOX-1: 2.8 ± 2.9%; LOX-1 + FD 1.7 ± 0.5%].

3.3 Reactive oxygen species

Since vascular dysfunction is associated with increased ROS production, we have examined the formation of ROS by chemiluminescence in mesenteric arteries. ROS formation was increased in WT + FD and LOX-1 + FD, and the increase

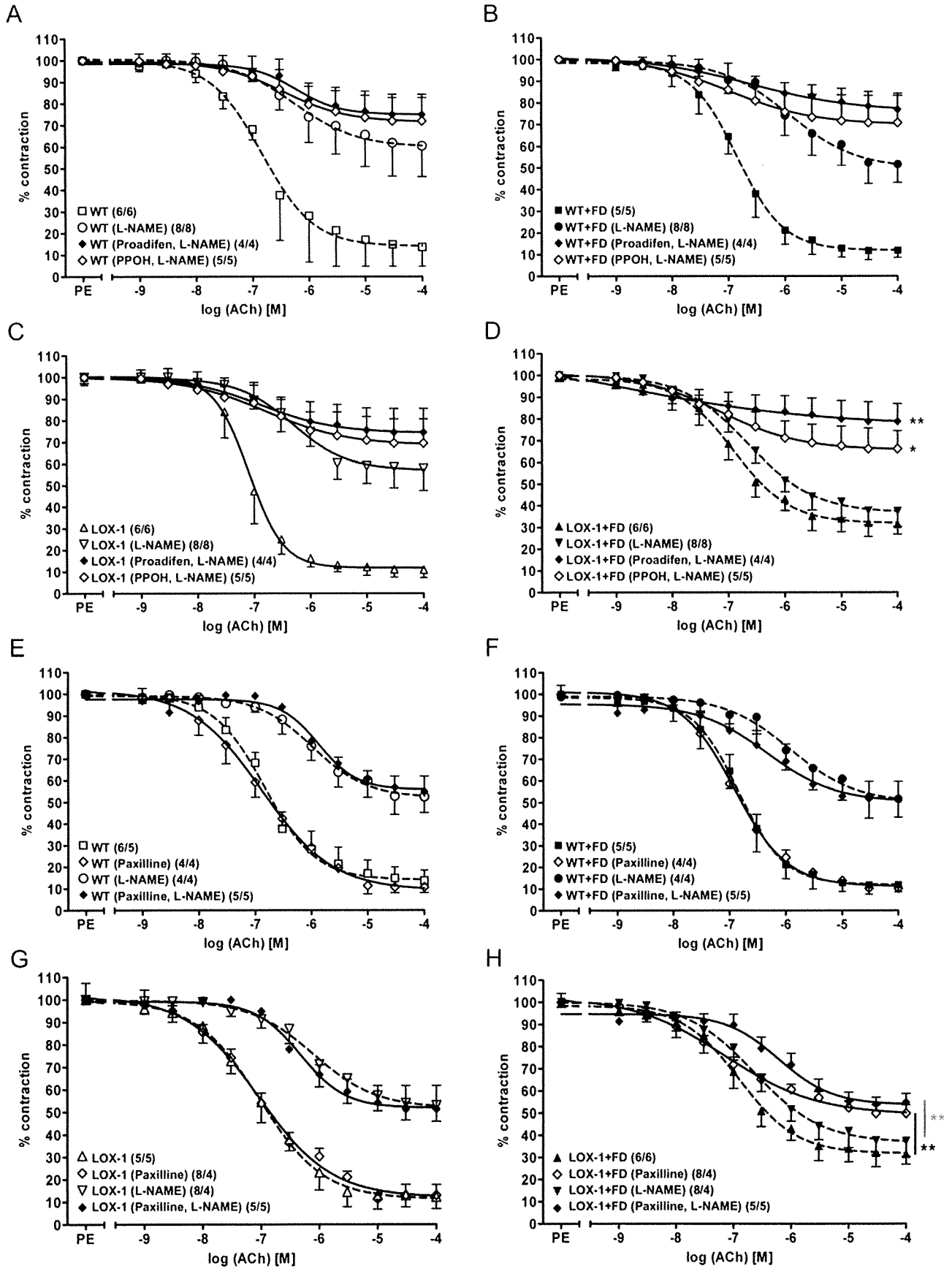

Figure 2 Concentration–response curve for acetylcholine in the presence of BK_{Ca} channel blocker and cytochrome P450 blocker in mesenteric arteries of WT and LOX-1 mice. Concentration–response curves (CRCs) for acetylcholine in the presence of cytochrome P450 blocker proadifen and epoxygenase blocker PPOH in arteries of (*A*) WT; (*B*) WT + FD; (*C*) LOX-1; (*D*) LOX-1 + FD in combination with L-NAME. CRCs for acetylcholine in the presence of BK_{Ca}-channel blocker paxilline on the arteries of (*E*) WT; (*F*) WT + FD; (*G*) LOX-1, and (*H*) LOX-1 + FD and in the presence and absence of L-NAME. $^{*}P < 0.05$ proadifen + L-NAME vs. L-NAME; $^{**}P < 0.01$ PPOH + L-NAME vs. L-NAME.

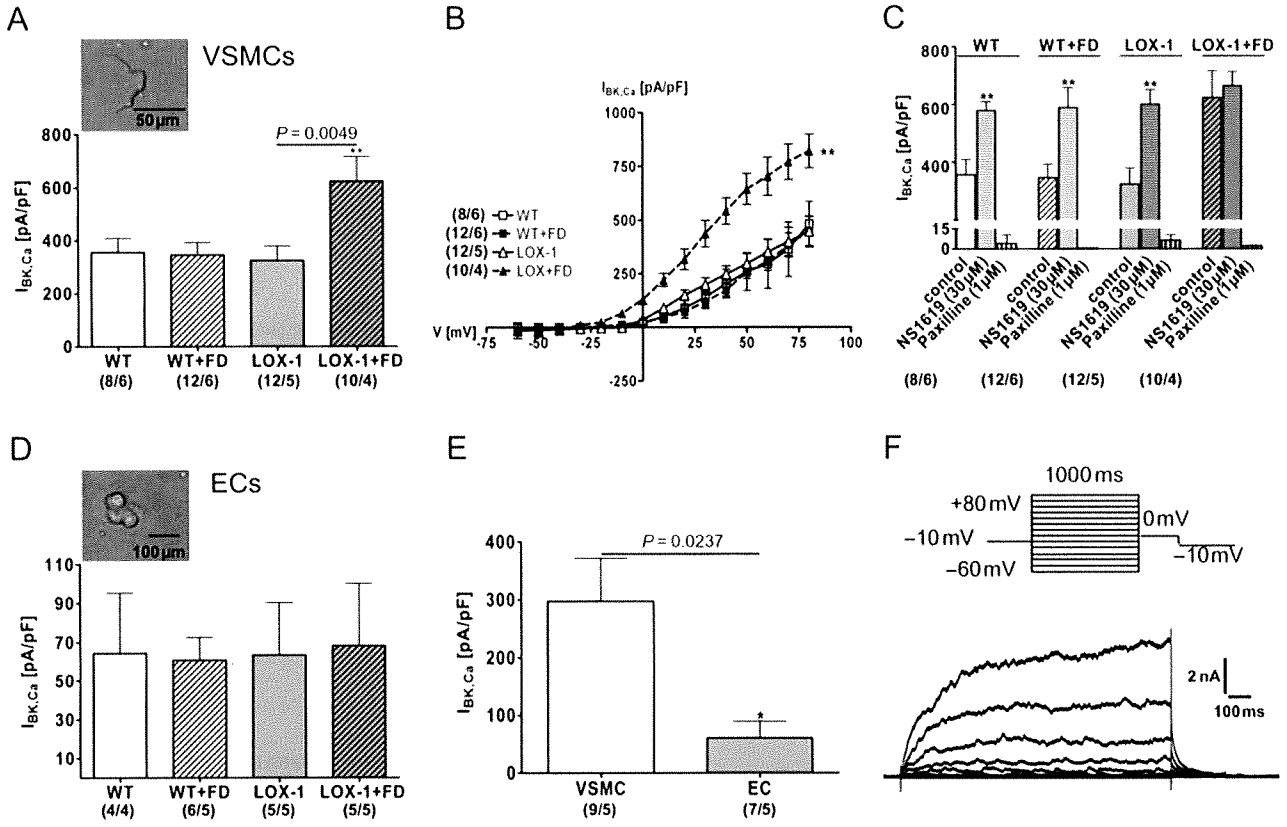

Figure 3 Characterization of electrophysiological properties in vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) of mesenteric arteries from WT and LOX-1 mice on standard and high-fat diet. (*A*) Current density in pA/pF of BK_{Ca} currents in VSMCs from WT, WT + FD, LOX-1, and LOX-1 + FD. (*B*) Current–voltage relationships of BK_{Ca} currents of VSMC from WT, WT + FD, LOX-1, and LOX-1 + FD. $^{**}P < 0.01$ standard diet vs. high-fat diet. (*C*) Current density in pA/pF of BK_{Ca} channels in VSMCs in response to clamp steps to + 60 mV under control condition, with NS1619 (30 µmol/L) and paxilline (1 µmol/L) in WT, WT + FD, LOX-1, and LOX-1 + FD. Please note the different scaling factors for the ordinate. $^{**}P < 0.01$ control vs. NS1619. (*D*) Current density in pA/pF of BK_{Ca} channels in ECs from WT, WT + FD, LOX-1, and LOX-1 + FD. (*E*) Comparison of the BK_{Ca} currents in VSMCs and ECs from WT. (*F*) (Above) Pulse protocol; (Below) Typical BK_{Ca} current tracings of a VSMC from WT.

was even higher in LOX-1 + FD than in WT + FD animals (see Supplementary material online, *Figure S4*). We have also determined superoxide anions, because this particular fraction of ROS has been previously shown to result in H_2O_2 formation and act directly as an EDHF component.[32] We found that H_2O_2 levels were significantly increased in LOX-1 + FD mice (56.2 ± 5.8%) compared with the other groups (WT: 33.2 ± 3.4%; WT + FD: 40.6 ± 8.3%; LOX-1: 39.8 ± 5.7%; see Supplementary material online, *Figure S4*).

3.4 BK_{Ca} currents

Our results provide evidence that EDHF formation is involved in the contractile responses and that EDHF has the largest impact in the LOX-1 + FD group. Since EDHF activates BK_{Ca} channels, we measured BK_{Ca} currents in smooth muscle and ECs in order to elucidate their contribution to the observed EDHF-mediated response in LOX-1 + FD mice.

All VSMCs exhibited robust BK_{Ca} currents. The current amplitude was completely suppressed by the selective BK_{Ca} channel-blocker paxilline (1 µmol/L).[33] Current densities of $I_{BK,Ca}$ in VSMCs at a test potential of +60 mV were significantly higher in LOX-1 + FD mice (623 ± 93 pA/pF) compared with WT (356 ± 54 pA/pF), WT + FD (346 ± 47 pA/pF), and LOX-1 (324 ± 54 pA/pF; *Figure 3A* and *B*). In LOX-1 + FD $I_{BK,Ca}$ voltage dependence was shifted to lower voltages suggesting that the current is already activated at more negative potentials. Moreover, opening of BK_{Ca}-channels with the selective channel activator NS1619 (30 µmol/L)[34] did not further increase current density in LOX-1 + FD mice, but increased the current in the other groups to almost the same levels as previously observed in LOX-1 + FD (*Figure 3C*). Interestingly, the $I_{BK,Ca}$ current densities in ECs were similar in all four mice groups (WT: 64 ± 31 pA/pF; WT + FD: 61 ± 12 pA/pF; LOX-1: 63 ± 27 pA/pF; LOX-1 + FD: 68 ± 32 pA/pF), but much smaller than in VSMCs (*Figure 3D* and *E*). Next, we examined the effects of three supposed EDHFs, i.e. H_2O_2, 11,12-EET, and 14,15-EET, on BK_{Ca} channel activity in WT mice. At + 60 mV H_2O_2 (1 µmol/L) significantly increased current density, but not to the same value as observed with NS1619 or in LOX-1 + FD mice. 11,12-EET and 14,15-EET (300 nmol/L each) activated $I_{BK,Ca}$ to the same maximum as NS1619 (*Figure 4A–C*) in WT mice and as the observed amplitude in LOX-1 + FD animals.

3.5 mRNA expression of BK_{Ca} channel subunits and eNOS

Differences in current densities could be due to different expression levels of the relevant channel subunits. Therefore, we have measured mRNA expression of the BK_{Ca}-channels using real-time PCR (*Figure 5A* and *B*). Both the pore-forming α-subunits and the accessory β1-subunits

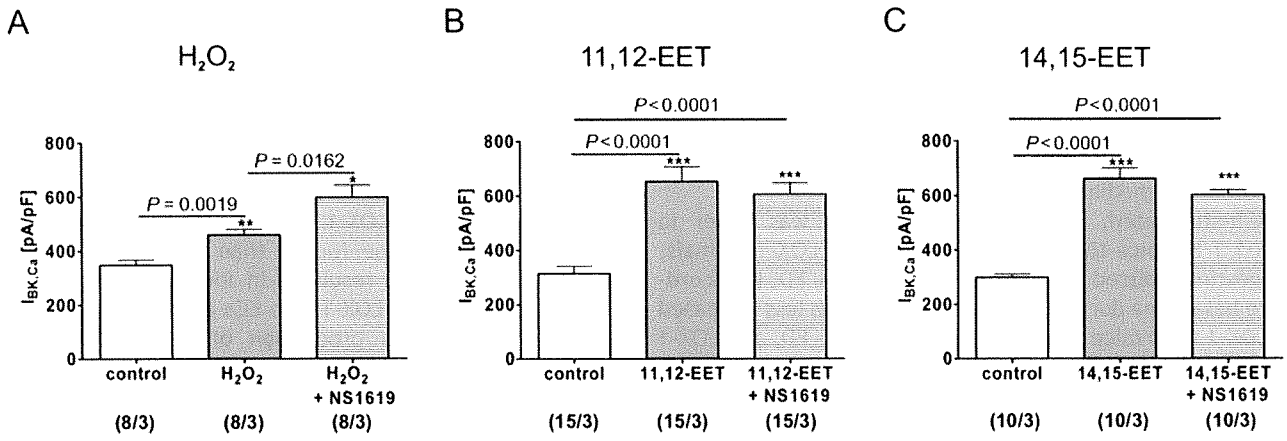

Figure 4 EDHF-mediated activation of BK_{Ca} currents in vascular smooth muscle cells (VMSCs) of mesenteric arteries from WT. (*A*) Hydrogen peroxide (1 μmol/L) activated the BK_{Ca} currents in VSMCs from WT, and even further increased with additional NS1619 (30 μmol/L). (*B*) 11,12-EET (300 nmol/L) significantly activated BK_{Ca} currents in VSMCs from WT to the same maximum like NS1619 (30 μmol/L). (*C*) Activation of BK_{Ca} currents by 14,15-EET (300 nmol/L) in VSMCs from WT to the similar values like NS1619 (30 μmol/L).

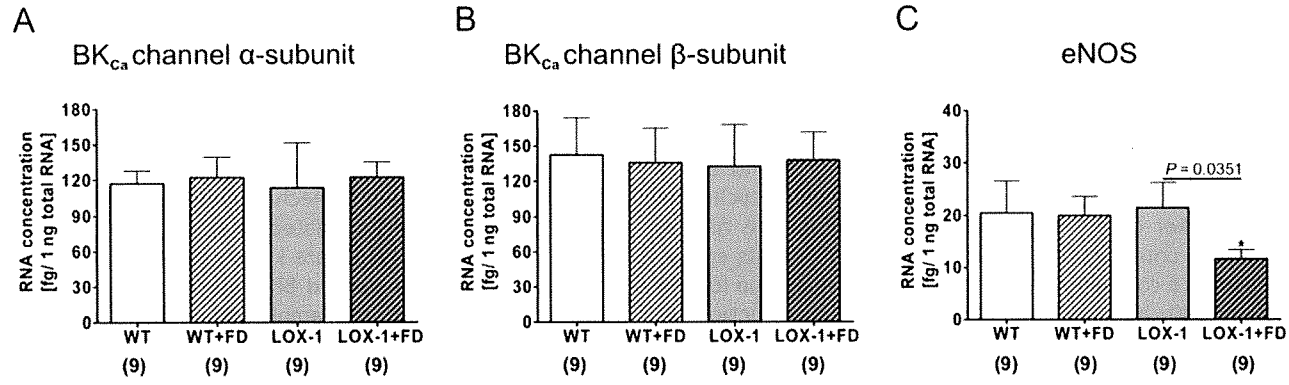

Figure 5 Expression of BK_{Ca} channel subunits and eNOS in mesenteric arteries. (*A*) BK_{Ca} channel α-subunit (KCNMA) mRNA expression was analysed by real-time PCR using an internal standard. (*B*) Amount of BK_{Ca} channel β_1-subunit (KCNMB1) mRNA was quantified by real-time PCR using an internal standard. (*C*) Expression of eNOS mRNA determined by real-time PCR using an internal standard. WT, wild-type; LOX-1, mice overexpressing LOX-1; FD, high-fat diet.

were expressed in similar amounts in all groups. In contrast mRNA expression of eNOS was lower in LOX-1 + FD mice compared with WT, WT + FD, and LOX-1 animals (*Figure 5C*). This finding is in agreement with the reduced NO-dependent vascular relaxation in this group of animals. In heart and kidney, we did not detect statistically significant differences in the mRNA and protein expression of the BK_{Ca} channel α- and β1-subunits and eNOS between all groups.

4. Discussion

The main findings of our study show that mice overexpressing the LOX-1 in combination with FD have markedly increased body weight, display impaired NO-mediated, and enhanced EDHF-dependent relaxation in mesenteric arteries, and have increased vascular ROS production in LOX-1 + FD compared with WT + FD animals. Furthermore, BK_{Ca} channel activity in VSMCs was higher in LOX-1 + FD, which could be increased by the EDHFs H_2O_2 and EETs. In contrast, endothelial BK_{Ca} channel activity was unchanged.

4.1 Body weight and plasma lipids

As expected, WT and LOX-1 overexpressing mice gained weight after FD. However, LOX-1 mice on standard diet were also heavier than their corresponding WT animals suggesting an impact of LOX-1 on weight control or metabolic status. Higher body weight in LOX-1 mice was not associated with a significant shift in plasma lipid parameters to a more harmful lipid profile as observed with FD in both WT and LOX-1 mice. In the latter two groups, elevated total plasma cholesterol, LDL, and HDL are in agreement with previous studies.[35,36] Furthermore the LDL/HDL quotient shifted to higher values suggesting an enhanced risk for endothelial dysfunction and atherosclerosis.[37]

4.2 Vascular constriction and relaxation

None of the animals had grossly visible atherosclerotic lesions. Therefore, we tested for vascular dysfunction as an early sign of cardiovascular disease by measuring contraction and endothelium-dependent as well as endothelium-independent relaxation of PE-preconstricted small resistance arterioles. The impact of oxLDL on vascular tone is not fully resolved. OxLDL enhances the basal tone in rabbit cerebral arteries.[38] In contrast, oxLDL has no effect on basal tone in rabbit aorta.[39] We observed no differences in basal vascular tone in the animal groups fed standard or FD. However, the maximum contractile response to PE was lower in LOX-1 + FD than in the other animals, but potencies

were similar. This difference in the vessel contraction between LOX-1 + FD mice and the other three animal groups was not observed in the presence of high, depolarizing potassium concentration or by the combination of PE and paxilline, suggesting a mechanism that involves potassium channel activity. BK_{Ca} channels also participate in ACh-/endothelium-mediated relaxation.[21–23]

ACh-stimulated, NO-mediated dilatation was lower in LOX-1 + FD mice than in WT, WT + FD, and LOX-1 animals, suggesting reduced NO availability in LOX-1 + FD. In accordance basal tone during L-NAME incubation was less increased in LOX-1 + FD mice compared with WT, WT + FD, and LOX-1 animals. Effectivity of L-NAME to eliminate NO production has been questioned because in some species, especially in pig and rabbit, endothelium-dependent/NO-mediated relaxation was resistant to eNOS-inhibitors.[40] In our experiments with mouse mesenteric arteries, however, relaxation persisting after inhibition of eNOS with L-NAME was fully suppressed with apamin and charybdotoxin, the combination of which is commonly used for EDHF block (data not shown). Possible explanations for decreased NO-mediated relaxation in mesenteric arteries of LOX-1 + FD mice are (i) that ROS production is elevated, (ii) that expression of eNOS mRNA is reduced, and (iii) that EDHF can compensate for the loss of NO.[41] In contrast to the reduced NO-mediated relaxation, the EDHF-dependent response was elevated in LOX-1 + FD mice compared with WT, WT + FD, and LOX-1 animals, but could be abolished by a general cytochrome P450 blocker or by an epoxygenase inhibitor. This finding suggests a potential role of cytochrome P450 and its epoxygenases as a compensatory mechanism that makes up for the loss of NO and subsequent endothelial dysfunction in LOX-1 + FD mice. This same amount of EDHF, which was abolished by the cytochrome P450 inhibitors, was also blocked by paxilline in the presence and absence of L-NAME suggesting an essential role of the BK_{Ca} channel in the EDHF-mediated relaxation. Block of cytochrome P450 or BK_{Ca} channels in LOX-1 + FD led to similar levels of persisting EDHF-mediated relaxation in all four groups. The nature of the persisting EDHF-mediated relaxation was not investigated in this study. As reported before, release of EDHF from ECs is associated with the endothelial activity of calcium-activated potassium channels of small and intermediate conductance,[21] but the activation of BK_{Ca} channels is involved in the EDHF-mediated relaxation of VSMCs. No relevant differences in endothelium-independent relaxation were detected, i.e. potency and efficacy of SNP were similar in all groups, suggesting comparable properties of VSMC-relaxation in mice on FD and standard diet. Interestingly, an EETs-mediated relaxation in response to 11,12-EET and 14,15-EET was significantly increased in mesenteric arteries with and without endothelium of LOX-1 + FD mice compared with WT, WT + FD, and LOX-1 animals (see Supplementary material online, *Figure S5*). This could be mediated by the compensatory enhanced activity of the BK_{Ca} channels in VSMCs of LOX-1 + FD mice. However, activation is not sufficient to compensate for the loss of NO in the LOX-1 + FD mice.

4.3 Vascular reactive oxygen species productions

Our results and several previous studies have shown that hypercholesterolaemia is associated with impaired endothelium-dependent relaxation in experimental and clinical studies.[1,42–44] Rapid degradation of endothelium-derived NO by high levels of ROS is thought to be a major mechanism underlying impaired endothelium-dependent vasodilatation under hypercholesterolaemic conditions. It should also be responsible for decreased endothelium-dependent relaxation.[45,46] In our experiments, ROS was indeed increased in LOX-1 + FD and WT + FD mice, and to a larger extent in the former than the latter. ROS leads to oxidation of LDL resulting in increased oxLDL levels. Uptake of oxLDL via LOX-1 into ECs activates NAD(P)H oxidases and enhances production of ROS,[47,48] starting a vicious cycle finally leading to endothelial dysfunction. However, exaggerated ROS production has not only deleterious effects. Growing evidence supports an import role of redox-sensitive signalling in vascular function.[49] Furthermore Shimokawa and Matoba[32] have suggested that the ROS product H_2O_2 could act as EDHF. H_2O_2 amount was higher in LOX-1 + FD mice than in WT, WT + FD, and LOX-1 animals, suggesting a potential role in the elevated EDHF-mediated relaxation.

4.4 Role of endothelium-derived hyperpolarizing factor and BK_{Ca} channel activity

In contrast to reduced NO-mediated responses, EDHF-mediated relaxation was most pronounced in LOX-1 + FD mice suggesting that this mechanism might partly compensate the impaired NO-mediated relaxation. Since different EDHFs, like EETs and H_2O_2 are known to activate BK_{Ca} channels in VSMCs,[21] we studied BK_{Ca} currents directly. At any activating potential, the amplitude of $I_{BK,Ca}$ was indeed larger in VSMCs from LOX-1 + FD than in the other groups. Moreover, $I_{BK,Ca}$ amplitude in LOX-1 + FD mice could not be further increased by the BK_{Ca} channel opener NS1619, suggesting that increased current amplitude resulted from enhanced channel open probability. This interpretation is supported by the fact that mRNA expression of the BK_{Ca} channel α- and β1-subunits was similar in all mice. Because of the limited availability of vascular tissue from the mesenteric arteries, no additional data on protein level could be obtained. However, similar mRNA and protein expression of BK_{Ca} channels have been demonstrated in human arteries and veins.[50] The $I_{BK,Ca}$ amplitudes in ECs were smaller than in VSMCs and did not reveal any differences between the four groups. Therefore BK_{Ca} channels in ECs do not contribute to vascular dysfunction in LOX-1 + FD mice. The activation of endothelial BK_{Ca}-channels contributes to the EDHF-mediated relaxation. Endothelial hyperpolarization can be transmitted directly via gap junctions to the VSMCs.[51,52] In addition, the K^+ outward current through BK_{Ca} channels increases the extracellular potassium concentration.[53] The subsequent activation of the inward-rectifier potassium channel (K_{IR}) and the Na^+/K^+ pump overcomes the minor depolarizing effects linked to the K^+ increase[54,55] and relaxes smooth muscle cells.

The possible EDHF H_2O_2 significantly increased $I_{BK,Ca}$ in VSMCs, although the level did not reach the maximum current amplitude of NS1619 or as in LOX-1 + FD mice. Therefore, H_2O_2 plays only a minor compensatory role in EDHF-mediated relaxation of this model. Recently, an inhibiting effect of H_2O_2 on cytochrome P450 has been

suggested, indicating a negative feedback mechanism of EET production.[56] This could not be confirmed in our experimental model.

Reduced NO levels have been reported to disinhibit cytochrome P450 and hence elevate two other possible EDHF components, 11,12-EET and 14,15-EET.[57–59] These two EETs were able to enhance $I_{BK,Ca}$ to similar maximum current amplitude as observed in the presence of NS1619 and in LOX-1 + FD. In agreement with previous findings in isolated renal arteries of hypercholesterolaemic rabbits,[18,19] we suggest that enhanced formation of EDHF represents a compensatory mechanism of the decreased NO-mediated vessel relaxation. In contrast, Urakami-Harasawa *et al.*[60] found a significant inhibition of endothelium-dependent hyperpolarization in isolated gastroepiploic arteries from atherosclerotic patients. These contradictory results could be explained by the longer duration of hypercholesterolaemic conditions and the progressive development of vascular diseases.

In the last few years, vascular BK_{Ca} channels were considered as potential therapeutic targets in the treatment of hypertension, endothelial dysfunction, and other cardiovascular diseases,[61] because aldosterone overexpression induces a nitric-oxide-independent coronary dysfunction with decreased VSMC BK_{Ca} expression and coronary BK_{Ca}-dependent relaxation.[62] However activation of LOX-1 in clinical manifestation of atherosclerosis could activate VSMC BK_{Ca} channels. In contrast, enhanced LOX-1 expression does not influence endothelial BK_{Ca} channels.

In conclusion, we have consistently detected significant changes in contractile and electrophysiological properties of small resistance vessels only in the combination of LOX-1 overexpression and FD. The endothelium-mediated relaxation via NO release was impaired, but partly compensated by the higher release of EDHF. The consequence of this compensatory mechanism was a higher $I_{BK,Ca}$ due to increased open probability of BK_{Ca} channels in VSMCs, but not in ECs. Our results clearly demonstrate that LOX-1 overexpression and FD cause functional changes in ECs and VSMCs of small resistance arteries leading to vascular dysfunction as an early sign of cardiovascular diseases.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

The authors would like to thank Dr Melinda Wuest for helpful discussion and Claudia Bodenstein for excellent technical assistance.

Conflict of interest: none declared.

Funding

This work was supported by the German Federal Ministry of Education and Research and the Dr. Robert Pfleger foundation.

References

1. Simionescu M. Implications of early structural-functional changes in the endothelium for vascular disease. *Arterioscler Thromb Vasc Biol* 2007; **27**:266–274.
2. Brevetti G, Schiano V, Chiariello M. Endothelial dysfunction: a key to the pathophysiology and natural history of peripheral arterial disease? *Atherosclerosis* 2008;**197**:1–11.
3. Doan AC, Meller N, McNamara CA. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arterioscler Thromb Vasc Biol* 2008;**28**:812–819.
4. Abularrage CJ, Sidawy AN, Aidinian G, Singh N, Weiswasser JM, Arora S. Evaluation of the microcirculation in vascular disease. *J Vasc Surg* 2005;**42**:574–581.
5. Vohra RS, Murphy JE, Walker JH, Ponnambalam S, Homer-Vanniasinkam S. Atherosclerosis and the Lectin-like OXidized low-density lipoprotein scavenger receptor. *Trends Cardiovasc Med* 2006;**16**:60–64.
6. Chen XP, Zhang TT, Du GH. Lectin-like oxidized low-density lipoprotein receptor-1, a new promising target for the therapy of atherosclerosis? *Cardiovasc Drug Rev* 2007;**25**:146–161.
7. Morawietz H. LOX-1 and atherosclerosis: proof of concept in LOX-1-knockout mice. *Circ Res* 2007;**100**:1534–1536.
8. Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y *et al.* An endothelial receptor for oxidized low-density lipoprotein. *Nature* 1997;**386**:73–77.
9. Draude G, Hrboticky N, Lorenz RL. The expression of the lectin-like oxidized low-density lipoprotein receptor (LOX-1) on human vascular smooth muscle cells and monocytes and its down-regulation by lovastatin. *Biochem Pharmacol* 1999;**75**:383–386.
10. Mehta JL, Chen J, Hermonat PL, Romeo F, Novelli G. Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1): a critical player in the development of atherosclerosis and related disorders. *Cardiovasc Res* 2006;**69**: 36–45.
11. Tatsuguchi M, Furutani M, Hinagata J, Tanaka T, Furutani Y, Imamura S *et al.* Oxidized LDL receptor gene (OLR1) is associated with the risk of myocardial infarction. *Biochem Biophys Res Commun* 2003;**303**:247–250.
12. Hattori H, Sonoda A, Sato H, Ito D, Tanahashi N, Murata M *et al.* G501C polymorphism of oxidized LDL receptor gene (OLR1) and ischemic stroke. *Brain Res* 2006;**1121**:246–249.
13. Kataoka H, Kume N, Miyamoto S, Minami M, Moriwaki H, Murase T *et al.* Expression of the lectin like oxidized low-density lipoprotein receptor-1 in human atherosclerotic lesions. *Circulation* 1999;**99**:3110–3117.
14. Inoue K, Arai Y, Kurihara H, Kita T, Sawamura T. Overexpression of lectin-like oxidized low-density lipoprotein receptor-1 induces intramyocardial vasculopathy in apolipoprotein E-null mice. *Circ Res* 2005;**97**:176–184.
15. Kuhlmann CR, Schäfer M, Li F, Sawamura T, Tillmanns H, Waldecker B *et al.* Modulation of endothelial Ca^{2+}-activated K^+ channels by oxidized LDL and its contribution to endothelial proliferation. *Cardiovasc Res* 2003;**60**:626–634.
16. Busse R, Fleming I. Vascular endothelium and blood flow. *Handb Exp Pharmacol* 2006;**176**:43–78.
17. Landmesser U, Drexler H. Endothelial function and hypertension. *Curr Opin Cardiol* 2007;**4**:316–320.
18. Brandes RP, Behra A, Lebherz C, Böger RH, Bode-Böger SM, Phivthong-Ngam L *et al.* N(G)-nitro-L-arginine- indomethacin-resistant endothelium-dependent relaxation in the rabbit renal artery: effect of hypercholesterolemia. *Atherosclerosis* 1997;**135**:49–55.
19. Honda H, Moroe H, Fujii H, Arai K, Notoya Y, Kogo H. Short term hypercholesterolemia alters N(G)-nitro-L-arginine and indomethacin-resistant endothelium relaxation by acetylcholine in rabbit renal artery. *Jpn J Pharmacol* 2001;**85**:203–206.
20. Köhler R, Hoyer J. The endothelium-derived hyperpolarizing factor: insights from genetic animal models. *Kidney Int* 2007;**72**:145–150.
21. Félétou M, Vanhoutte PM. Endothelium-derived hyperpolarizing factor: where are we now? *Arterioscler Thromb Vasc Biol* 2006;**26**:1215–1225.
22. Michaelis UR, Fleming I. From endothelium-derived hyperpolarizing factor (EDHF) to angiogenesis: Epoxyeicosatrienoic acids (EETs) and cell signaling. *Pharmacol Ther* 2006;**111**:584–595.
23. Tanaka Y, Koike K, Toro L. MaxiK channels roles in blood vessel relaxation induced by endothelium-derived relaxing factors and their molecular mechanisms. *J Smooth Muscle Res* 2004;**40**:125–153.
24. Münzel T, Afanas'ev IB, Kleschyov AL, Harrison DG. Detection of superoxide in vascular tissue. *Arterioscler Thromb Vasc Biol* 2002;**22**:1761–1768.
25. Bolann BJ, Ulvik RJ. Release of iron from ferritin by xanthine oxidase. Role of the superoxide radical. *Biochem J* 1987;**243**:55–59.

26. Neidhold S, Eichhorn B, Kasper M, Ravens U, Kaumann AJ. The function of alpha- and beta-adrenoceptors of the saphenous artery in caveolin-1 knockout and wild-type mice. *Br J Pharmacol* 2007;**150**:261–270.
27. Wuest M, Eichhorn B, Braeter M, Strugala G, Michel MC, Ravens U. Muscarinic receptor expression and receptor-mediated detrusor contraction: comparison of juvenile and adult porcine tissue. *Pflugers Arch* 2008; **456**:349–358.
28. Jiang B, Wu L, Wang R. Sulphonylureas induced vasorelaxation of mouse arteries. *Eur J Pharmacol* 2007;**577**:124–128.
29. Matsumoto T, Kakami M, Kobayashi T, Kamata K. Gender differences in vascular reactivity to endothelin-1 (1–31) in mesenteric arteries from diabetic mice. *Peptides* 2008;**29**:1338–1346.
30. Busse R, Edwards G, Félétou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. *Trends Pharmacol Sci* 2002;**23**: 374–380.
31. Campbell WB, Falck JR. Arachidonic acid metabolites as endothelium-derived hyperpolarizing factors. *Hypertension* 2007;**49**:590–596.
32. Shimokawa H, Matoba T. Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. *Pharmacol Res* 2004;**49**:543–549.
33. Li HF, Chen SA, Wu SN. Evidence for the stimulatory effect of resveratrol on Ca^{2+}-activated K^+ current in vascular endothelial cells. *Cardiovasc Res* 2000;**4**:1035–1045.
34. Bentzen BH, Nardi A, Calloe K, Madsen LS, Olesen SP, Grunnet M. The small molecule NS11021 is a potent and specific activator of Ca^{2+}-activated big-conductance K^+channels. *Mol Pharmacol* 2007;**72**: 1033–1044.
35. Libinaki R, Heffernan M, Jiang WJ, Ogru E, Ignjatovic V, Gianello R *et al.* Effects of genetic and diet-induced obesity on lipid metabolism. *IUBMB Life* 1999;**48**:109–113.
36. Gallou-Kabani C, Vigé A, Gross MS, Rabès JP, Boileau C, Larue-Achagiotis C *et al.* C57BL/6J and A/J mice fed a high-fat diet delineate components of metabolic syndrome. *Obesity* 2007;**15**:1996–2005.
37. Cannon CP. High-density lipoprotein cholesterol and residual cardiometabolic risk in metabolic syndrome. *Clin Cornerstone* 2007;**8**:14–12.
38. Xie H, Bevan JA. Oxidized low-density lipoprotein enhances myogenic tone in the rabbit posterior cerebral artery through the release of endothelin-1. *Stroke* 1999;**30**:2423–2429.
39. Galle J, Mameghani A, Bolz SS, Gambaryan S, Görg M, Quaschning T *et al.* Oxidized LDL and its compound lysophosphatidylcholine potentiate AngII-induced vasoconstriction by stimulation of RhoA. *J Am Soc Nephrol* 2003;**14**:1471–1479.
40. Ge ZD, Zhang XH, Fung PC, He GW. Endothelium-dependent hyperpolarization and relaxation resistance to N(G)-nitro-L-arginine and indomethacin in coronary circulation. *Cardiovasc Res* 2000;**46**:547–556.
41. Huang A, Sun D, Smith CJ, Connetta JA, Shesely EG, Koller A *et al.* In eNOS knockout mice skeletal muscle arteriolar dilation to acetylcholine is mediated by EDHF. *Am J Physiol Heart Circ Physiol* 2000;**278**:762–768.
42. Freiman PC, Mitchell GG, Heistad DD, Armstrong ML, Harrison DG. Atherosclerosis impairs endothelium-dependent vascular relaxation to acetylcholine and thrombin in primates. *Circ Res* 1986;**58**:783–789.
43. Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* 1990;**344**:160–162.
44. Golino P, Piscione F, Willerson JT, Cappelli-Bigazzi M, Focaccio A, Villari B *et al.* Divergent effects of serotonin on coronary-artery dimensions and blood flow in patients with coronary atherosclerosis and control patients. *N Engl J Med* 1991;**324**:641–648.
45. Saran M, Michel C, Bors W. Reaction of NO with $O^{2\cdot-}$ implications for the action of endothelium-derived relaxing factor (EDRF). *Free Radic Res Commun* 1990;**10**:221–226.
46. Huie RE, Padmaja S. The reaction of no with superoxide. *Free Radic Res Commun* 1993;**18**:195–199.
47. Rueckschloss U, Galle J, Holtz J, Zerkowski HR, Morawietz H. Induction of NAD(P)H-oxidases by oxidized low-density lipoprotein in human endothelial cells: antioxidative potential of hydroxymethylglutaryl coenzyme A reductase inhibitor therapy. *Circulation* 2001;**104**:1767–1772.
48. Stielow C, Catar RA, Muller G, Wingler K, Scheurer P, Schmidt HH *et al.* Novel Nox inhibitor of oxLDL-induced reactive oxygen species formation in human endothelial cells. *Biochem Biophys Res Commun* 2006;**344**: 200–205.
49. Lehoux S. Redox signalling in vascular responses to shear and stretch. *Cardiovasc Res* 2006;**71**:269–279.
50. Wareing M, Bai X, Seghier F, Turner CM, Greenwood SL, Baker PN *et al.* Expression and function of potassium channels in the human placental vasculature. *Am J Physiol Regul Integr Comp Physiol* 2006;**291**:437–446.
51. Sandow SL, Tare M, Coleman HA, Hill CE, Parkington HC. Involvement of myoendothelial gap junctions in the action of endothelium-derived hyperpolarizing factor. *Circ Res* 2002;**90**:1108–1113.
52. Dora KA, Sandow SL, Gallagher NT, Takano H, Rummery NM, Hill C *et al.* Myoendothelial gap junctions may provide the pathway for EDHF in mouse mesenteric artery. *J Vasc Res* 2003;**40**:480–490.
53. Haddy FJ, Vanhoutte PM, Félétou M. Role of potassium in regulating blood flow and blood pressure. *Am J Physiol* 2005;**290**:R546–R552.
54. Prior HM, Webster N, Quinn K, Beech DJ, Yates MS. K^+-induced dilation of a small renal artery: no role for inward rectifier K^+ channels. *Cardiovasc Res* 1998;**37**:780–790.
55. Nelson MT, Quayle JM. Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol* 1995;**268**:799–822.
56. Larsen BT, Gutterman DD, Sato A, Toyama K, Campbell WB, Zeldin DC *et al.* Hydrogen peroxide inhibits cytochrome P450 epoxygenases: interaction between two endothelium-derived hyperpolarizing factors. *Circ Res* 2008;**102**:59–67.
57. Yang Q, Yima AP, He GW. The significance of endothelium-derived hyperpolarizing factor in the human circulation. *Curr Vasc Pharmacol* 2007;**5**: 85–92.
58. Spector AA, Norris AW. Action of epoxyeicosatrienoic acids on cellular function. *Am J Cell Physiol* 2007;**292**:996–1012.
59. Archer SL, Gragasin FS, Wu X, Wang S, McMurtry S, Kim DH *et al.* Endothelium-derived hyperpolarizing factor in human internal mammary artery is 11,12-epoxyeicosatrienoic acid and causes relaxation by activating smooth muscle BK(Ca) channels. *Circulation* 2003;**107**: 769–776.
60. Urakami-Harasawa L, Shimokawa H, Nakashima M, Egashira K, Takeshita A. Importance of endothelium-derived hyperpolarizing factor in human arteries. *J Clin Invest* 1997;**100**:2793–2799.
61. Eichhorn B, Dobrev D. Vascular large conductance calcium-activated potassium channels: Functional role and therapeutic potential. *Naunyn Schmiedebergs Arch Pharmacol* 2007;**376**:145–155.
62. Ambroisine ML, Favre J, Oliviero P, Rodriguez C, Gao J, Thuillez C *et al.* Aldosterone-induced coronary dysfunction in transgenic mice involves the calcium-activated potassium (BK_{Ca}) channels of vascular smooth muscle cells. *Circulation* 2007;**116**:2435–2443.

Mediation of Electronegative Low-Density Lipoprotein Signaling by LOX-1

A Possible Mechanism of Endothelial Apoptosis

Jonathan Lu, Jun-Hai Yang, Alan R. Burns, Hsin-Hung Chen, Daming Tang, Jeffrey P. Walterscheid, Shinichi Suzuki, Chao-Yuh Yang, Tatsuya Sawamura, Chu-Huang Chen

Abstract—The lectin-like oxidized LDL receptor LOX-1 mediates endothelial cell (EC) uptake of experimentally prepared copper-oxidized LDL (oxLDL). To confirm the atherogenic role of this receptor cloned against copper-oxLDL, we examined whether it mediates EC uptake of L5, an electronegative LDL abundant in dyslipidemic but not normolipidemic human plasma. Hypercholesterolemic (LDL-cholesterol, >160 mg/dL) human LDL was fractionated into L1–L5, increasingly electronegative, by ion-exchange chromatography. In cultured bovine aortic ECs (BAECs), L5 upregulated LOX-1 and induced apoptosis. Transfection of BAECs with LOX-1–specific small interfering RNAs (siLOX-1) minimized baseline LOX-1 production and restrained L5-induced LOX-1 upregulation. Internalization of labeled L1–L5 was monitored in BAECs and human umbilical venous ECs by fluorescence microscopy. LOX-1 knockdown with siLOX-1 impeded the endocytosis of L5 but not L1–L4. In contrast, blocking LDL receptor with RAP (LDL receptor–associated protein) stopped the internalization of L1–L4 but not L5. Although chemically different, L5 and oxLDL competed for EC entry through LOX-1. Via LOX-1, L5 signaling hampered Akt phosphorylation and suppressed EC expression of fibroblast growth factor-2 and Bcl-2. L5 also selectively inhibited Bcl-xL expression and endothelial nitric oxide synthase phosphorylation but increased synthesis of Bax, Bad, and tumor necrosis factor-α. Blocking Akt phosphorylation with wortmannin increased LOX-1 expression, suggesting a modulatory role of Akt in LOX-1 synthesis; L5 upregulated LOX-1 by dephosphorylating Akt. Because endothelial nitric oxide synthase and Bcl-2 activities are Akt-dependent, L5 impairs Akt-mediated growth and survival signals in vascular ECs by way of LOX-1. Thus, the L5/LOX-1 complex may play a critical role in atherogenesis and illuminate important targets for disease intervention. **(*Circ Res*. 2009;104:619-627.)**

Key Words: apoptosis ■ atherosclerosis ■ endothelium ■ lipoproteins ■ receptors

The lectin-like oxidized low-density lipoprotein (LDL) receptor LOX-1 (the type D scavenger receptor) was initially cloned from bovine aortic endothelial cells (BAECs) in 1997 by Sawamura et al via its ability to bind LDL oxidized ex vivo by copper (oxLDL).[1] Today, the atherothrombotic designation of LOX-1 is manifold, as has been reviewed.[2,3] LOX-1 is considered the major receptor for oxLDL in human and various animal vascular endothelial cells (ECs). In human atherosclerotic lesions, it is expressed in intimal smooth muscle cells and lipid-laden macrophages, as well as in plaque neovasculature.[4] LOX-1 plays a role in oxLDL-induced apoptosis of vascular smooth muscle cells and in the production of matrix metalloproteinases; hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins) inhibit its expression in atheromas of Watanabe heritable hyperlipidemic rabbits.[5] In LOX-1 knockout mice, binding of oxLDL to aortic ECs was decreased and endothelium-dependent vasorelaxation was preserved after oxLDL treatment.[6]

The role of LOX-1 as an atherogenesis-related signaling transducer has yet to be confirmed, however, through naturally occurring modified LDL. Identifying such atherogenic particles is a priority in the sustained research initiative against the death and disability of cardiovascular disease. Small, dense LDL and the electronegatively charged subfraction of LDL in human blood are good suspects, and oxidative mechanisms are a key focus as their possible molecular modus operandi. The latter has been of interest since 1988, when Avogaro et al first characterized LDL(−) after its dichotomic separation by anion-exchange chromatography.[7] Whether lipid peroxidation is required for its formation remains in question; several routes independent of oxidation

Original received January 8, 2008; resubmission received October 27, 2008; revised resubmission received December 31, 2008; accepted January 7, 2009.

From the Department of Medicine (J.L., A.R.B., H.-H.C., D.T., J.P.W., C.-Y.Y., C.-H.C.), Baylor College of Medicine, Houston, Tex; Cardiovascular Research Laboratory (J.-H.Y.), School of Medicine, University of California, Los Angeles; College of Optometry (A.R.B.), University of Houston, Tex; Department of Cardiovascular Surgery (S.S.), Yokohama City University Medical Center, Japan; Department of Bioscience (T.S.), National Cardiovascular Center Research Institute, Osaka, Japan; and Department of Medicine (C.-H.C.), China Medical University Hospital, Taichung, Taiwan.

Correspondence to Chu-Huang Chen, MD, PhD, 6565 Fannin St, MS A-601, Houston, TX 77030. E-mail cchen@bcm.tmc.edu

Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.108.190116**

have been proposed.[8–10] Despite its minimal oxidation relative to oxLDL, LDL(−) can induce a spectrum of proinflammatory and cytotoxic responses in cultured vascular cells.[11,12] High concentrations of LDL(−) have been associated with major risk factors for cardiovascular disease, including hypercholesterolemia[13] and type 2 diabetes mellitus.[14]

Apparently more toxic still is L5, which is the most electronegative of the LDL subfractions separable by high-capacity ion-exchange chromatography according to charge that we first described in 2003.[11,15] L5 is present in hypercholesterolemic subjects and those with type 2 diabetes but not in healthy subjects with clinically normal lipid concentrations.[16] Distinct from L1–L4, it has marked potency in inducing EC apoptosis.[11]

In the present study, we extended our work with L5 to determine whether it signals through LOX-1. We examined that mechanism in coordination with the pathway of fibroblast growth factor (FGF)2, a potent antiapoptotic and prosurvival protein that functions by stimulating the phosphatidylinositol 3′-kinase (PI3K)-Akt axis.[17] We previously showed that L5 induces EC apoptosis in part by disrupting FGF2 autoregulation along the FGF2-PI3K-Akt loop.[18] Because FGF2 functions by activating downstream kinases and effectors (including Akt, Bcl-2, Bad, Bax, Bcl-xL, and endothelial nitric oxide synthase [eNOS]), we tested whether L5 dysregulates those FGF2-regulated targets through LOX-1. To better understand the atherogenic processes within hypercholesterolemic patients, we also examined how L5 upregulates LOX-1 to transduce its signaling.

Materials and Methods

Cell Culture

Primary BAECs (Cambrex) were used after 3 or 4 passages and maintained in DMEM (Invitrogen) containing 10% FBS. Primary human umbilical venous ECs (HUVECs), maintained in EGM medium (Cambrex), were cultured from umbilical cords of newborn infants and used after the first passage.[19] The content of FBS was reduced to 5% during LDL treatments. The LOX-1–neutralizing antibody TS20 was provided by T.S. All procedures were approved by the Baylor College of Medicine Institutional Review Board.

LDL Isolation and oxLDL Preparation

To prevent contamination and experimental oxidation, 50 mU/mL aprotinin, 1% ampicillin/streptomycin, and 5 mmol/L EDTA were added to human plasma samples immediately after collection. LDL particles were isolated from hypercholesterolemic (LDL-cholesterol, >160 mg/dL) volunteers by sequential potassium bromide density centrifugation to remove chylomicrons, very-low-density lipoprotein, and intermediate-density lipoprotein fractions, yielding LDL at a final density of 1.019 to 1.063 g/mL.[20] OxLDL was prepared by incubating the dialyzed portion of L5-free LDL, obtained from healthy volunteers, with 5 μmol/L $CuSO_4$ at 37°C for 24 hours.[20]

Ion-Exchange Purification of L5

Whole LDL was equilibrated by dialysis in a column loaded with buffer A, comprising 20 mmol/L Tris HCl, pH 8.0, 0.5 mmol/L EDTA, and 0.01% NaN_3. Approximately 100 mg of LDL material was injected onto a UnoQ12 anion-exchange column (Bio-Rad) by using an LCC-500 programmable fast-protein liquid chromatography pump (Pharmacia) and eluted according to a multistep sodium chloride gradient as previously described.[15]

Apoptosis Measurements

Apoptosis was assessed by the Vybrant Apoptosis Assay kit (Molecular Probes), with visualization by a Zeiss Axiovert 200 fluorescence microscope and filters to capture digitally images based on Hoechst 33342, propidium iodide, and calcein AM staining of nuclear or apoptotic DNA.[11] Cytoplasmic histone-associated DNA fragmentation was examined by using the Cell Death Detection ELISA Assay (Roche) according to the protocol of the manufacturer.

LOX-1 Small Interfering RNA Transfection

Complementary antisense constructs of the bovine LOX-1 receptor were custom designed (Dharmacon) from GenBank accession no. NM_174132. The set, designed to disrupt the lectin-recognition domain of LOX-1, was composed of the following sequences: 5′-GAACCTGAATCTCCAAGAA, to clamp bases 366 to 384; 5′-GAAAGAGGCAGCAAACTAT, to complement bases 390 to 408; 5′-CCAGGTCTCTGATCTCATA, to bind bases 171 to 189; and 5′-AGAAGGAACTCAAAGAAAT, to target bases 284 to 302 of LOX-1 mRNA. For a positive control, silencing efficiency was determined by transfection with siCONTROL cyclophilin B small interfering (si)RNA. For a negative control, primary BAEC cultures were transfected with the siCONTROL nontargeting siRNA pool, which is a mixture of DNA constructs that produce siRNAs but do not bind to cellular mRNA. Primary BAECs were transfected in 6-well plates at 70% confluence. The transfection was executed according to the recommended protocol of 100 nmol/L siRNA mixed with Oligofectamine (Invitrogen). After transfection, the supernatant was replaced with 5% FBS DMEM, and cells were exposed to experimental treatments.

LDL Labeling With 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate and 3,3′-Dioctadecyloxacarbocyanine Perchlorate

Lipophilic dyes 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 3, 3′-dioctadecyloxacarbocyanine perchlorate (DiO) were purchased from Invitrogen. DiI was used to label LDL subfractions. In brief, DiI-LDL subfractions (L1–L5) were freshly prepared before use, as described by Pitas et al[21] L1–L5 were diluted to 1 mg/mL with PBS and incubated with 80 μmol/L DiI at 37°C overnight. The labeled L1–L5 were then purified by ultracentrifugation at a density of 1.063 g/mL and subsequently dialyzed against PBS-EDTA (0.5 mmol/L), with all steps protected from light. DiO was used to label oxLDL with the same procedure.

Fluorescence Monitoring of L1–L5 Internalization

The DiI-LDL subfractions were normalized by protein content and added to primary BAECs at a concentration of 50 μg/mL. Various amounts of DiO-oxLDL were also used for receptor binding analysis. Internalization was observed using a Zeiss Axiovert 200 fluorescence microscope to record the positions of DiI-LDL and DiO-oxLDL with respect to bright field images in overlay. To antagonize LDL binding to LDL receptors, cells were pretreated with a final concentration of 2 μmol/L human LDL receptor (LDLR)-associated protein (RAP) (EMD Biosciences).

Western Blot Analysis

To measure protein levels by Bradford assay, cells were solubilized in NET-N lysis buffer. Electrophoresis was conducted with 10% SDS-PAGE by using 10 μg of each cell lysate.[20] The separated proteins were transferred to nitrocellulose paper (Bio-Rad) and blocked with SuperBlock (Pierce). Monoclonal mouse anti–LOX-1 (provided by T.S.), polyclonal rabbit anti-FGF2, monoclonal mouse anti-Bcl-2, polyclonal goat anti–tumor necrosis factor (TNF)-α (R&D systems), polyclonal rabbit anti–phospho-Akt (Ser473), polyclonal rabbit anti-Akt, monoclonal rabbit anti-p-eNOS, monoclonal

rabbit anti-eNOS, monoclonal mouse anti-Bax, polyclonal rabbit anti-Bad and anti-Bcl-xL (Cell Signaling Technology), and monoclonal mouse anti–β-actin (Sigma-Aldrich) antibodies were used to probe membranes to evaluate relative quantities of specific proteins before and after treatments. Secondary anti-rabbit, -goat, and -mouse IgG antibodies were conjugated to horseradish peroxidase (Amersham Biosciences). Signals were recorded on Kodak films by using ECL Plus chemiluminescent reagents (Amersham Biosciences). The results were normalized to those of β-actin.

Semiquantitative RT-PCR

The mRNA-silencing efficiency of transfected siRNA was evaluated by semiquantitative RT-PCR (Invitrogen SuperScript III One-Step RT-PCR kit). Total RNA was isolated from BAECs by using the RNeasy Midi Kit (Qiagen). Bovine LOX-1 primers (forward: 5′-GTACCCTGCTGCTGTGA; reverse: 5′-TTGACAA CCCCATCCAGA) and bovine cyclophilin B primers (forward: 5′-GTACTTTGACCTGCGAA; reverse: 5′-GCCCTATTCCTTGGCAA) were designed to amplify the respective regions.

Statistical Analysis

The significance of differences was assessed by a paired Students *t* test with Bonferroni correction. Probability values of $P<0.05$ were considered significant. Results are expressed as mean values±SEM.

Results

L5 Isolation and Activity

Consistent with our previous report,[11] L5 was present in hypercholesterolemic (Figure 1A) but not normocholesterolemic (Figure 1B) human plasma. In BAECs incubated with 50 μg/mL each LDL preparation for 24 hours, neither LDL from healthy donors nor L1 from hypercholesterolemic subjects showed detectable apoptotic activity, whereas BAEC apoptosis was substantially greater after exposure to L5 (Figure 1A and 1C). The apoptotic activity of L5 was quenched, in a concentration-dependent manner, by the addition of 10 μg/mL TS20, a LOX-1–neutralizing antibody (Figure 1C).

Subfractionated LDL Internalization With Regard to LDL Receptor and LOX-1

We typically evaluate apoptotic activity in ECs after an overnight incubation with LDL subfractions. However, internalization of the LDL fractions should be an early event in the apoptosis cascade.[22] Therefore, we monitored intracellular fluorescence by deconvolution microscopy from the outset of treating HUVECs with 50 μg/mL DiI-L1 or DiI-L5. At 30 minutes, both DiI-L1 and DiI-L5 were visible intracellularly. Pretreatment with TS20 to neutralize LOX-1 activity blocked L5 endocytosis but had no effect on L1 (Figure 2A).

To examine how the chromatographically separated LDL particles were internalized with regard to LDLR, we used recombinant human RAP to selectively antagonize LDLR activity.[23] DiI-labeled L1 and L5 were applied to BAECs, and the cells were photographed after overnight incubation. In the negative control (−RAP), cells internalized L1 and L5 equally well. In contrast, RAP pretreatment (+RAP) obstructed the internalization of L1–L4 but did not prevent internalization of L5 (Figure 2B; L2–L4 not shown). Although L5 fits within the physical characteristics of LDL,[16] these results show that it is not a ligand for LDLR.

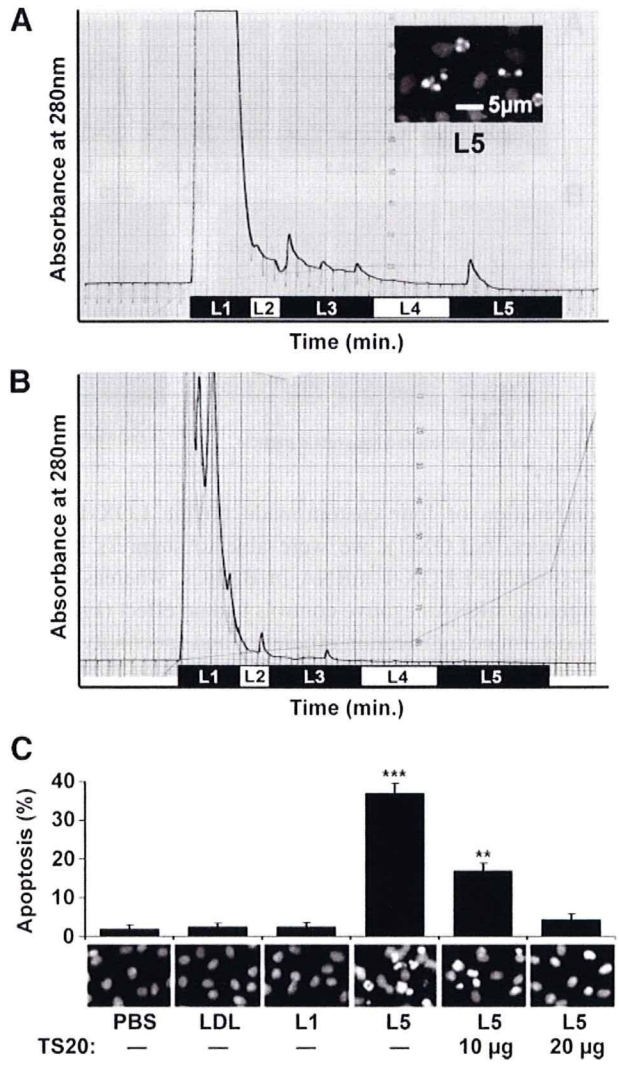

Figure 1. Presence of electronegative LDL subfractions in human hypercholesterolemic plasma and apoptotic activity of L5. A and B, Representative fast-protein liquid chromatography chromatograms of hypercholesterolemic LDL, yielding L5 (A), and normocholesterolemic LDL, where L5 is absent (B). C, BAECs exhibiting condensed, fragmented nuclei by epifluorescence microscopy were considered to be apoptotic. Percentage of cells undergoing apoptosis was evaluated in 6 samples. $^{**}P<0.01$, $^{***}P<0.001$ vs PBS.

DiO-oxLDL was used as a positive control and a competitive ligand of L5 to LOX-1. To avoid massive apoptosis, BAECs were treated with 25 μg/mL of either DiI-L5 or DiO-oxLDL in initial experiments. At 24 hours, both could be easily detected inside their respective cells (Figure 2C). In cells loaded with 25 μg/mL DiI-L5, addition of DiO-oxLDL exhibited a progressive increase of colocalization of both particles in a concentration-dependent manner. When DiO-oxLDL was increased to 25 μg/mL, the combined toxicity of L5 and oxLDL resulted in marked apoptosis. Because oxLDL is also internalized by LOX-1,[1] the results suggest that L5 and oxLDL compete for EC entry through this particular receptor (Figure 2C).

Characterization of LOX-1–Specific siRNAs

Before using siRNA in experiments to support our hypothesis, we tested its activity in our model system. Using a

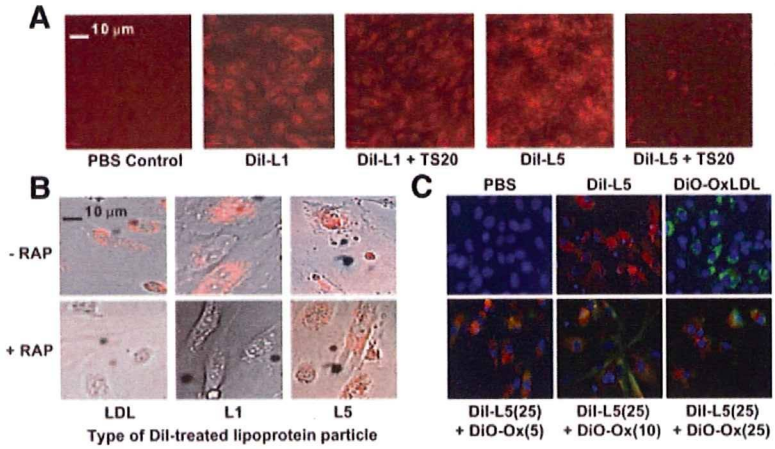

Figure 2. Internalization of L5 and oxLDL by LOX-1 and endocytosis of L1 by LDLR. A, Internalization of DiI-L1 and DiI-L5 was viewed in HUVECs with and without TS20 neutralization of LOX-1. B, Subconfluent BAECs were incubated with 50 μg/mL DiI-labeled L1 and L5, and particle internalization was monitored after 24 hours by fluorescence microscopy in cells with and without RAP peptide antagonism of LDLR. C, Internalization of DiI-L5 and DiO-oxLDL, alone (25 μg/mL) or in combinations at concentrations indicated, was monitored in BAECs at 24 hours.

combination of four custom-made bovine LOX-1–specific constructs (siLOX-1), we were able to suppress basal and L5-stimulated LOX-1 mRNA in BAECs, whereas the nontargeting, siRNA negative controls had no effect (Figure 3A). To determine transfection efficiency and specificity, we used cyclophilin B siRNAs and RT-PCR to measure cyclophilin B in BAECs. Two siRNA controls, without specific mRNA targets, were used to observe the effects of siRNA production. With the RNA-induced silencing complex (RISC)-free and nontargeting template sets, there was some inhibition of cyclophilin B mRNA, likely attributable to the transfection process itself. However, the cyclophilin B–positive control potently deleted the mRNA to a level below the detection by RT-PCR (Figure 3B). The effectiveness of siRNA treatment was confirmed by Western blot analysis, which showed a significant reduction of LOX-1 expression compared with control (Figure 3C).

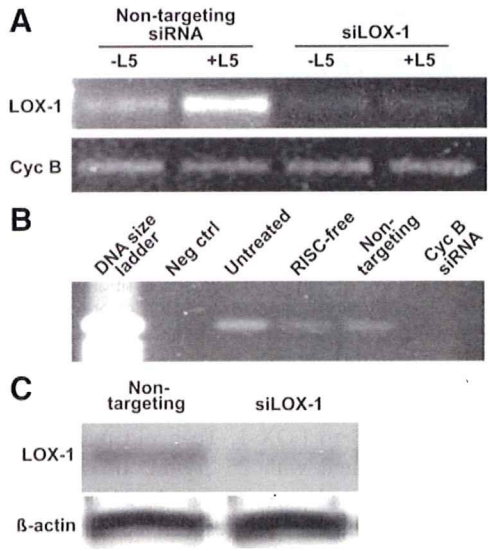

Figure 3. Characterization of LOX-1 siRNA silencing. A, Results of RT-PCR showing relative levels of LOX-1 mRNA in BAECs treated with L5 and siLOX-1. Cyclophilin B (Cyc B) was amplified as an internal control. B, RT-PCR controls using cyclophilin B to determine transfection efficiency and specificity. The negative control is a reaction without the addition of a template. The untreated lane represents cells without transfection or empty liposomal treatment. RISC-free corresponds to cells treated with constructs that prevent RISC assembly. The nontargeting lane is a set of specific siRNAs developed not to bind to any product of the human genome. The cyclophilin B control is a set of siRNAs that target cyclophilin B mRNA. C, Western blot of LOX-1 protein before and after siLOX-1 treatment. As an internal control, β-actin was referenced for protein normalization.

Subfractionated LDL Internalization With Regard to LOX-1

In experiments parallel to our studies with LDLR, we incubated BAECs with 50 μg/mL DiI-labeled L1–L5 and used siLOX-1 to specifically block its cellular expression. All the LDL subfractions were equally internalized among cells transfected with a pool of nontargeting siRNAs (Figure 4, top row). In LOX-1–silenced BAECs, all but DiI-L5 were internalized (Figure 4, bottom row). These findings indicate that L5 internalization requires LOX-1 expression, whereas the less electronegative LDL subfractions rely on the LDL receptor.

Comparison of oxLDL and L5 Activity in Modulating LOX-1 and FGF2

Much of the present literature on LDL oxidation is based on results that use the experimentally prepared oxLDL for eliciting cellular responses.[24] Copper-oxLDL is well characterized, and more easily obtained than pathologically derived L5, so we used it as a positive control for our assays. We treated BAECs with 50 μg/mL L5 or oxLDL and determined the amount of LOX-1 protein by Western blot. Both L5 and oxLDL enhanced LOX-1 expression to a similar extent (Figure 5A). BAECs transfected with siLOX-1 maintained a significantly reduced baseline LOX-1 and easily resisted the stimulatory effects of L5 and oxLDL.

Because L5 causes apoptosis, we hypothesized that LOX-1 activation inhibits the expression of FGF2, a crucial factor for EC growth and survival. We measured FGF2 protein in L5- or oxLDL-treated BAECs with or without TS20 inactivation of LOX-1. L5 and oxLDL were equally effective in reducing FGF2 protein production, yet this activity was prevented by TS20 (Figure 5B). Therefore, LOX-1 is an inducible receptor for oxLDL and L5, which downregulates FGF2 expression when activated.

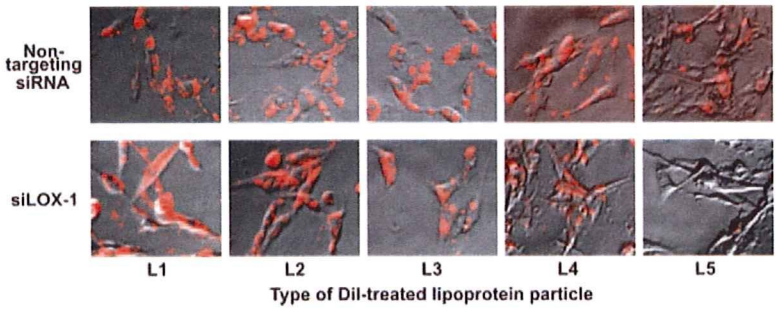

Figure 4. LDL subfractional binding preferences for LOX-1. BAECs transfected with nontargeting or specific LOX-1 siRNAs were incubated with charge-separated, DiI-labeled LDL fractions. Internalization was monitored by fluorescence microscopy.

Role of LOX-1 in L5 Signaling and Mechanism of L5-Induced LOX-1 Expression

The signals directed by FGF2 work through the PI3K/Akt pathway, ultimately to raise Bcl-2 levels and discourage apoptosis while promoting EC growth.[17] Because of the importance of Akt and Bcl-2 in maintaining EC health, we examined at which points L5 and LOX-1 disrupt this survival loop. BAECs were treated with 50 μg/mL L5 for 24 hours, and the lysates were assayed for FGF2, phosphorylated Akt, and Bcl-2 by Western blot analysis. Cells treated with L5 displayed a significant reduction of FGF2 protein, whereas cells transfected with siLOX-1 were free of such L5 effects (Figure 6A). Cells treated with 20 ng/mL FGF2 showed a significant increase of phosphorylated Akt relative to total Akt protein, whereas cells treated with L5 were unable to maintain a basal level (Figure 6B). However, treatment with siLOX-1 prevented a further reduction in phosphorylated Akt when challenged with L5. In the final step, L5 restrained Bcl-2 expression, but the addition of FGF2 stimulated Bcl-2 expression above basal levels (Figure 6C). Cells transfected with siLOX-1 maintained a steady amount of Bcl-2, even when stimulated with L5.

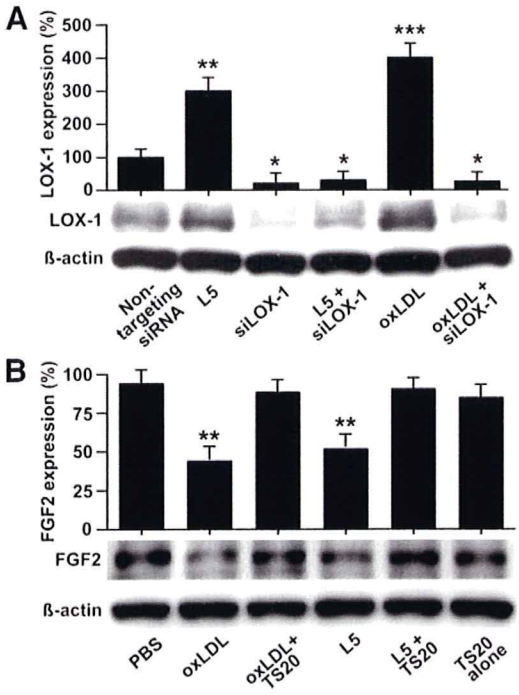

Figure 5. Comparison of oxLDL and L5 in modulation of LOX-1 and FGF2. A, Western blot of BAECs treated with 50 μg/mL L5 or oxLDL, in combinations with siLOX-1 knockdown. The bar graph shows LOX-1 intensity normalized to β-actin. B, Western blot of BAECs treated with 50 μg/mL L5 or oxLDL, in combinations with TS20. The bar graph relates FGF2 intensity to β-actin. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs nontargeting control or IgG control (n=3).

L5 signaling was accompanied by augmented LOX-1 production. Because LOX-1 was upregulated during Akt dephosphorylation in L5-exposed cells (Figure 5), we tested whether baseline LOX-1 expression is negatively modulated by Akt. The findings that hindering Akt activation with PI3K inhibitor wortmannin increased LOX-1 expression in a concentration-dependent manner verified the hypothesis (Figure 6D). Thus, by dephosphorylating Akt (Figure 6D), L5 activated LOX-1 expression that is normally suppressed by Akt.

To further examine the role of LOX-1 in L5 signaling, we performed additional siRNA transfection studies. In nontransfected BAECs, treatment with 50 μg/mL L5 for 24 hours resulted in apoptotic nuclear changes as expected, whereas cells that had been transfected with siLOX-1 were highly resistant to the toxic effects of L5 (Figure 7A and 7B). Transfection with siLOX-1 or nontargeting siRNA alone did not affect cell survival. The apoptotic effect of L5 was accomplished by its ability to selectively downregulate prosurvival proteins (Bcl-2, Bcl-xL, eNOS, p-eNOS) (Figures 6C and 7C) while concomitantly upregulating proapoptotic factors (Bax, Bad, TNF-α) (Figure 7C).

Discussion

LOX-1 is highly expressed in vivo in large human arteries, particularly at the predilection sites of atherosclerosis.[1,25] One of the prototypical endothelial scavenger receptors that bind to negatively charged molecules, LOX-1 has emerged as the principal receptor that mediates oxLDL uptake in the vascular wall in both mammals and humans.[1,25,26] Thus, it may be the most important scavenger receptor in the development of atherothrombotic disease, given that high susceptibility to lipid peroxidation is believed to be a key characteristic of atherogenic LDL.[24] In LOX-1 studies, the high conservation among species of its lectin-like domain at the long, extracellular C terminus[1] facilitates the use of human oxLDL in animal EC models.[27] Our present studies demonstrated that LOX-1 also mediates the actions of pathophysiologically derived human

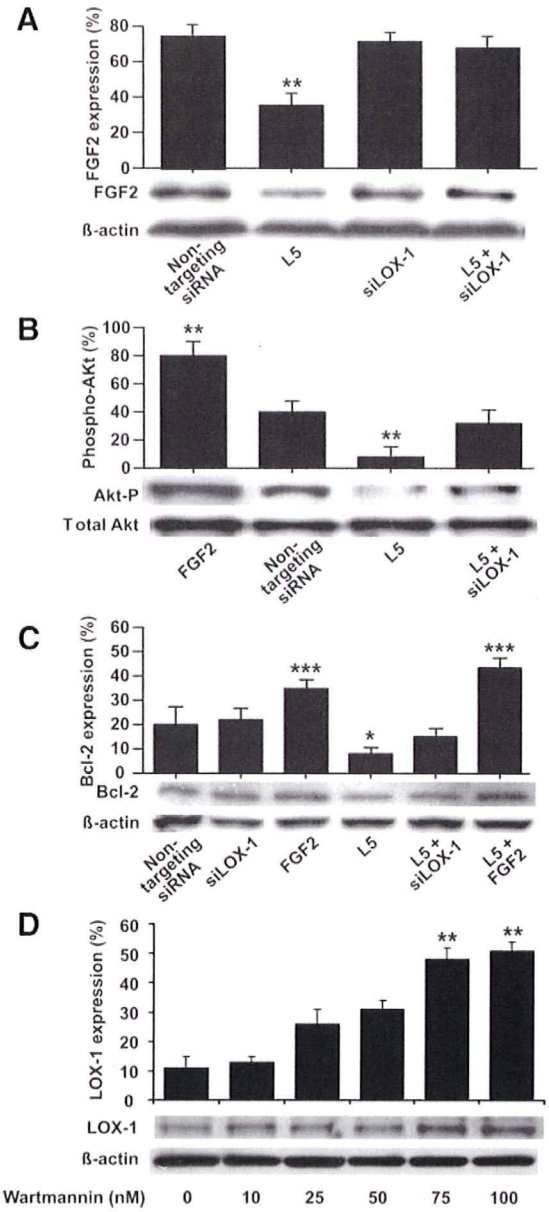

Figure 6. Role of LOX-1 in L5 signaling and mechanism of L5-induced LOX-1 expression. A, FGF2 protein concentrations were assayed by Western blot analysis 24 hours after BAECs were treated with 50 μg/mL L5, with or without siLOX-1 blockade. B, The degree of Akt phosphorylation was measured by normalization to total Akt in BAECs treated with 20 ng/mL FGF2 or 50 μg/mL L5, with or without siLOX-1 transfection. C, Bcl-2 protein levels were assayed by Western blot analysis and normalized to β-actin. BAECs had been treated with 20 ng/mL FGF2, 50 μg/mL L5, or a combination of L5 and FGF2, with or without siLOX-1 transfection. D, LOX-1 expression in BAECs was analyzed by Western blot, in the absence or presence of the PI3K inhibitor wortmannin in graded concentrations. *$P<0.05$, **$P<0.01$ vs nontargeting siRNA control; ***$P<0.001$ vs L5 alone (n=3).

electronegative LDL, perhaps a native atherogenic lipoprotein species.

Our experiments were performed using 5 LDL subfractions, L1 to L5. L5, the most electronegative subfraction, accounts for a smaller proportion (1% to 3%)[11,15] of LDL than does the dichotomically separated LDL(−).[7,9] L5 decreases intracellular FGF2 production in vascular ECs, thus contributing to apoptosis and impaired angiogenic capacity.[11] In addition, it induces monocyte–EC adhesion,[15] a step that precedes the subendothelial deposition of inflammatory cells and modified lipids in atherogenesis. Unlike its normal LDL counterpart, LDL(−) also induces a spectrum of atherogenic responses and production inflammatory chemokines from ECs.[28–31]

In this study, LOX-1 protein levels were increased by 3-fold when the cells were exposed to L5. Exposing ECs to oxLDL yielded a similar effect. Baseline protein expression of LOX-1 was greatly suppressed in BAECs transfected with siLOX-1. Receptor knockdown also completely prevented its upregulation by L5 or oxLDL. A growing body of studies shows that a variety of agents, including oxLDL, TNF-α, angiotensin II, and endothelin 1, affect LOX-1 at both the mRNA and protein levels.[32–34]

DiI-labeled LDL subfractions L1–L5 could be visualized inside normal BAECs after 24 hours of incubation. Labeling with DiI, a lipophilic fluorescence probe with excitation and emission wavelengths of 520 and 578 nm,[35] preserves the functional properties of LDL, including its affinity to LDLR and subsequent interactions with cells.[36] That LOX-1 knockdown prevented the internalization of L5 exclusively indicates that L5 was the only subfraction endocytosed by LOX-1, whereas the other subfractions were internalized by other receptors. Neutralizing LOX-1 with TS20 was equally effective in blocking L5 entry into the cells. L4 entry was minimally obstructed in LOX-1–silenced cells, suggesting that a small portion of L4 particles may exhibit L5-like properties, as explained above.

RAP, known for its potent antagonistic effect against members of the LDLR family,[37] blocked the entry of L1–L3 into BAECs but had no effect on L5 internalization. Again, there was a partial effect on L4. In combination, these findings indicate that L5 is an extreme and pure form of LDL(−) that is endocytosed specifically by LOX-1, not by the normal LDL receptor. The more electropositive subfractions L1 to L3 are internalized by LDLR but not LOX-1. Most L4 particles enter the ECs through LDLR, with a small portion entering the cells through LOX-1 owing to possible amalgamation.

Because our original observation was made at 24 hours after the cells had been treated with L5, the rest of our assays were completed at 24 hours for consistency and comparability. However, the initial internalization of these particles may occur much sooner. In a series of experiments using HUVECs, we found that the uptake of L1 and L5 may be complete as early as 30 minutes. Neutralizing LOX-1 with TS20 inhibited the entry of L5 but not L1. This further confirms the mediator role of LOX-1 in L5 internalization in both human and bovine ECs.

LOX-1 mediates oxLDL-induced EC apoptosis.[38] Here, neutralizing LOX-1 with TS20 attenuated the apoptotic effect of L5. The role of LOX-1 was confirmed by gene silencing of this receptor by a LOX-1–specific siRNA cocktail. The efficiency of siRNA transfection can be influenced by factors including siRNA design, transfection reagents, and RISC

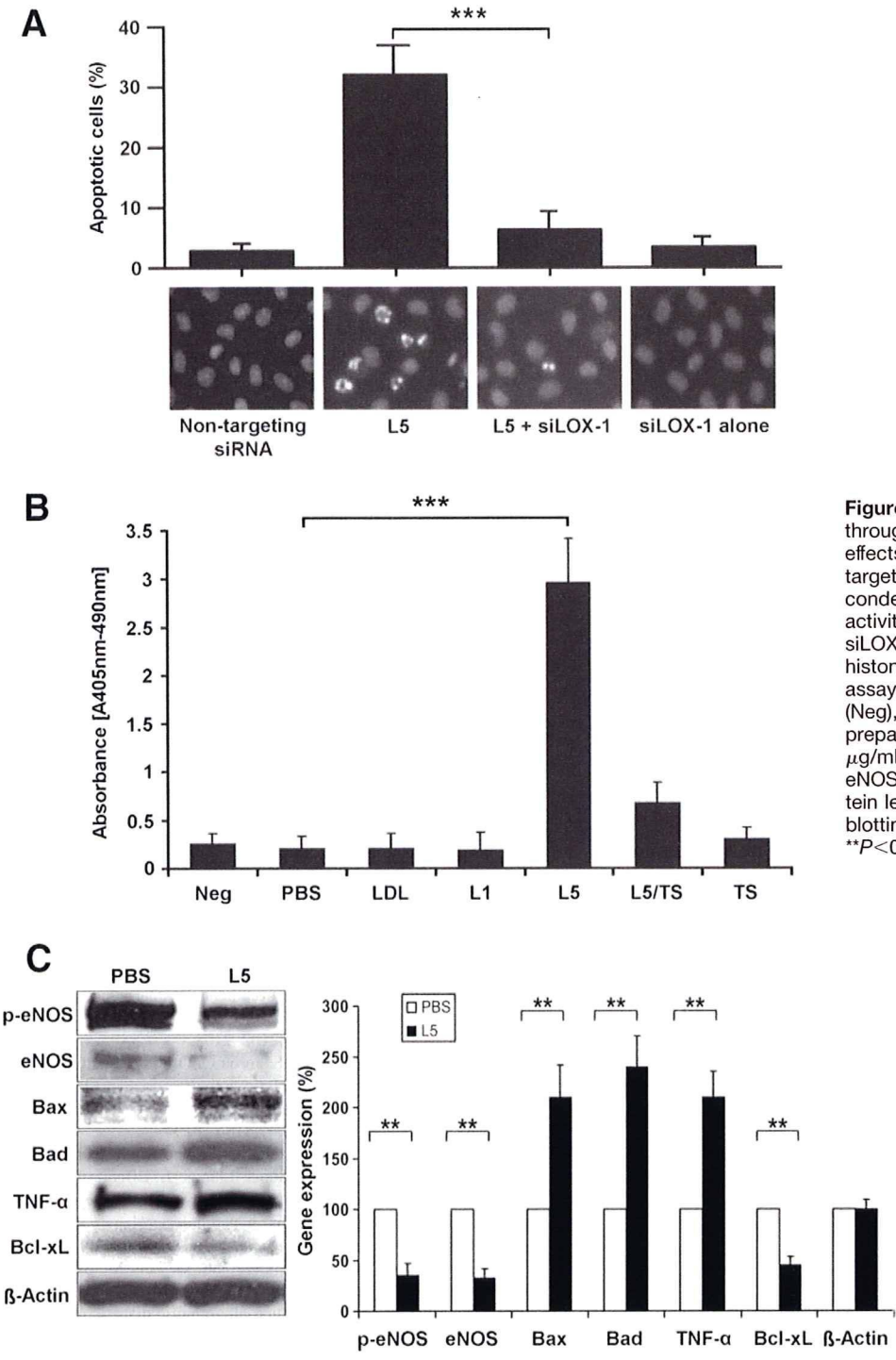

Figure 7. L5 induces EC apoptosis through LOX-1 and exerts opposite effects on prosurvival vs proapoptotic targets. A, Fluorescence microscopy of condensed nuclear staining shows the activity of L5 in BAECs with and without siLOX-1 transfection. B, Cytoplasmic histone-associated DNA fragmentation assayed in BAECs treated with nothing (Neg), PBS, LDL (50 μg/mL for all LDL preparation), L1, L5, L5 with TS20 (10 μg/mL), or TS20 alone. C, p-eNOS, eNOS, Bax, Bad, TNF-α, and Bcl-xL protein levels were evaluated by Western blotting and normalized to β-actin. $^{**}P<0.01$, $^{***}P<0.001$ (n=3).

formation.[39] The positive control cyclophilin B siRNA demonstrated near-complete silencing, and the designed LOX-1 siRNA demonstrated 80% silencing efficiency, indicating a sufficient delivery of siRNA. To test whether L5 and oxLDL compete for the same receptor, we challenged BAECs with DiI-L5 and DiO-oxLDL. The results indicated that L5 and oxLDL are both internalized by LOX-1 in a competitive manner.

In agreement with our previous findings,[11,20] both L5 and oxLDL reduced the protein level of prosurvival FGF2×40% to 50%. Because of the mediator role of LOX-1 in L5-induced EC apoptosis, it is not surprising to find that L5-induced FGF2 downregulation could be prevented by blockade or knockdown of this receptor. One of the early effectors of FGF2 is Akt, whose activation through phosphorylation by PI3K signaling is stimulated by exogenous or autocrine production of FGF2.[40] Opposite to its stimulatory effect on FGF2 transcription through the FGF2-PI3K-Akt autoregulatory loop,[18] Akt was found to retain LOX-1 expression to a minimum at the baseline. By means of dephosphorylating Akt, L5 lifts this suppression and augments LOX-1 production.

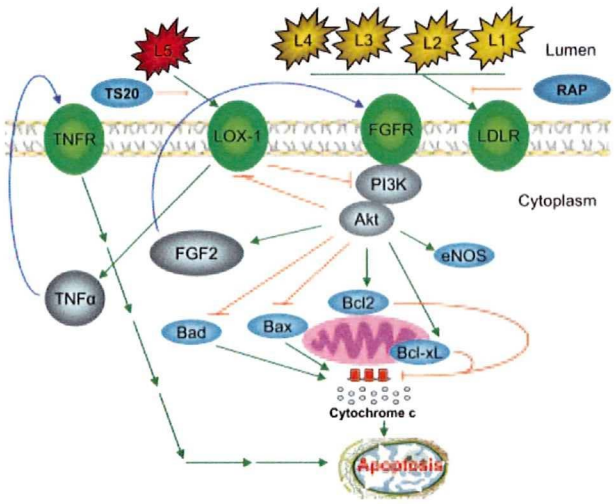

Figure 8. Schematic summary of L5 signaling through LOX-1 in vascular endothelial cells. L5 signals and is internalized through LOX-1, whereas L1–L4 are endocytosed via LDLR. Green arrows indicate stimulation; blue arrows indicate direction of transportation; red arrowheads indicate release from mitochondria; red lines with end bar indicate inhibition. RAP, LDLR inhibitor; TS20, LOX-1–neutralizing antibody.

The antiapoptotic protein Bcl-2 represents a relatively downstream effector of FGF2.[11] The opposing stimulatory effects of FGF2 and inhibitory effects of L5 on Akt phosphorylation and Bcl-2 expression support the hypothesis that L5 induces EC apoptosis by inhibiting FGF2-dependent survival mechanisms. The preservation of Akt phosphorylation and Bcl-2 expression in LOX-1 knockdown cells indicates that L5 interrupts the normal FGF2–Akt–Bcl-2 signaling by means of activating LOX-1. Additionally, L5 suppressed Bcl-xL, eNOS, and p-eNOS while upregulating Bax, Bad, and TNF-α. Taken together, by way of LOX-1, L5 selectively suppresses prosurvival mechanisms but concomitantly activates proapoptotic effectors to force the cells into apoptosis (Figure 8).

Ectopic expression of LOX-1 facilitates the removal of plasma oxLDL and attenuates atherosclerosis development in apolipoprotein E–deficient mice.[41] Our finding that the naturally occurring L5 signals through LOX-1 suggests a significant role of the L5-LOX-1 axis in atherogenesis.

Acknowledgments

We are grateful to Suzanne Simpson for editorial assistance.

Sources of Funding

This work was supported by American Diabetes Association research grants 9630095N (to C.-H.C.), 1-04-RA-13 (to C.-H.C.), and 7-03-RA-108 (to C.-Y.Y.); an Atorvastatin Research Award (to C.-H.C.); a grant from the Philip Morris External Research Program (to C.-H.C.); an Independent Medical Grant from Pfizer Pharmaceuticals (to C.-H.C.); a Research Fund from Asia Chemical Corporation (Houston, Tex) (to C.-H.C.); National Eye Institute grant EY017120 (to A.R.B.); NIH grants HL63364 (to C.-Y.Y.) and a fellowship from the American Heart Association Texas Affiliate (to J.P.W.). D.T. is supported by NIH training grant T32, HL07812-10 (to Dr. Joel D. Morrisett, Baylor College of Medicine). NIH training grant T32 (to D.T.); and a fellowship from the American Heart Association Texas Affiliate (to J.P.W.).

Disclosures

None.

References

1. Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, Tanaka T, Miwa S, Katsura Y, Kita T, Masaki T. An endothelial receptor for oxidized low-density lipoprotein. *Nature*. 1997;386:73–77.
2. Mehta JL, Chen J, Hermonat PL, Romeo F, Novelli G. Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1): a critical player in the development of atherosclerosis and related disorders. *Cardiovasc Res*. 2006;69:36–45.
3. Chen XP, Du GH. Lectin-like oxidized low-density lipoprotein receptor-1: protein, ligands, expression and pathophysiological significance. *Chin Med J (Engl)*. 2007;120:421–426.
4. Kataoka H, Kume N, Miyamoto S, Minami M, Moriwaki H, Murase T, Sawamura T, Masaki T, Hashimoto N, Kita T. Expression of lectinlike oxidized low-density lipoprotein receptor-1 in human atherosclerotic lesions. *Circulation*. 1999;99:3110–3117.
5. Hofnagel O, Luechtenborg B, Eschert H, Weissen-Plenz G, Severs NJ, Robenek H. Pravastatin inhibits expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in Watanabe heritable hyperlipidemic rabbits: a new pleiotropic effect of statins. *Arterioscler Thromb Vasc Biol*. 2006;26:604–610.
6. Mehta JL, Sanada N, Hu CP, Chen J, Dandapat A, Sugawara F, Satoh H, Inoue K, Kawase Y, Jishage K, Suzuki H, Takeya M, Schnackenberg L, Beger R, Hermonat PL, Thomas M, Sawamura T. Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet. *Circ Res*. 2007;100:1634–1642.
7. Avogaro P, Bon GB, Cazzolato G. Presence of a modified low density lipoprotein in humans. *Arteriosclerosis*. 1988;8:79–87.
8. Sanchez-Quesada JL, Perez A, Caixas A, Rigla M, Payes A, Benitez S, Ordonez-Llanos J. Effect of glycemic optimization on electronegative low-density lipoprotein in diabetes: relation to nonenzymatic glycosylation and oxidative modification. *J Clin Endocrinol Metab*. 2001;86:3243–3249.
9. Sanchez-Quesada JL, Benitez S, Ordonez-Llanos J. Electronegative low-density lipoprotein. *Curr Opin Lipidol*. 2004;15:329–335.
10. Asatryan L, Hamilton RT, Isas JM, Hwang J, Kayed R, Sevanian A. LDL phospholipid hydrolysis produces modified electronegative particles with an unfolded apoB-100 protein. *J Lipid Res*. 2005;46:115–122.
11. Chen CH, Jiang T, Yang JH, Jiang W, Lu J, Marathe GK, Pownall HJ, Ballantyne CM, McIntyre TM, Henry PD, Yang CY. Low-density lipoprotein in hypercholesterolemic human plasma induces vascular endothelial cell apoptosis by inhibiting fibroblast growth factor 2 transcription. *Circulation*. 2003;107:2102–2108.
12. Bancells C, Benitez S, Villegas S, Jorba O, Ordonez-Llanos J, Sanchez-Quesada JL. Novel phospholipolytic activities associated with electronegative low-density lipoprotein are involved in increased self-aggregation. *Biochemistry*. 2008;47:8186–8194.
13. Sanchez-Quesada JL, Otal-Entraigas C, Franco M, Jorba O, Gonzalez-Sastre F, Blanco-Vaca F, Ordonez-Llanos J. Effect of simvastatin treatment on the electronegative low-density lipoprotein present in patients with heterozygous familial hypercholesterolemia. *Am J Cardiol*. 1999;84:655–659.
14. Moro E, Zambon C, Pianetti S, Cazzolato G, Pais M, Bittolo Bon G. Electronegative low density lipoprotein subform (LDL-) is increased in type 2 (non-insulin-dependent) microalbuminuric diabetic patients and is closely associated with LDL susceptibility to oxidation. *Acta Diabetol*. 1998;35:161–164.
15. Yang CY, Raya JL, Chen HH, Chen CH, Abe Y, Pownall HJ, Taylor AA, Smith CV. Isolation, characterization, and functional assessment of oxidatively modified subfractions of circulating low-density lipoproteins. *Arterioscler Thromb Vasc Biol*. 2003;23:1083–1090.
16. Yang CY, Chen HH, Huang MT, Raya JL, Yang JH, Chen CH, Gaubatz JW, Pownall HJ, Taylor AA, Ballantyne CM, Jenniskens FA, Smith CV. Pro-apoptotic low-density lipoprotein subfractions in type II diabetes. *Atherosclerosis*. 2007;193:283–291.
17. Chen CH. Platelet-activating factor acetylhydrolase: is it good or bad for you? *Curr Opin Lipidol*. 2004;15:337–341.
18. Lu J, Jiang W, Yang JH, Chang PY, Walterscheid JP, Chen HH, Marcelli M, Tang D, Lee YT, Liao WS, Yang CY, Chen CH. Electronegative LDL impairs vascular endothelial cell integrity in diabetes by disrupting fibroblast growth factor 2 (FGF2) autoregulation. *Diabetes*. 2008;57:158–166.

19. Frankel DJ, Pfeiffer JR, Surviladze Z, Johnson AE, Oliver JM, Wilson BS, Burns AR. Revealing the topography of cellular membrane domains by combined atomic force microscopy/fluorescence imaging. *Biophys J.* 2006;90:2404–2413.
20. Chen CH, Jiang W, Via DP, Luo S, Li TR, Lee YT, Henry PD. Oxidized low-density lipoproteins inhibit endothelial cell proliferation by suppressing basic fibroblast growth factor expression. *Circulation.* 2000;101:171–177.
21. Pitas RE, Innerarity TL, Weinstein JN, Mahley RW. Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. *Arteriosclerosis.* 1981;1:177–185.
22. Zeng Y, Tao N, Chung KN, Heuser JE, Lublin DM. Endocytosis of oxidized low density lipoprotein through scavenger receptor CD36 utilizes a lipid raft pathway that does not require caveolin-1. *J Biol Chem.* 2003;278:45931–45936.
23. Bu G, Marzolo MP. Role of rap in the biogenesis of lipoprotein receptors. *Trends Cardiovasc Med.* 2000;10:148–155.
24. Witztum JL, Steinberg D. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? *Trends Cardiovasc Med.* 2001;11:93–102.
25. Shi X, Niimi S, Ohtani T, Machida S. Characterization of residues and sequences of the carbohydrate recognition domain required for cell surface localization and ligand binding of human lectin-like oxidized LDL receptor. *J Cell Sci.* 2001;114:1273–1282.
26. Chen M, Inoue K, Narumiya S, Masaki T, Sawamura T. Requirements of basic amino acid residues within the lectin-like domain of LOX-1 for the binding of oxidized low-density lipoprotein. *FEBS Lett.* 2001;499:215–219.
27. Chen M, Narumiya S, Masaki T, Sawamura T. Conserved C-terminal residues within the lectin-like domain of LOX-1 are essential for oxidized low-density-lipoprotein binding. *Biochem J.* 2001;355:289–296.
28. Benitez S, Camacho M, Bancells C, Vila L, Sanchez-Quesada JL, Ordonez-Llanos J. Wide proinflammatory effect of electronegative low-density lipoprotein on human endothelial cells assayed by a protein array. *Biochim Biophys Acta.* 2006;1761:1014–1021.
29. Benitez S, Perez A, Sanchez-Quesada JL, Wagner AM, Rigla M, Arcelus R, Jorba O, Ordonez-Llanos J. Electronegative low-density lipoprotein subfraction from type 2 diabetic subjects is proatherogenic and unrelated to glycemic control. *Diabetes Metab Res Rev.* 2007;23:26–34.
30. Gambino R, Pisu E, Pagano G, Cassader M. Low-density lipoproteins are more electronegatively charged in type 1 than in type 2 diabetes mellitus. *Lipids.* 2006;41:529–533.
31. Ziouzenkova O, Asatryan L, Sahady D, Orasanu G, Perrey S, Cutak B, Hassell T, Akiyama TE, Berger JP, Sevanian A, Plutzky J. Dual roles for lipolysis and oxidation in peroxisome proliferation-activator receptor responses to electronegative low density lipoprotein. *J Biol Chem.* 2003;278:39874–39881.
32. Aoyama T, Fujiwara H, Masaki T, Sawamura T. Induction of lectin-like oxidized LDL receptor by oxidized LDL and lysophosphatidylcholine in cultured endothelial cells. *J Mol Cell Cardiol.* 1999;31:2101–2114.
33. Chen M, Masaki T, Sawamura T. LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. *Pharmacol Ther.* 2002;95:89–100.
34. Moriwaki H, Kume N, Kataoka H, Murase T, Nishi E, Sawamura T, Masaki T, Kita T. Expression of lectin-like oxidized low density lipoprotein receptor-1 in human and murine macrophages: upregulated expression by TNF-alpha. *FEBS Lett.* 1998;440:29–32.
35. Reynolds GD, St Clair RW. A comparative microscopic and biochemical study of the uptake of fluorescent and 125I-labeled lipoproteins by skin fibroblasts, smooth muscle cells, and peritoneal macrophages in culture. *Am J Pathol.* 1985;121:200–211.
36. Stephan ZF, Yurachek EC. Rapid fluorometric assay of LDL receptor activity by DiI-labeled LDL. *J Lipid Res.* 1993;34:325–330.
37. Li Y, Lu W, Schwartz AL, Bu G. Receptor-associated protein facilitates proper folding and maturation of the low-density lipoprotein receptor and its class 2 mutants. *Biochemistry.* 2002;41:4921–4928.
38. Li D, Mehta JL. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. *Arterioscler Thromb Vasc Biol.* 2000;20:1116–1122.
39. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 2001;411:494–498.
40. Chen CH, Poucher SM, Lu J, Henry PD. Fibroblast growth factor 2: from laboratory evidence to clinical application. *Curr Vasc Pharmacol.* 2004;2:33–43.
41. Ishigaki Y, Katagiri H, Gao J, Yamada T, Imai J, Uno K, Hasegawa Y, Kaneko K, Ogihara T, Ishihara H, Sato Y, Takikawa K, Nishimichi N, Matsuda H, Sawamura T, Oka Y. Impact of plasma oxidized low-density lipoprotein removal on atherosclerosis. *Circulation.* 2008;118:75–83.