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OxLDL-Dependent Activation of Arginase II Is Dependent on the LOX-1 Receptor and Downstream RhoA Signaling

Sungwoo Ryoo^{1,4}, Anil Bhunia¹, Fumin Chang¹, Artin Shoukas², Dan E Berkowitz^{1,2,*}, and Lewis H Romer^{1,2,3,*}

¹Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins Medical Institutions, Baltimore, MD

²Department of Biomedical Engineering, The Johns Hopkins Medical Institutions, Baltimore, MD

³Department of Pediatrics and Cell Biology, The Johns Hopkins Medical Institutions, Baltimore, MD

⁴Department of Biology Program, Kangwon National University, Chuncheon, Korea

Abstract

Aims—Arginase II regulates NOS activity by competing for the substrate L-arginine. Oxidized LDL (OxLDL) is a proatherogenic molecule that activates arginase II. We tested the hypotheses that OxLDL-dependent arginase II activation occurs through a specific receptor, and via a Rho GTPase effector mechanism that is inhibited by statins.

Methods and Results—Arginase II activation by OxLDL was attenuated following preincubation with the LOX-1 receptor-blocking antibody JTX92. This also prevented the dissociation of arginase II from microtubules. LOX-1^{-/-} mice failed to exhibit the increased arginase II activity seen in WT mice fed a high cholesterol diet. Furthermore, endothelium from LOX^{-/-} mice failed to demonstrate the diet-dependent reduction in NO and increase in ROS that were observed in WT mice. OxLDL induced Rho translocation to the membrane and Rho activation, and these effects were inhibited by pretreatment with JTX92 or statins. Transfection with siRNA for RhoA, or inhibition of ROCK both decreased OxLDL-stimulated arginase II activation. Preincubation with simvastatin or lovastatin blocked OxLDL-induced dissociation of arginase II from microtubules and prevented microtubule depolymerization.

Conclusions—This study provides a new focus for preventive therapy for atherosclerotic disease by delineating a clearer path from OxLDL through the endothelial cell LOX-1 receptor, RhoA, and ROCK, to the activation of arginase II, downregulation of NO, and vascular dysfunction.

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Correspondence: Lew Romer, M.D., Blalock 904 - Pediatric ACCM, Johns Hopkins University School of Medicine / 600 North Wolfe Street, Baltimore, MD 21287-4904, Tel: 410-955-7610, Fax: 410-502-5312, LRomer@jhmi.edu.

*Denotes equal contribution

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Disclosures

Dan E. Berkowitz is a scientific founder and consultant for Arginex Inc., a biotechnology company dedicated to the development of therapeutics targeting arginase in diseases in which endothelial dysfunction is an important contributing factor.

Keywords

atherosclerosis; OxLDL; LOX-1; endothelium; arginase; nitric oxide; Rho

1. Introduction

It is now well established that endothelial arginase constrains the activity of endothelial nitric oxide synthase (eNOS) by substrate depletion, thereby reducing nitric oxide (NO) bioavailability and contributing to vascular diseases including hypertension, aging, and atherosclerosis¹⁻⁵.

We recently demonstrated that Oxidized LDL (OxLDL), the primary pathogenic lipid in atherogenesis, activates endothelial cell arginase II⁶. This consequently leads to a decrease in endothelial NO production. Furthermore, atherogenic-prone apolipoprotein E-null (ApoE^{-/-}) mice treated with arginase inhibitor exhibited restored NO bioavailability, reactive oxygen species (ROS) production, endothelial function, and arterial stiffness to the WT phenotype⁵.

While there is evidence that Rho and Rho-Kinase (ROCK) might be involved in the activation of arginase⁴, the signal transduction pathways leading to this increase in activity remain poorly understood. The lectin-like OxLDL scavenger receptor has been shown to be critical in the OxLDL-dependent activation of arginase II in endothelial cells (EC)⁷ and the downregulation of endothelial cell NO production. This has been elegantly shown using LOX-1^{-/-} mice, in which a high cholesterol diet (HC) had attenuated impact on endothelial dysfunction⁸, inflammatory response⁹ and NO production¹⁰.

We tested the hypothesis that arginase activation is dependent on the LOX-1 receptor and that the downstream signaling linking the engagement of this receptor to up regulation of arginase II activity in vascular endothelial cells involves RhoA. We demonstrate that endothelial arginase II activation by OxLDL is dependent upon the LOX-1 receptor and downstream Rho/ROCK signaling, and that statins inhibit this process. These findings may, in part, explain a new mechanism for the pleiotropic effects of statins with regard to NO signaling and endothelial protection.

2. METHODS

2.1 Cell Culture

Human aortic endothelial cells (HAEC) were purchased from Cascade Biologics (Portland, OR) and maintained in Medium M200 containing low serum growth supplement according to the supplier's protocol. Confluent HAEC were incubated with starvation medium (M200 plus only 0.5 % fetal bovine serum) for 24 hours prior to OxLDL treatment.

2.2 Chemicals and Reagents

JTX92 antibody and LOX-1^{-/-} mice were obtained from Dr. Tatsuya Sawamura (National Cardiovascular Center Research Institute, Osaka, Japan). OxLDL, prepared by reaction with CuSO₄, was purchased from Intracel Co (Frederick, MD). Statins were purchased from Calbiochem (Darmstadt, Germany). All other chemicals were obtained from Sigma unless otherwise stated.

2.3 Arginase Activity Measurement

Arginase activity was measured by determination of urea content using α -isonitrosopropiophenone as described previously^{6, 11}. Briefly, supernatants of extracted cell lysates were prepared by incubation with lysis buffer (50 mM Tris-HCl, pH7.5, 0.1 mM EDTA and protease inhibitors) for 30 min at 4°C and centrifugation for 20 minutes at $14,000 \times g$ at 4°C. Serum-starved HAEC were treated with statins (10 μ M, 30 minutes) before OxLDL stimulation. Assays on aortic vessel samples were performed following homogenization in lysis buffer.

2.4 Tubulin Depolymerization Assay

Tubulin depolymerization was evaluated by a simple method, as described previously by Giannakakou et al^{6, 12}. In short, cells were washed twice with PBS and lysed at 37°C for 5 minutes in the dark with 150 μ l of hypotonic buffer (20 mM Tris-HCl, pH 6.8, 1mM MgCl₂, 2mM EGTA, 0.5% NP-40, and 2mM PMSF). Lysis was followed by centrifugation at $14,000 \times g$ for 10 minutes at room temperature. Supernatants containing soluble (cytosolic) tubulin were transferred to fresh tubes. Pellets containing cytoskeletal (polymerized) tubulin were resuspended in 150 μ l of hypotonic buffer and centrifugation was repeated as above. The cytosolic and the cytoskeletal fractions were analyzed using arginase activity assays and by Western blotting for tubulin and arginase II content.

2.5 Measurement of nitric oxide and ROS with DAF and DHE

All experimental procedures using mice were approved by the Institutional Animal Care and Use Committee at The Johns Hopkins University School of Medicine. 10-week-old male LOX-1^{-/-} or wild type C57Bl/6 mice were fed normal (ND) or high cholesterol (HC) diet (1.25% cholesterol, 0% cholate; Research Diets) for 6–8 weeks. Aortic rings were prepared for use in biochemical assays for arginase activity or for labeling with a fluorescent-probe to superoxide (DHE, 1 μ mol/L, 5 minutes) and NO(DAF, 5 μ mol/L, 5 minutes). Images were acquired using a Nikon TE-200 epifluorescence microscope. In some cases, aortas were incubated with OxLDL for 30 minutes after loading the probes. Fluorescence intensity was measured as previously described^{13, 14}.

2.6 Western Blot Analysis

Cells were lysed in SDS sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 10% Glycerol) and then sonicated for 5 seconds to reduce sample viscosity. After protein content was quantified using the Pierce Coomassie protein reagent (Fisher), each sample was resolved by 10% SDS-PAGE, transferred to PVDF membrane (Bio-Rad), analyzed with antibodies according to the supplier's protocol, and visualized with peroxidase and an enhanced-chemiluminescence system (Pierce). Normalization was performed using anti- β -tubulin antibody (BD bioscience, 1:1,000). Densitometry analysis of bands was performed with NIH ImageJ¹¹.

2.7 Rho Activity Assay

Briefly, the cDNA of the RhoA-binding domain (RBD) from human Rhotekin (amino acids 7–89) (gift from Keith Burridge, University of North Carolina) was expressed in *E. coli* as a glutathione S-transferase fusion protein, purified, and immobilized on glutathione-sepharose beads as previously described¹⁵. Protein lysates were obtained with lysis buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10 μ g/ml each of aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 200 μ M vanadate, and cleared at $15,000 \times g$ for 5 minutes. Approximately 800 μ g of proteins were rotated for 30 minutes with 50 μ g of GST-RBD. Samples were then washed three times with buffer containing 50 mM Tris, pH7.4, 10 mM MgCl₂, 150 mM

NaCl, 1% Triton X-100 and protease inhibitors, and then immunoblotted with RhoA monoclonal antibody (Santa Cruz Biotechnology, Inc.).

2.8 Membrane Fractionation

Treated cells were homogenized in Tris buffer (25 mM, pH 7.4, with protease inhibitors (Roche Co.), 250 mM NaCl, 0.1% β -mercaptoethanol, and 3 mM EDTA) and centrifuged at $1,000 \times g$ for 10 minutes to remove cell debris and unbroken cells. The supernatants were subjected to centrifugation ($21,000 \times g$, 45 minutes, 4°C) to separate membrane and cytosolic fractions. The cytosolic and membrane fractions containing 20 μg protein were used for Western blot analysis for RhoA.

2.9 Transient transfection of siRNA

Transient transfection of RhoA-siRNA (Santa Cruz Biotechnology) was performed with oligofectamine reagent according to instructions provided by the supplier (Invitrogen). In brief, 8 μL of oligofectamine was added to 17 μL Opti-MEM reduced serum medium (Gibco), incubated for 5 minutes at room temperature, mixed with 180 μL Opti-MEM medium containing siRNA, and further incubated for 15 minutes. The siRNA-oligofectamine complex was then overlaid on cells (each well, 1 mL serum-free media, in a 6 well culture dish). After incubation for 6 hours, the serum concentration in the endothelial growth medium was returned to a 1X serum concentration by adding 3X media of 0.5 mL per well. Transfected cells were then cultured for 36 hours and then serum-starved for 24 hours prior to stimulation with OxLDL.

2.10 Immunofluorescence

Following plating and treatment HAEC were fixed and permeabilized with 3% paraformaldehyde and 0.5% Triton X-100 in PBS for 2 minutes, followed by 20 minutes of 3% paraformaldehyde alone. Samples were prepared for immunofluorescence analysis by incubating with rabbit polyclonal antisera against arginase II (Santa Cruz Biotechnology, 1:50) and a mouse monoclonal antibody against β -tubulin (BD Biosciences, 1:50) for 30 minutes at 37°C . They were then rinsed in tris-buffered saline and incubated with Cy5-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG (Chemicon, Temecula, CA). Images were acquired using a Nikon TE-200 epifluorescence microscope (with a $60\times$ objective), and collected using Openlab software (Improvision, Lexington, MA) and an internally cooled 12-bit CCD camera (CoolsnapHQ, Photometrics, Tucson, AZ).

2.11 Statistics

Each graph represents cumulative data from between 3–5 independent experiments, and each individual assay was performed in triplicate. Statistical significance was determined by one way ANOVA (mean $\pm$ SEM) with a post-hoc test or t-test (mean $\pm$ SEM) (Graphpad Prism 4 software).

3. RESULTS

3.1 OxLDL affects arginase II activation and interaction with microtubules through the LOX-1 receptor

OxLDL stimulation of HAEC induced an increase in arginase II activity from 179.7 ± 35.8 pmol urea/mg protein/min in untreated controls to 328 ± 50.7 (Figure 1A). This increased arginase activity was blocked by preincubation for 1 hour with monoclonal antibody JTX-92 (10 $\mu\text{g}/\text{mL}$) against the lectin-like OxLDL receptor (LOX-1). We previously demonstrated that arginase activation by OxLDL is associated with a movement of arginase from the detergent insoluble fraction to the soluble fraction, and microtubule depolymerization⁶. The

JTX92 antibody attenuated the dissociation of arginase from the cytoskeleton and into the soluble fraction, and nearly obviated OxLDL-induced tubulin depolymerization (Figure 1B).

3.2 EC from LOX-1^{-/-} mice do not show an increase in arginase II activity after a high cholesterol diet, and exhibit attenuated changes in NO and superoxide production after OxLDL

We next tested whether the absence of LOX-1 receptors prevented HC diet induced increases in vascular arginase activity (Figure 2A). Both WT and LOX-1^{-/-} mice were fed a HC diet for 6 weeks. Aortas were excised and arginase activity measured. As seen in Figure 2A, a HC diet in WT mice induced a marked increase in vascular arginase activity from 216.2±16 pmol urea/mg protein/min to 483.6±51. This was significantly reduced in the presence of the arginase inhibitor 2(S)-amino-6-boronohexonic acid (ABH) (10μmol/L). On the other hand, arginase activity in the LOX-1^{-/-} mice was not altered by the HC diet and inhibition with ABH had no effect.

We have previously demonstrated that arginase inhibition in ApoE^{-/-} mice prevented OxLDL-dependent eNOS uncoupling and increased ROS production⁵. Therefore, we next tested the role of the LOX-1 receptor on NO production and ROS generation in aorta of HC fed mice. We used en-face, endothelial side up aortic strips loaded with fluorescent indicators (Figures 2B and 2C). Vascular endothelium from WT mice fed the HC diet demonstrated a 33% reduction in NO production, while no change was seen with HC diet in the EC from LOX-1^{-/-} mice. Similarly, vascular endothelium from HC-fed WT mice produced ~25% more ROS. This augmented ROS activity was absent in the strips from LOX-1^{-/-} mice. Treatment with ABH (10 μmol/L, 10 minutes) showed the additional effect of direct arginase inhibition (Figure 2A) on NO production (Figure 2B) and ROS generation (Figure 2C), respectively. These data indicate that arginase-mediated eNOS uncoupling and ROS accumulation are induced by a HC diet and mediated by the LOX-1 receptor (Figure 2D).

To further confirm that the endothelium was the source of the ROS, DHE fluorescence was measured in both intact (E+) and de-endothelialized (E-) aortic strips from WT and LOX-1^{-/-} mice. In the presence of OxLDL, E+ rings generated a significant increase in DHE fluorescence which was reduced to baseline when the endothelium was removed. As predicted, presence or absence of the endothelium had no effect on ROS production in LOX-1^{-/-} mice. Pre-incubation with L-NAME (100μmol/L) prevented OxLDL- stimulated ROS production in WT E+ samples, indicating that eNOS was required for this ROS generation (Figure 2E). Additionally, data from experiments using apocyanin indicated that NADPH oxidase may also contribute to ROS production in EC following OxLDL exposure (data not shown).

3.3 OxLDL induces RhoA activation and translocation to the membrane in HAEC

Cytoskeletal reorganization in vascular cells is regulated in large measure by changes in the activation of Rho GTPase, which associates with the cell membrane and its substrates upon activation¹⁶⁻¹⁸. This led us to examine RhoA activation in response to OxLDL using a Rhotekin pull-down assay (Figure 3). Exposure to OxLDL-induced RhoA activation as early as 1 minute, while activity gradually increased through 30 minutes. Positive and negative controls were cell lysates incubated with GTPγS and GDP, respectively. Cytosolic and membrane fractions of cell homogenates were then studied to determine the extent to which activated RhoA associated with the plasma membrane following exposure of HAEC to OxLDL. We documented that RhoA activation by OxLDL resulted in translocation to the EC plasma membrane within 30 minutes, and that preincubation with JTX92 (for 1 hour)

blocked this redistribution. These findings are consistent with the conclusion that OxLDL effects on RhoA are mediated through the LOX-1 receptor.

3.4 OxLDL stimulation of arginase II activity is RhoA-dependent

RhoA-specific siRNA was then used to interrogate the role of RhoA in OxLDL-induced arginase activation. siRNA transfection successfully reduced the RhoA expression in HAEC to less than 50% of control (Figure 4A). Although the siRNA alone did not change baseline arginase activity, knockdown of RhoA expression markedly attenuated the augmentation of arginase activity in HAEC that were exposed to OxLDL to less than 10% of the amount that was seen in untransfected controls (Figure 4B). Since Rho kinase (ROCK) is a major Rho effector in vascular wall cells¹⁸, we next examined the effects of the ROCK inhibitor Y27632 in this system. Pretreatment of HAEC with this inhibitor (10 μ M, 1 hour) ablated the induction of arginase activity by OxLDL (Figure 4C). Additionally, ROCK inhibition blocked Ox-LDL-mediated ROS production (Figure 4D). These findings implicate ROCK in the downstream signaling that mediates OxLDL-induced changes in arginase activity.

3.5 Statins block OxLDL-induced dissociation of arginase from microtubules

Statins have been shown to have beneficial effects on vascular endothelium in studies of a variety of pathophysiological conditions, and some of these effects are due to the inhibition of RhoA^{19, 20}. We therefore tested the effects of statins on OxLDL-stimulated arginase activation in EC. Pre-incubation of HAEC with either lovastatin or simvastatin (both used at 10 μ M for 30 minutes) resulted in nearly complete inhibition of arginase activation in OxLDL-stimulated HAEC (Figure 5A). Furthermore, simvastatin preincubation blocked the tubulin depolymerization, the arginase II movement from the insoluble cytoskeletal fraction to the soluble fraction, and the co-linearity of arginase II with β -tubulin on immunofluorescence analysis that all appear to be integral parts⁶ of OxLDL-induced arginase II activation in HAEC (Figures 5B and 5C). Lovastatin also blocked arginase activation and the depression of NOx in ApoE^{-/-} mice that were fed a HC diet (Figures 5D and 5E).

4. Discussion

4.1 LOX receptor and downstream signaling

Our previous reports have defined an intriguing and novel focus for targeted therapeutic drug design for atherosclerotic vascular disease – endothelial arginase II, the most abundant arginase in HAEC^{5, 6, 21}. We have demonstrated that OxLDL stimulation activates arginase II in human aortic endothelial cells through the dissociation of arginase from the microtubule cytoskeleton, that ApoE^{-/-} mice develop elevated arginase activity on a high cholesterol diet, and that arginase inhibitors decrease atherogenesis. A detailed understanding of the molecular mechanisms that transduce atherogenic stimuli into arginase activation is now required in order to optimize the translational benefits of these insights. In this current report we define a signaling cascade that begins with either the direct exposure of HAEC to OxLDL or with murine ingestion of a high cholesterol diet. We demonstrate that both of these atherogenic stimuli initiate critical cellular events through the LOX-1 receptor on the aortic endothelium. Downstream transduction of these pathogenic inputs triggers the activation of RhoA and ROCK with consequences that include the activation and subcellular redistribution of arginase II, changes in the microtubule cytoskeleton, reduced NO production, and the generation of injurious ROS.

4.2 LOX-1 receptor and endothelial dysfunction

Our data indicate that the LOX-1 receptor is the predominant transmembrane signal transducer in OxLDL-induced arginase activation in HAEC, and that blocking of LOX-1 receptor with JTX92 inhibits tubulin depolymerization induced by OxLDL. Sawamura and coworkers demonstrated that this lectin-like OxLDL receptor - a 50 kDa, type II transmembrane glycoprotein in the class E scavenger receptor subgroup - is a critical mediator of atherogenesis^{22–24}. In ECs, LOX-1 mediates the negative effect of OxLDL on the Akt-protein kinase B-eNOS axis by inhibiting PI 3kinase-mediated phosphorylation of Akt²⁵. The resulting decreased availability of NO is the central mechanism underlying endothelial dysfunction in atherosclerosis. LOX-1 also provides an avenue for increased superoxide production in EC. This occurs through OxLDL induced and PKC- α -mediated changes in eNOS function^{25, 26}. Additionally, LOX-1 provides a specific docking location for monocytes on the HAEC surface, with resulting increases in membrane-associated p47^{phox}, production of ROS, and phosphorylation of NF- κ B in the HAEC²⁷. Our data yield further insight into the molecular basis of the results of OxLDL binding to LOX-1.

4.3 Arginase II and vascular disease

Arginase II competitively inhibits NOS via use of the common substrate L-Arginine^{2, 11, 28–31}. The activation and expression of endothelial arginase II can be induced by a variety of vascular insults including OxLDL, LPS, TNF- α , IFN- γ , 8-bromo-cGMP, and hypoxia^{6, 31–34}. Arginase inhibition actively augments NO production, and has had reportedly beneficial effects on cardiac and vascular wall function in atherogenesis and aging^{2, 8, 11, 35, 36}. The current work establishes links between arginase II activation and additional dysregulation mechanisms in EC from a common receptor through downstream signaling intermediates, and invites a new understanding of potential therapeutic approaches.

4.4 Statin has pleiotrophic beneficial effects on the cardiovascular system

Several studies have implicated the statins as positive modulators of vascular function through salutary effects on endothelium, including decreased LOX-1 receptor expression, and increased expression of the eNOS protein via enhanced stability of eNOS mRNA by 3' polyadenylation^{37–39 40 41}. An additional specific molecular mechanism underlying the favorable impact of statins on EC and upon vascular tone is the prevention of the translocation of RhoA to the plasma membrane where it interacts with downstream effectors. This effect is mediated by the decrease in geranylgeranyl pyrophosphate production by the inhibitory effect of statins on HMG-CoA reductase, and the subsequent limitation of RhoA isoprenylation at the carboxy terminus^{16, 42}. By way of this negative modulation of RhoA activation and effector stimulation, statins may improve the stability of mRNA for eNOS¹⁹. Insights from the current work elucidate additional downstream molecular consequences of statin therapy via arginase inhibition. Focus on arginase inhibition in future therapeutic drug design could obviate some of the myopathic consequences of HMG-CoA inhibition with statins⁴³.

5. Conclusions

This study delineates a clearer path from atherogenic stimuli in the endothelial environment to the activation of arginase II and EC dysfunction. OxLDL induces dysfunction in vascular endothelia that requires signaling via the LOX-1 receptor. Necessary downstream signaling from this receptor includes the activation of RhoA. RhoA and ROCK are involved in the subsequent activation of endothelial arginase II. Arginase II activation then leads to the downregulation of NO and the release of ROS. These insights provide new impetus for the development of safe and effective preventive therapies for atherosclerotic disease.

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Figure 1. Arginase II activation and dissociation from microtubules following OxLDL treatment are LOX-1-dependent

A. The OxLDL (50 $\mu\text{g/mL}$)-induced increase in arginase enzyme activity (*untreated vs. OxLDL, $p < 0.05$; $n = 4$ independent experiments) was inhibited by preincubation (10 $\mu\text{g/mL}$) with LOX-1 blocking antibody, JTX92 (OxLDL vs. OxLDL + anti-LOX-1, # $p < 0.05$; $n = 4$ independent experiments) (means $\pm$ SEM are shown).

B. OxLDL-induced arginase II dissociation from the cytoskeleton into the soluble fraction was blocked by JTX92 (*untreated vs. OxLDL, $p < 0.01$, # OxLDL vs. OxLDL + anti-LOX-1, $p < 0.01$, $n = 9$). Densitometry data (means $\pm$ SEM) are shown in the panel below the western blot.

Figure 2. Arginase activation, and the production of both NOx and ROS are altered in LOX-1^{-/-} mice

A. A high cholesterol diet (HC) increased arginase activity in the aorta of WT mice (* vs. WT + normal diet (ND), $p < 0.05$), but in not in LOX-1^{-/-} mice (# vs. WT+ND, NS). The increase noted in WT EC was blocked by ABH. ($n = 5$ mice for each experimental group) (means $\pm$ SEM are shown for all panels).

B. NO production in aorta was reduced in WT mice, but not LOX-1^{-/-} mice, that were fed a HC diet (* WT+ND vs. WT+HC, $p < 0.05$)(** LOX-1^{-/-}+ND vs. LOX-1^{-/-}+HC, NS). The arginase inhibitor ABH increased aortic NO production in mice fed HC from both genotypes

(# WT+HC vs. WT+HC+ABH, $p<0.05$, ## LOX-1^{-/-}+ND vs. LOX-1^{-/-}+HC+ABH, $p<0.05$) (n = 5 mice for each experimental group).

C. HC diet increased the rate of ROS production in aortas from WT (* WT+ND vs. WT+HC, $p<0.05$) but not LOX-1^{-/-} mice (**LOX-1^{-/-}+ND vs. LOX-1^{-/-}+HC, NS). Arginase inhibition with ABH decreased the rate of ROS production in both WT and LOX-1^{-/-} (# WT+HC vs. WT+HC+ABH, $p<0.05$; ## LOX-1^{-/-}+HC vs. LOX-1^{-/-}+HC+ABH, $p<0.05$) (n = 5 mice for each experimental group).

D. ROS production was increased in intact aortas (E+) of WT mice pre-incubated with Ox-LDL (** vs WT E⁺ control, $p<0.001$) (n = 5) but not in those of LOX-1^{-/-} mice. ROS production was completely abolished in endothelium denuded aortas (E-) (n = 5 mice for each experimental group) (# vs WT E⁺ + Ox-LDL), $p<0.001$).

E. The Ox-LDL-mediated increase in aortic ROS production was abrogated by L-NAME in WT mice (** vs control, $p<0.01$ and ## vs Ox-LDL, $p<0.01$) .

B.

Figure 3. OxLDL stimulation activated Rho A

A. OxLDL stimulation rapidly increased RhoA activity, and the increase was sustained for 30 minutes (* vs 0 min, $p < 0.05$ by ANOVA; $n = 4$). Densitometry analysis of the data is shown below the Western blot (means $\pm$ SEM). Cell lysates incubated with GTP γ S or GDP were used as positive and negative controls, respectively.

B. Fractionation of cells by centrifugation was used to quantify RhoA relocation to the membrane fraction upon activation. Pretreatment of anti-LOX-1 antibody blocked the translocation of RhoA from the cytosol to the plasma membrane ($n = 3$).

Figure 4. Rho A-dependent ROCK activation was associated with OxLDL-stimulated arginase activation

A. Transfection of siRNA using oligofectamine inhibits the protein amount of Rho A (* vs. untreated, $p < 0.05$, $n = 3$; # vs. OxLDL, $p < 0.05$; $n = 3$) (means $\pm$ SEM are shown in all three panels).

B. siRNA knockdown of Rho blocks arginase activation by OxLDL stimulation (* vs. untreated, $p < 0.01$, $n = 4$; # vs. OxLDL, $p < 0.01$; $n = 4$).

C. Inhibition of the Rho effector ROCK with the specific inhibitor Y27632 (10 μ M, 1 hour) resulted in significant attenuation of arginase activity (* vs. control, $p < 0.05$, # vs. OxLDL, $p < 0.05$; $n = 3$).

D. Rho inhibitor abrogates Ox-LDL mediated ROS production in mouse aorta. Ox-LDL (50 μ g/ml) was added in mouse aorta pre-incubated with Rho inhibitor, Y27632, (10 μ M) for 1 hour and ROS was measured using DHE method (** vs. control, $p < 0.01$, # vs. OxLDL, $p < 0.05$; $n = 3$).

Figure 5. Statins blocked the induction of arginase activity and preserved the co-linearity of arginase II with b-tubulin after OxLDL exposure

A. Incubation of HAEC with either lovastatin or simvastatin inhibited OxLDL-induced increases in arginase activity (* OxLDL vs. untreated, $p<0.01$; # statin + OxLDL vs. OxLDL alone, $p<0.01$; means $\pm$ SEM; $n=6$).

B. Simvastatin attenuated arginase dissociation from the microtubule cytoskeleton and β -tubulin depolymerization after OxLDL stimulation (* OxLDL vs. untreated $p<0.05$; # simvastatin + OxLDL vs. OxLDL alone, $p<0.05$, $n=6$). Normalized densitometry data (means $\pm$ SEM) are shown below the Western blot.

C. HAEC were prepared for immunofluorescence analysis of arginase II (red) and beta-tubulin (green) localization after no treatment (control, A), 5 minutes of OxLDL (50 mg/mL)(B), or OxLDL after preincubation with simvastatin (10 mM for 30 minutes). OxLDL disrupted the colinearity of ArgII and tubulin, indicating ArgII release from the microtubule scaffold.

(C). Statin treatment blocked the decrease in linear organization of arginase II and the microtubule collapse seen with OxLDL treatment. Scale bar ~ 10 μ m. 4x insets are shown in the upper left of each panel that correspond with the smaller area with dashed perimeter in each respective panel.

D. Lovastatin inhibited arginase activation in aortas from ApoE^{-/-} mice fed a high fat (HC) diet (* WT, ND vs. ApoE^{-/-}, HC; # ApoE^{-/-}, HC vs. ApoE^{-/-}, HC + Lovastatin; $n=6$ for each group).

E. Lovastatin inhibited the attenuation of NOx production in aortas from ApoE^{-/-} mice fed a high fat (HC) diet (* WT, ND vs. ApoE^{-/-}, HC; # ApoE^{-/-}, HC vs ApoE^{-/-}, HC + Lovastatin; $n=6$ for each group).

Figure 6. Proposed downstream LOX-1 receptor signaling from OxLDL to arginase-mediated endothelial dysfunction

The atherogenic stimulus OxLDL is ligated by the LOX-1 receptor (1). Putative downstream events include the engagement of signaling intermediates that activate RhoA. Rho effectors ROCK and mDia1 may then liberate ArgII from sequestration by mitochondria and the microtubule cytoskeleton, leading to ArgII activation and L-arginine catabolism. This results in eNOS uncoupling (2), decreased NO production, an increase in available ROS, and subsequent vascular endothelial dysfunction (3).