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OxLDL-dependent activation of arginase II is dependent on the LOX-1 receptor and downstream RhoA signaling

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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-1$^{-/-}$ 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.

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 atherosclerosis [1–5].

We recently demonstrated that Oxidized LDL (OxLDL), the primary pathogenic lipid in atherogenesis, activates endothelial cell arginase II [6]. 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 phenotype [5].

While there is evidence that Rho and Rho-Kinase (ROCK) might be involved in the activation of arginase [4], 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 dysfunction [8], inflammatory response [9] and NO production [10].

We tested the hypotheses 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

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statins inhibit this process. These findings may, in part, explain a new mechanism for the pleotropic 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 h 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 $CuSO_4$, 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, pH 7.5, 0.1 mM EDTA and protease inhibitors) for 30 min at 4 °C and centrifugation for 20 min at 14,000 × *g* at 4 °C. Serum-starved HAEC were treated with statins (10 μM, 30 min) 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 min in the dark with 150 μL of hypotonic buffer (20 mM Tris–HCl, pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40, and 2 mM PMSF). Lysis was followed by centrifugation at 14,000 × *g* for 10 min at room temperature. Supernatants containing soluble (cytosolic) tubulin were transferred to fresh tubes. Pellets containing cytoskeletal (polymerized) tubulin were resuspended in 150 μL 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 μmol/L, 5 min) and NO (DAF, 5 μmol/L, 5 min). Images were acquired using a NikonTE-200 epifluorescence microscope. In some cases, aortas were incubated with OxLDL for 30 min 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 s 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, 1:1000). Densitometry analysis of bands was performed with NIH ImageJ [11].

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 described [15]. Protein lysates were obtained with lysis buffer 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10 μg/mL each of aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 20 mM vanadate, and cleared at 15,000 × *g* for 5 min. Approximately 800 μg of proteins were rotated for 30 min with 50 μg of GST-RBD. Sample was then washed three times with buffer containing 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 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 1000 × *g* for 10 min to remove cell debris and unbroken cells. The supernatants were subjected to centrifugation (21,000 × *g*, 45 min, 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 min at room temperature, mixed with 180 μL Opti-MEM medium containing siRNA, and further incubated for 15 min. 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 h, the serum concentration in the endothelial growth medium was returned to a 1× serum concentration by adding 3× media of 0.5 mL per well. Transfected cells were then cultured for 36 h and then serum-starved for 24 h 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 min, followed by 20 min of 3% paraformaldehyde alone. Samples were prepared for immunofluorescence analysis by incubating with rabbit polyclonal antisera against arginase II (Santa Cruz