OxLDL Triggers Retrograde Translocation of Arginase 2 in Aortic Endothelial Cells via ROCK and Mitochondrial Processing Peptidase

Deepesh Pandey¹, Anil Bhunia¹, Young Jun Oh¹, Fumin Chang¹, Yehudit Bergman¹, Jae Hyung Kim¹, Janna Serbo¹,², Tatiana N. Boronina⁶, Robert N. Cole⁶, Jennifer Van Eyk⁷, Alan T. Remaley⁸, Dan E. Berkowitz¹,², Lewis Romer¹,²,³,⁴,⁵

¹Anesthesiology and Critical Care Medicine; ²Biomedical Engineering; ³Cell Biology; ⁴Pediatrics; ⁵Center for Cell Dynamics; ⁶Mass Spectrometry and Proteomics Facility; ⁷Departments of Medicine and Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21287-4904, and; ⁸Cardiovascular-Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD.

D.E.B. and L.R. contributed equally to this study.

Running title: Arginase 2 Translocation by OxLDL

Subject codes:
[134] Pathophysiology
[95] Endothelium/vascular type/nitric oxide
[96] Mechanisms of atherosclerosis/growth factors

Address correspondence to:
Dr. Lew Romer
Johns Hopkins University School of Medicine
Charlotte R. Bloomberg Children’s Center
Suite 6318-C / Pediatric ACCM
1800 Orleans Street
Baltimore, MD 21287-4904
Tel: 410-955-7610
Fax: 410-502-5312
LRomer@jhmi.edu

In May 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.87 days.
ABSTRACT

**Rationale:** Increased arginase activity contributes to endothelial dysfunction by competition for L-arginine substrate and reciprocal regulation of NOS. The rapid increase in arginase activity in human aortic endothelial cells (HAEC) exposed to oxidized LDL is consistent with post-translational modification or subcellular trafficking.

**Objective:** To test the hypotheses that OxLDL triggers reverse translocation of mitochondrial Arginase 2 (Arg2) to cytosol and Arg2 activation, and that this process is dependent upon mitochondrial processing peptidase (MPP), LOX-1 receptor and ROCK.

**Methods and Results:** OxLDL triggered translocation of Arg2 from mitochondria to cytosol in HAEC and in murine aortic intima with a concomitant rise in arginase activity. All of these changes were abolished by inhibition of MPP or by its siRNA-mediated knockdown. ROCK inhibition and the absence of the LOX-1 receptor in KO mice also ablated translocation. Amino-terminal sequencing of Arg2 revealed 2 candidate mitochondrial targeting sequences, and deletion of either of these confined Arg2 to the cytoplasm. Inhibitors of MPP or LOX-1 receptor KO attenuated OxLDL-mediated decrements in endothelial-specific NO production and increases in superoxide generation. Finally, Arg2−/− mice bred on an ApoE−/− background showed reduced plaque load, reduced ROS production, enhanced NO, and improved endothelial function as compared with ApoE−/− controls.

**Conclusion:** These data demonstrate dual distribution of Arg2, a protein with an unambiguous MTS, in mammalian cells, and its reverse translocation to cytoplasm by alterations in the extracellular milieu. This novel molecular mechanism drives OxLDL-mediated arginase activation, eNOS uncoupling, endothelial dysfunction, and atherogenesis.

**Keywords:** Atherogenesis, endothelial dysfunction, endothelial nitric oxide synthase, oxidized low-density lipoprotein, mitochondria, arginase, mitochondrial process

**Nonstandard Abbreviations and Acronyms:**
- ABH: amino-2-borono-6-hexanoic acid
- Arg2: arginase 2
- eNOS: endothelial nitric oxide synthase
- HAEC: human aortic endothelial cells
- MPP: mitochondrial processing peptidase
- OxLDL: oxidized low-density lipoprotein
- ROCK: rho kinase
- ROS: reactive oxygen species
- SNP: sodium nitroprusside
INTRODUCTION

Accumulating evidence suggests that oxidized low density lipoprotein (OxLDL) plays an important role in the pathogenesis of atherosclerosis, and this is supported by its abundance in atherosclerotic lesions. A major target for OxLDL-mediated vascular injury in atherogenesis is the aortic intima. Most of these effects occur through OxLDL interaction with its lectin-like oxidized low density lipoprotein receptor-1 (LOX-1). Endothelium plays a major role in the regulation of vascular homeostasis by modulating vasomotor tone, and vascular smooth muscle cell growth and migration. Impaired endothelial function is considered to be an early and critical event in atherosclerosis, causing abnormalities in the arterial wall and plaque formation.

An emerging paradigm in NO biology is the concept that Arg reciprocally regulates NOS activity by competing for L-arginine substrate, and effectively inhibiting NO-dependent processes by depleting the substrate pool available for NO biosynthesis. Furthermore, since L-arginine is spatially confined to a minimum of 3 distinct subcellular pools that are regulated by different transporters and enzymes, local concentrations of L-arginine may limit the activity of the different NOS isoforms that are also spatially segregated within the cell. Arg1 and Arg2 are distinct isoforms encoded by different genes. Arg1 has been referred to as the hepatic isoform and catalyzes the final step of the urea cycle, although its expression can be induced in a wide variety of cells and tissues by hypoxia and LPS. Arg2 is the principal form in EC, and has been referred to as the extrahepatic isoform. It provides ornithine for polyamine synthesis, thereby controlling cell proliferation and differentiation. Arg2 also exhibits broad tissue distribution, and is inducible by a variety of factors including LPS, TNF-α, and hypoxia. The role of Arg2 in endothelial dysfunction has recently been extended to other disorders in animal models including aging, ischemia-reperfusion, hypertension, balloon vascular injury, ischemia reperfusion injury, and atherosclerosis. Our laboratories have demonstrated that OxLDL increases Arg2 activity in HAEC in a rapid, dose-dependent manner that leads to impaired endothelial NO synthesis. However, the signaling pathways that connect Ox-LDL exposure to Arg2 in EC remain incompletely defined. Thrombin increases Arg2 activity in EC via the Rho/ROCK signaling pathway, and RhoA may also be important in Ox-LDL-mediated Arg2 activation in EC. Another clue regarding its regulation is that Arg2 contains a putative mitochondrial targeting sequence in its amino terminus. Arg2 is thought to be largely, but not exclusively, confined to mitochondria in quiescent vascular EC, and we have shown that it constrains endothelial NOS activity. However, the mechanism by which mitochondrial Arg2 constrains the activity of (predominantly) cytosolic eNOS is unclear. In the current study, we demonstrate that Arg2 release from mitochondria to cytoplasm triggers NO-dysregulated vascular dysfunction in rapid response to OxLDL exposure.

METHODS

Detailed methods may be found in the online supplement, which includes following:

Arginase activity assay was determined in lysates of HAEC and murine aortic tissue by measuring urea production.

For plaque quantification, vessels were stained with Sudan IV. Aortic roots were then paraffin-embedded, and cross sections were stained with hematoxylin-eosin to evaluate the atherosclerotic lesion area. Image analysis was done with Image J, version 1.42n. Some aortic segments were permeabilized with 0.5% Triton X-100 in 3% paraformaldehyde and then cut open to expose the intima and Arg2 was imaged using a Zeiss 710-NLO confocal unit and Zeiss Zen software.
Ad-shNontargeted, Ad-MPPα, Ad-MPPβ, Ad-shArg1 and Ad-shArg2 encoded viruses were generated using a pAdBLOCK-iT kit (Life Sciences). An adenoviral construct containing GFP Arg2 was constructed by subsequent subcloning of C-terminally GFP epitope-tagged Arg2 into PENTR1a and then into the PDEST destination vector.

For proteomics studies, cytosol and mitochondria were fractionated and immunoprecipitated from EC using Arg2 polyclonal antibody. Gel bands corresponding to both control and Ox-LDL treated samples were excised, digested with Lys-C and subjected to mass spectrometry analysis. Protein identification by liquid chromatography tandem mass spectrometry (LCMS/MS) analysis of peptides was performed using an LTQ ion trap MS (Thermo Fisher Scientific) interfaced with a 2D nanoLC system (Eksigent, Dublin, CA).

Superoxide and NO measurements were determined using the Luminol analog L-012, and a Siever’s NO analyzer, respectively.

MPP activity was determined by measuring PINK1 cleavage.

RESULTS

**OxLDL activates endothelial Arg2 by triggering its translocation from the mitochondria to the cytosol.**

Previously, we have shown that exposure of HAEC to OxLDL rapidly increases Arg2 activity 24, 26. This time course is consistent with translocation between subcellular compartments and/or post-translational modification(s). Serum-starved HAEC were incubated with 50 μg/mL OxLDL for 2 hours. Ox-LDL exposure promoted robust translocation of Arg2 from mitochondria to the cytosol (Figure 1A). To further confirm that Arg2 is predominantly targeted to mitochondria, FLAG-tagged Arg2 was expressed in HAEC cells (Figure 1C, 1D). Immunofluorescence studies in unstimulated HAEC cells transfected with FLAG-tagged Arg2 also demonstrated that the majority of Arg2 is located in mitochondria (Figure 1E). Arginase activity was also measured in both mitochondrial and cytoplasmic fractions (Figure 1B). Interestingly, arginase activity was substantially increased in the cytosolic fraction of OxLDL-treated HAEC as compared with the activity of mitochondrial Arg2 in untreated cells.

Although we and others have identified Arg2 as the predominant arginase isoform in HAEC, some reports indicate that Arg1 is expressed in endothelial cells 29. To determine the contribution of individual Arginase isoforms to total arginase activity in HAEC, we individually silenced Arg1 and Arg2 using adenovirally mediated shRNA transduction. Arg2 silencing inhibited total arginase activity to less than 25% of control levels, whereas Arg1 knockdown had very little effect on total HAEC arginase activity (Supplemental Figure IA). Furthermore, we did not detect any Arg1 expression in quiescent HAEC. These findings suggest that the OxLDL-induced increase in total arginase activity in HAEC is almost entirely mediated by Arg2.

To examine OxLDL-triggered translocation of Arg2 from mitochondria to cytosol in real time, we constructed an Arg2 with a C-terminus epitope GFP tag, and employed live cell imaging with and without OxLDL (Supplemental Figure II and Supplemental Movies I-III). These data corroborated the biochemical findings and demonstrated the dynamics of OxLDL-induced Arg2 cytosolic translocation.

Changes in mitochondrial membrane potential have been shown to be strongly linked to the decompartmentalization of mitochondrial proteins 30, however no change in mitochondrial membrane potential was observed in intact HAEC in response to OxLDL using the fluorescent probe TMRM (Supplemental Figure III).
OxLDL-mediated cytosolic release and activation of endothelial Arg2 are dependent upon the LOX-1 receptor and Rho Kinase activity.

We have previously demonstrated that OxLDL-mediated increases in EC Arg activity are dependent on the LOX-1 receptor 26. Here we measured Arg2 quantity and activity in cytosolic and mitochondrial fractions of primary cultures of aortic endothelial cells isolated from wild type and LOX-1 receptor knock out mice (LOX-1−/−) in response to OxLDL. OxLDL failed to induce cytosolic release and activation of Arg2 in aortic EC from LOX-1−/− mice (Figure 2A and B). This supports our previous finding that LOX-1 mediates acute OxLDL-induced increases in the activity of Arg2 26.

Our previous data 26 have demonstrated that RhoA and ROCK activation are downstream events in OxLDL-dependent augmentation of Arg2 activity in EC. To define the role of Rho signaling in OxLDL-dependent decompartmentalization and activation of Arg2, we pre-incubated HAEC with the specific Rho kinase (ROCK) inhibitor Y-27632 (10 μM Y-27632, 2 hours) which obviated both Ox-LDL-induced cytosolic translocation of Arg2 and the associated increase in arginase activity (Figure 2C and D).

Truncated Arg2 (without the MTS) localizes to the cytoplasm of HAEC.

Most nuclear-encoded proteins that are destined to be incorporated into mitochondria possess mitochondrial targeting sequences (MTS). Although it has been shown that Arg2 resides predominantly in mitochondria 27, the MTS for Arg2 is still undefined and bioinformatics-based predictions of MTS of dual targeted genes have shown to be inaccurate 31. Identification and characterization of the Arg2 MTS was undertaken in order to understand the dual targeting of this protein, and its regulation by the OxLDL stimulus. We employed mass spectrometry and N-terminal sequencing using Edman’s degradation analysis. MS analysis indicated that an immunoprecipitated (IP) cytosolic Arg2 from OxLDL-treated HAEC is cleaved at the His amino acid residue at position 24 (Figure 3A). MS analysis of an IP of Arg2 from the cytosolic fraction of HAEC that were treated with OxLDL provided 30% amino acid sequence coverage at 95% confidence, and yielded the fragmentation spectrum shown in Figure 3A. Since the N-terminus of this Arg2 peptide is not a LysC cleavage site, the N-terminal position of this sequence indicates that the Arg2 parent protein was cleaved (by an endogenous peptidase), leaving HSVAVIGAPFSQGQK as the N-terminal peptide, rather than the traditional LysC site (SVHSVAVIGAPGSQGQK). To further investigate the amino acid(s) that form the N-terminus of full-length Arg2, Edman degradation analysis was carried out on immuno-enriched C-terminal Flag-Arg2 isolated from 293 cells. The N-terminal amino acid sequences obtained were from two peptides, VHSVAVIG, and GQKRKGVEH. These data indicate potential cleavage sites between amino acids 22 and 23 and 35 and 36, respectively (Figure 3B). The second of these potential cleavage sites detected with Edman degradation was consistent with canonical sequences associated with cleavage by the mitochondrial processing peptidase (MPP), as it included arginine residues at positions -2 and -3 relative to the candidate site of cleavage 32. To evaluate whether deletion of these putative MTS had functional consequences for Arg2 localization, we generated two N-terminal truncation mutants of Arg2 (deletion of resides 1-22 or 1-40) as shown in Figure 4A. Deletion of either Arg2 amino acids 1-22 or 1-40 resulted in almost exclusive cytoplasmic distribution of Arg2 (Figure 4B and C). A point mutation of the MPP cleavage site at position 36 in Arg2 that was predicted by N-terminal sequencing significantly inhibited the translocation of Arg2 to mitochondria and attenuated Arginase activity.

OxLDL-induced translocation and activation of Arg2 in HAEC involves the Mitochondrial Processing Peptidase (MPP).

The MTS and putative MPP cleavage site in the N-terminus of human Arg 2 that we identified by mass spectrometry and Edman degradation analysis are regions of Arg2 that are highly conserved among species (Figure 5A). Given the redistribution of mitochondrial Arg2 following OxLDL stimulation, we examined whether MPP, and therefore MTS cleavage, contributes to OxLDL-evoked reverse translocation of
Arg2 from mitochondria to the cytosol. O-phenanthroline (Oph), a biochemical inhibitor of MPP, prevented the induction of Arg activity (Figure 5B) and completely blocked OxLDL-induced cytosolic release of Arg2 (Figure 5C). Interestingly, the competitive Arg inhibitor 2(S)-amino-6-boronhexanoic acid (ABH), while inhibiting Arginase activity, did not prevent Ox-LDL-induced Arg2 translocation to the cytosol (Figure 5B and 5C). Additionally, MPPα knock down blocked cytosolic redistribution of Arg2 with OxLDL exposure, and prevented Arg2 activation in response to OxLDL (Figure 5D and 5E). On the other hand, control siRNA had no effect on MPP expression, or on the distribution or activity of Arg2. The siRNA for MPP knocked down MPPα subunit protein abundance by more than 50% in HAEC (Figure 5F).

Immunofluorescence localization experiments were then conducted in native vascular endothelial cells in aortic strips from transgenic mice with Tie2 promoter-driven endothelial-specific Arg2 overexpression (Figure 6A). Arg2 was confined to distinct perinuclear granular structures consistent with mitochondria in control samples. Stimulation of the aortic intima with OxLDL resulted in Arg2 redistribution to a diffuse blush consistent with cytoplasmic redistribution, and this was prevented in samples in which MPP was inhibited by OPH. The same redistribution pattern was observed in Arg2-GFP-transduced HAEC (Figure 6B and Supplemental Figure VIII). Additionally, while Arg2 was confined to the mitochondria in quiescent HAEC, OxLDL stimulation led to cytoplasmic translocation, a process that was inhibited by shRNA knockdown of either MPPα or MPPβ in HAEC (Figure 6B).

**Inhibition or knockdown of MPP prevents OxLDL-mediated vascular dysfunction and eNOS uncoupling.**

OxLDL-incubated aortic rings transduced with 100 MOI of control shRNA (NT, nontargeted) exhibited attenuated relaxation responses to acetylcholine (endothelial dysfunction). This was substantially improved in aortic rings that were transduced with either 100 MOI of adenoviruses with shRNA for MPPα or for MPPβ when added 24 hours prior to OxLDL stimulation (Figure 6C and 6D). MPPα and MPPβ knockdown in these vessels was verified by immunoblotting with MPPα and MPPβ specific antibodies (Supplemental Figure V).

The process termed “eNOS uncoupling” occurs under a number of pathologic conditions in which the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, but leads to an increase in superoxide rather than NO production. OxLDL has been shown to decrease the bioavailability of NO by eNOS uncoupling. This may occur in endothelial cells due to depletion of L-arginine substrate available for eNOS in presence of activated Arg2. We tested the effects of inhibiting MPP, and its role in the downstream cytosolic translocation and activation of Arg2, on eNOS uncoupling by OxLDL exposure. MPP inhibition blocked the increased ROS (reactive oxygen species) generation and the decreased NO production seen in aortas from WT (LOX-1+/+) mice after OxLDL exposure (Figure 7A and 7B). OxLDL-induction of ROS production in aortas that were not treated with MPP inhibitors was inhibited by L-NAME (100 μM), suggesting that eNOS was the source of ROS (via eNOS uncoupling) in these vessels. OxLDL had no effect on NO production and ROS generation in aortas from LOX-1−/− mice. In addition, endothelium-denuded (E-) strips demonstrated very little ROS production suggesting that the endothelium is the primary source of the OxLDL-dependent ROS (Figure 7C). ROS was measured using the luminol analog L-012, and Nitrite accumulation was measured using a Siever’s NO analyzer to determine NO production. The specificity of L-012 for superoxide was examined by treating cells with the superoxide scavenger, SOD (20U/mL). SOD nearly abolished signals from HAEC loaded with L-O12 indicating that chemiluminescence was specific to superoxide (Figure 7D). OxLDL significantly increased superoxide production in HAEC, which was further shown to be sensitive to SOD. Adenoviral knockdown of either Arg 2 or MPPα with human specific shRNA significantly reduced OxLDL-dependent increases in ROS (Fig 7E), and MPPα knockdown obviated the drop in NO that was caused by OxLDL (Fig 7F).

In order to determine whether reduced levels of L-arginine contribute to OxLDL-mediated eNOS uncoupling, we measured the concentration of L-arginine in both the cytosolic and mitochondrial...
compartments of HAEC in the presence and absence of OxLDL using a Biochrom-20 amino acid analyzer. OxLDL significantly diminished cytosolic L-arginine concentrations (Supplemental Fig IV). Inhibition of MPP with Oph prior to OxLDL exposure prevented this OxLDL-dependent decrease in cytosolic L-arginine.

**OxLDL increase MPP activity and is dependent on ROCK.**

We next used recent data identifying PINK1 cleavage as a metric for MPP activity to interrogate the effects of ROCK and of OxLDL on MPP activity. FLAG-PINK1 cDNA alone or FLAG-PINK1 together with constitutively active (CA) or dominant negative (DN) mutants of ROCK were expressed in HAEC (Supplemental Figure VI). Cleaved PINK1 increased in the presence of CA-ROCK, whereas PINK cleavage did not change when control GFP cDNA or DN-ROCK were co-expressed. The negligible effect observed with DN-ROCK may be attributed to its lower expression level compared to active mutant (third segment of Supplemental Figure VI, panel A). HAEC expressing FLAG-PINK1 were also treated with OxLDL alone or in combination with the ROCK inhibitor Y27632 (Supplemental Figure VI, panel B). OxLDL up regulated PINK1 cleavage (and thus MPP activation), and this was inhibited by Y27632. Taken together, these findings suggest a signaling cascade that is initiated by OxLDL and extends through RhoA and ROCK to MPP.

**Arg2 knock out reduces atheromatous plaque burden, improves endothelial function, enhances NO and reduces ROS in ApoE⁻/⁻ mice.**

Endothelial dysfunction and inflammation are two critical events that drive atherosclerosis. To investigate the role of Arg2 in endothelial inflammation, we examined OxLDL-dependent NFκB activity in the presence or absence of ABH. NFκB activity was determined by measuring luciferase activity that was driven by an NFκB response element. OxLDL increased NFκB activity and this was significantly attenuated by ABH (Figure 8A).

Aortic atheromatous plaque burden was then determined in ApoE⁻/⁻ and Arg2⁻/⁻/ApoE⁻/⁻ double knockout mice (DKO) mice (created by backcrossing the Arg2⁻/⁻ on an ApoE⁻/⁻ background) that were fed an atherogenic diet for 12 weeks. Plaque thickness was significantly reduced in DKO mice compared to ApoE⁻/⁻ mice (178.6±25.6 vs. 416.7±52.5 μm, p < 0.01; Figure 8B, top and bottom panels) as was the percentage of total aortic surface area that was covered by plaque (38.4±3.8% vs. 52.0±2.8%, n=6, p < 0.01; Figure 8C, top and bottom panels). No plaque was detected in WT or Arg2⁻/⁻ mice.

We next determined vascular reactivity in response to acetylcholine (endothelial-dependent) or sodium nitroprusside (endothelial-independent) in isolated aortas from ApoE⁻/⁻ and DKO mice that were fed a high cholesterol diet/ high fat diet. Endothelial-dependent vascular relaxation in response to acetylcholine was significantly improved in aortas from DKO mice as compared with aortas from ApoE⁻/⁻ mice (log EC50 = -5.68 ± 0.31, vs. -5.13 ± 0.53 mol/L, p < 0.05, n=6). Acetylcholine-mediated relaxation responses in vessels from WT and Arg2⁻/⁻ mice were significantly greater than in either the ApoE⁻/⁻ or the DKO samples (log EC50, -5.79±0.23 and -6.05±0.29 mol/L, respectively, p < 0.05, n=6 ) (Figure 8D). eNOS inhibition with L-NAME (100 μM) abolished the relaxation in response to acetylcholine in all groups, suggesting that the differences seen between groups were due to eNOS activity (Supplemental Figure VII). SNP-mediated relaxation (endothelium-independent responses) in samples from WT and Arg2⁻/⁻ mice were not significantly different than those seen in ApoE⁻/⁻ or DKO mice (Figure 8E).

We next tested whether genetic deletion of Arg2 ameliorated changes in NO and ROS production that were seen in atheroprone ApoE⁻/⁻ mice that were fed a high cholesterol diet. Aortic intima from DKO animals exhibited improved NO production and lower ROS levels compared with those measured in samples from ApoE⁻/⁻ mice (Supplemental Figure IX). The atherogenic diet did not change NO production in Arg2⁻/⁻ mice (P=0.36).
DISCUSSION

In the current study, we show that OxLDL triggers rapid reverse translocation of Arg2 from mitochondria to cytoplasm in EC. This retrograde translocation of Arg2 to cytosol significantly augments its activity, and is dependent upon processing by MPP. Furthermore, using LOX-1 KO mice and Rho kinase inhibitors, we demonstrate that OxLDL-mediated activation and movement of Arg2 to the cytoplasm is mediated through the LOX-1 receptors on HAEC and requires Rho kinase-mediated signaling. Finally, we show that eNOS uncoupling is a biologically critical consequence of these changes in Arg2 localization and activity, with subsequently reduced NO production and increased generation of ROS.

Many mitochondrial proteins are encoded by nuclear genes, synthesized in the cytosol as large precursor proteins with N-terminal MTSs, and imported into mitochondria. During or after import, precursor proteins may be cleaved by MPP leaving a functional protein. The positively charged MTS are recognized directly by mitochondrial surface receptors and also facilitate translocation into mitochondria by interacting with the membrane electrical potential that exists across the mitochondrial inner membrane. Some proteins, such as fumarase, may undergo bidirectional trafficking between cytosol and mitochondria based upon processing by MPP.

Our data indicate that Arg2 is dual-targeted to mitochondria and the cytosol, thereby adding Arg2 to a number of other proteins that share this distinction – up to one quarter of the mitochondrial proteome in some species. There are at least four potential mechanisms by which a single translation product may be present in dual compartments. These include an ambiguous targeting sequence recognized by more than one organelle, multiple targeting sequences; differential targeting accessibility; and reverse translocation - a mechanism that may be dependent upon processing of a MTS in the mitochondria. Our data implicate the latter mechanism in the subcellular trafficking of Arg2. Reverse translocation-based dual targeting of proteins to mitochondria and cytosol in response to altered cellular environment or stress (such as OxLDL exposure) has not been previously described in mammalian cells, and represents a novel mechanism of cellular regulation in health and disease. Data supporting this mechanism in the current study include the demonstrated dependence of localization on both an intact MTS and on the activity of MPP, as the inhibition of MPP in HAEC by either pharmacological or siRNA approaches abolished the cytosolic release and activation of Arg2 in response to OxLDL.

We used experimental data from mass spectrometry and N-terminal sequencing using Edman’s degradation analysis followed by genetic mutations to identify and characterize candidates for the MTS and the MPP cleavage site. Differences in the N-terminal residues of cleaved Arg2 that were found by MS and Edman’s degradation (HSVAV vs. VHSVAV, respectively) may be explained by post-MPP cleavage “trimming” by other mitochondrial peptidases. Our findings that mutations of the predicted MPP cleavage site inhibited mitochondrial localization of Arg2 and attenuated its activity suggest that processing of Arg2 in mitochondria is a requirement for its activation. This proposed mechanism of dual localization has been characterized in yeast, where the Kreb’s cycle enzyme fumarase, is distributed between the mitochondria and cytoplasm based upon metabolic cues from the glyoxyxlate shunt. The enzyme is first targeted to the mitochondria by an MTS (24 amino acids at the N-terminal sequence) that is processed by MPP. A population of the enzyme then moves back onto the cytoplasm in a process that is regulated by protein folding. One possible mechanism for reverse translocation of Arg2 in the current study is regulation of Arg2 folding by a chaperone protein such as HSP60 - a mitochondrial protein that is released into the cytoplasm in response to endothelial activation with exposure to OxLDL and which we have shown to be a binding partner for Arg2 (unpublished observation).

Despite our finding that the majority of Arg2 is confined to the mitochondria, arginase activity in the cytosol and mitochondrial fractions were comparable. This finding suggests that cytosolic Arg2 is more active than mitochondrial Arg2. This could clarify some controversies regarding the “arginine paradox” - the
increased production of NO with addition of extracellular arginine despite saturated levels of intracellular L-arginine. Our findings that the single translation product of Arg2 could be localized in two separate compartments upon cellular stress, and that Arg2 can constrain eNOS activity supports the concept that specific subcellular pools of L-arginine do exist. We speculate that Arg2 is in closer proximity to binding and/or regulatory proteins that enhance its function after its translocation to cytosol. Recent studies demonstrate that arginine succinate lyase (ASL) enhanced NO production by NOS through recycling citrulline to L-arginine and maintaining the integrity of the NOS complex. Similarly, cytosolic ASL could recycle L-citrulline to L-arginine and provide substrate for Arg2 and maintain its structural complex in an active conformation.

Activation of Rho kinase has been linked to the acceleration of atherosclerosis in a mouse model. Furthermore, we have demonstrated that RhoA and ROCK are critical to Ox-LDL-dependent increases in EC Arg activity. In the current study, we present data demonstrating that inhibition of ROCK with Y-27632 prevented rises in both cytosolic Arg2 content and activity following Ox-LDL stimulation. These new data suggest that ROCK facilitates Ox-LDL-evoked activation and redistribution of Arg2.

Although it is assumed that the mechanism of eNOS uncoupling by arginase upregulation is in part related to arginase-dependent decrements in L-arginine availability, changes in L-arginine concentrations in response to Ox-LDL have not been measured in individual cellular compartments. Our data demonstrate that Ox-LDL significantly diminished cytosolic L-arginine concentrations (Supplemental Figure IV). These findings are consistent with our hypothesis that Arg2, which is normally confined to the mitochondria, changes to a cytosolic distribution with OxLDL stimulation, and uncouples eNOS in the cytosol. Further, inhibition of MPP both attenuated the increase in cytoplasmic arginase activity and prevented an OxLDL-dependent decrease in cytosolic L-arginine (Figure 5 and Supplemental Figure IV).

In conclusion, the reverse translocation of Arg2 from its MTS-directed sequestration in mitochondria to the cytosol in response to OxLDL apparently underlies a novel mechanism for OxLDL-mediated increases in Arg activity in HAEC. This study identifies MPP as a new subject for targeted therapeutics for atherosclerosis and other vascular disorders in which Arg2 interrupts NO production and promotes the generation of injurious ROS by uncoupling eNOS.

**SOURCES OF FUNDING**
This work was supported by AHA Postdoctoral Fellowship to D.P. (13POST16810011) and HL089668 from NHLBI to D.B. and L.R., by NSF MCB-0923661 to L.R., and by a Clinical Translational Science Award at Johns Hopkins University (JVE) and NIH S10RR024550.

**ACKNOWLEDGMENTS**
The authors are grateful to Shawna Lewis, Lakshmi Santhanam, Zongming Fu and Nagababu Enikah for technical assistance, and to David Fulton of the Vascular Biology Center at Georgia Regents University in Augusta, Georgia for Arg2 cDNA constructs.

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


17. Collado B, Sanchez-Chapado M, Prieto JC, Carmen MJ. Hypoxia regulation of expression and angiogenic effects of vasoactive intestinal peptide (VIP) and VIP receptors in Incap prostate cancer cells. Mol Cell Endocrinol. 2006;249:116-122


DOI: 10.1161/CIRCRESAHA.115.304262 10


40. Stein I, Peleg Y, Even-Ram S, Pines O. The single translation product of the fum1 gene (fumarase) is processed in mitochondria before being distributed between the cytosol and mitochondria in saccharomyces cerevisiae. Mol Cell Biol. 1994;14:4770-4778
43. Teixeira PF, Glaser E. Processing peptidases in mitochondria and chloroplasts. Biochim Biophys Acta. 2013;1833:360-370

DOI: 10.1161/CIRCRESAHA.115.304262
FIGURE LEGENDS

Figure 1. Ox-LDL triggers cytosolic release of activated Arg2: HAEC were incubated with 50μg/ml Ox-LDL for 2h. Cell lysates were fractionated by differential centrifugation. A. The quantity of Arg2 was measured in cytosolic (C) and mitochondrial fractions (M) by western blotting. Cell lysates were also immunoblotted for LDH and MnSOD to verify fractionation. B. Arginase activity was measured in the separated C and M fractions. C and D. 293 cells were transfected with C-terminal FLAG epitope-tagged Arg2 and subjected to cytosolic and mitochondrial fractionation. Fractionated lysates were probed with FLAG antibody (C), and with cytochrome C antibody (D). E. FLAG-Arg2-transfected unstimulated HAEC were stained with mitotracker red CMXRos (red), anti-Flag primary antibody, AlexaFlour 488 mouse (green) secondary, and DAPI (blue) and analyzed by immunofluorescence. * denotes p<0.05 vs Control group (CON, panel B).

Figure 2. Ox-LDL-mediated activation and cytosolic release of Arg2 require LOX-1 and Rho kinase signaling. A and B: Endothelial cells were isolated from WT and LOX-1 knockout (LOX-1−/−) murine aortas and incubated with 50μg/ml Ox-LDL for 2h. Mitochondrial and cytosolic fractions were separated for measurements of Arginase activity (A) and immunoblotting for Arg2 (B). C and D: In other experiments, HAEC were exposed to either OxLDL alone or OxLDL with Y-27632. Mitochondrial and cytosolic fractions were used for arginase activity measurements (C), or immunoblotting for Arg2 (D). * denotes P<0.05 vs control group.

Figure 3. Cytosolic Arg2 is truncated. A) Collision-induced dissociation (CID) fragmentation spectra of a 762.73 [M+2H] ion from a tryptic in-gel digest of Arg2 that was immunoprecipitated from the cytoplasm of OxLDL-treated HAEC using an anti-Arg2 polyclonal antibody. B) Cell lysates from Flag-tagged Arg2-transfected 293 cells were immunoprecipitated with Flag monoclonal antibody and subjected to N-terminal Edman degradation analysis.

Figure 4. The MTS of Arg2 is located on N-terminus and includes amino acids 1-22 or 1-40. HAEC were transfected with Full length Arg2, or either the 1-22 or the 1-40 N terminal deletion mutants, and analysis of A) western blotting, B) subcellular fractionation of full length and 1-22 truncated Arg2, and C) immunofluorescence using anti-FLAG antibody (green) and mitotracker red are shown. Full length Arg2 was localized almost exclusively to mitochondria and both truncated mutants were predominantly cytosolic (DAPI-labeled nuclei (blue) are shown in the merged images). Scale bar denotes ~40 microns.

Figure 5. Pharmacologic inhibition or siRNA-mediated knockdown of MPP prevent activation and release of Arg2 to cytosol. A) N-terminal Arg2 protein sequences from several species were aligned utilizing the Clustal W method and Mac Vector software. B and C: Serum-starved HAEC were pre-incubated with 5mM O-phenanthroline or 100nM ABH for 30 min to inhibit MPP or arginase activity, respectively. Cells were then incubated with 50μ/ml Ox-LDL for 2h and then fractionated. Arginase activity (B), and Arg2 protein expression (C) were measured in the mitochondrial (M) and cytosolic (C) fractions. D-F: MPP-specific siRNA (10nM) was transfected into HAEC. Serum-starved cells were then treated with OxLDL for 2h, and subjected to fractionation. Arginase activity (D) and Arg2 expression (E) were measured in C and M fractions. Knockdown efficiency of the MPP siRNA was determined by immunoblotting for MPP in both untreated and OxLDL-treated HAEC (F). * indicates p<0.05 vs. Control cytosolic group, and # denotes p<0.05 vs. Control mitochondrial group.

Figure 6. OxLDL-mediated redistribution of Arg2 and impairment of endothelial function are prevented by biochemical inhibition of MPP and by MPPα or MPPβ knockdown. A) Confocal images from aortic strips isolated from Arg2-overexpressing mice are shown: Left panel shows untreated controls; Middle panel shows effects of 50μg/mL of OxLDL; Right panel shows effects of 1mM of Oph for 30m prior to the 2h treatment with 50μg/mL OxLDL. Data are representative of three independent experiments.
B) After transduction with non-targeted, MPPα shRNA or MPPβ shRNA, HAEC were further transduced with GFP-tagged Arg2 (C-term). 24 hours later cells were incubated in presence or absence of 50μg/ml Ox-LDL for 2 hours and subjected to immunofluorescence for GFP-Arg2 and Mitotracker. C and D) Murine aortic rings were incubated with 100 MOI of nontargeted, MPPα or MPPβ shRNA adenoviruses and treated 24 hours later with or without Ox-LDL (50μg/mL). Dose-response effects of ACh (C) and SNP (D) on vascular relaxation were then determined. * indicates p<0.05 vs. Control (NT/Ox-LDL).

**Figure 7. Ox-LDL-mediated eNOS uncoupling is dependent upon MPP and intact endothelium.** Aortic strips from wild type or LOX-1-/- mice were exposed to OxLDL alone, OxLDL with 5mM Oph, or OxLDL plus L-NAME (100μM). A) ROS production was measured as the slope of DHE fluorescence. B) NO production was measured as a slope of DAF fluorescence. C) ROS production was also studied in parallel in murine aortic specimens with intact (E+) or mechanically denuded (E-) intimal endothelium. D) HAEC production of ROS was determined by L-012 chemiluminescence following incubation with or without SOD (20U/mL), and expressed as relative light units (RLU). E) HAEC were incubated with 100 MOI of nontargeted, MPPα or MPPβ shRNA adenoviruses. 24 hours after transduction, media was replaced with fresh media with or without OxLDL (50μg/mL). After 48 hours, the production of ROS was determined in presence or absence of SOD (20U/mL) via L-012 chemiluminescence. F) HAEC were incubated with 100 MOI of nontargeted shRNA or MPPα shRNA adenoviruses. Cells were treated the next day with control medium or OxLDL (50μg/mL). After 48 hours, the production of NO was determined by measuring nitrite levels with Siever’s NO analyzer, means ± S.E are shown, n=6. * indicates p<0.05 vs. Con, # denotes p<0.05 vs. OxLDL.

**Figure 8. Arg2 inhibition ameliorates atherogenesis in ApoE knockout mice.** A) HAEC expressing NFkB-LUC were incubated with OxLDL (50μg/mL) alone or with ABH (200μM) for 8 hours, and luciferase activity was determined using chemiluminescence. B) Quantitative graph (upper panel) of plaque formation in histological sections of ascending aortas (lower panel). C) Cumulative quantitative assessment (upper panel) of the plaque area determined by pixel count of Sudan IV-stained areas of the lipid-rich intraluminal lesions in longitudinally opened aortas (lower panel). D and E) Dose-response effects of Ach (D) and SNP (E) on vascular relaxation in isolated aortas from ApoE-/- mice and Dbl-KO (ArgII-/-/ApoE-/-) mice fed with HC diet were determined. * indicates P<0.05 vs Con, ** indicates P<0.001, # indicates P<0.05 vs OxLDL without ABH.
Novelty and Significance

What Is Known?

- Arginase 2 (Arg2) inhibits nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS) by competing for the common substrate L-arginine.
- In quiescent endothelial cells Arg2 is confined predominantly to the mitochondria.
- Activation of endothelial cells by the atherogenic stimulus oxidized low density lipoprotein (OxLDL) leads to a rapid increase in arginase activity, and this results in eNOS uncoupling and contributes to endothelial dysfunction.
- Pharmacologic inhibition of arginase improves NO production and endothelial function, and reduces plaque burden in atheroprone ApoE−/− mice.

What New Information Does This Article Contribute?

- oxLDL induces reverse translocation of Arg2 reverse from mitochondria to the cytoplasm where Arg2 constrains eNOS activity.
- OxLDL-mediated translocation of Arg2 is dependent upon the presence of a mitochondrial targeting sequence in Arg2, and the mitochondrial processing peptidase.
- Knockout of Arg2 in atherogenic ApoE−/− mice recouples eNOS to its substrate L-arginine, improves endothelial function, and reduces atherosclerotic plaque burden.

Arginase is an important negative regulator of eNOS because of its competition for the common substrate L-arginine. Arginase upregulation contributes to the pathobiology of vascular diseases including atherosclerosis, erectile dysfunction, and pulmonary and systemic hypertension. OxLDL-mediated injury to endothelium causes 2 distinct events that contribute to increased Arg2: a decreased check on Arg2 transcription by HDAC 2; and a rapid increase in Arg2 activity. In this paper we report that OxLDL triggers rapid Arg2 translocation from the mitochondria of EC (where it is confined in the quiescent state) to their cytosol. This process requires the LOX-1 receptor, Rho kinase, and MPP. Arg2 translocation leads to eNOS uncoupling, decreased NO production, and impaired endothelial-dependent vasorelaxation. Finally Arg2−/− mice bred on an ApoE−/− background exhibit enhanced eNOS function, improved endothelium-mediated vasoreactivity, and reduced plaque load. These findings suggest a novel mechanism for rapid Arg2 activation in response to EC injury; and reveal a signaling pathway by which a single gene product with an unambiguous mitochondrial targeting sequence undergoes dual compartmentalization.
FIGURE 2
Panel A) N-terminal amino acid sequence by N-terminal Edman Degradation
(Intact full length Arginase 2 is blocked at the N-terminus and so will not be sequenced, only degraded or cleaved forms with free N-termini will be sequenced by this technique)

<table>
<thead>
<tr>
<th>Sequence round</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>H</td>
<td>S</td>
<td>V</td>
<td>A</td>
<td>V</td>
<td>I</td>
<td>G</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td></td>
<td>O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Panel B) Human Arginase 2, with potential mitochondrial targeting sequence(s)

```
MSLEGSLSRL LQTRVHSILK KSVBSVAVIG APFGQGCCDK QVERGPAAIR EAGNKLKLSS
LGCHLXDFQD LQFTPFPKEDD LYNMLLVNPR SGELCLGELA EVVQRATVSG YCSVTVGDDW
SLAIGTSGH ARMCPDCLCV WDRAHADINT PLTSSGNNH GQPSVPLRE LQDKVQPLPG
FSWIKPCISS ASIVYGLRDI VPDEHFLIKC NYDIQYFSPR DIDIRGELQKV MERFDLLIG
KRQRPIHLSF DIIDAPFVTLA PATQTPVVG G L T R E G T Y I A E E I N T G L L S A L D L V E V N F Q
LATSEEAKT TANLAVVDVA SSFGQYREGG HIVYDDLFTP SSPESENQDA RVRI
```
Figure 4
FIGURE 5
A. CONTROL | OxLDL | OxLDL + OPH

RED = Arginase2  
Blue = DAPI (nucleus)

B. CONTROL | OxLDL | OxLDL + shMPPα | OxLDL + shMPPβ

GFP-Arg2

Mitotracker

C. Ach D/R

% Relaxation vs Log[Ach]

D. SNP D/R

% Relaxation vs Log[SNP]

FIGURE 6
Figure 7
Figure 8.
OxLDL Triggers Retrograde Translocation of Arginase 2 in Aortic Endothelial Cells via ROCK and Mitochondrial Processing Peptidase

Deepesh Pandey, Anil Bhunia, Young Jun Oh, Fumin Chang, Yehudit Bergman, Jae H Kim, Janna Serbo, Tatiana N Boronina, Robert N Cole, Jennifer Van Eyk, Alan Remaley, Dan E Berkowitz and Lewis H Romer

Circ Res. published online June 5, 2014;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2014/06/05/CIRCRESAHA.115.304262

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/06/05/CIRCRESAHA.115.304262.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIALS

Pandey et.al., OxLDL Triggers Retrograde Translocation of Arginase 2

Deepesh Pandey¹
Anil Bhunia¹
Young Jun Oh¹
Fumin Chang¹
Yehudit Bergman¹
Jae Kim Hyung¹
Janna Serbo¹,²
Tatiana N. Boronina⁶
Robert N. Cole⁶
Jennifer Van Eyk⁷
Alan T. Remaley³
Dan E. Berkowitz¹,²*
Lewis Romer¹,²,³,⁴,⁵*

Departments of ¹Anesthesiology and Critical Care Medicine, ²Biomedical Engineering, ³Cell Biology, ⁴Pediatrics, and the ⁵Center for Cell Dynamics, ⁶Mass Spectrometry and Proteomics Facility, ⁷Departments of Medicine and Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21287-4904, and ⁶Cardiovascular-Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD.

* Denotes equal contribution
MATERIALS AND METHODS

Cell culture and transfection
HAEC were maintained in ECM culture medium (Science Cell Research Laboratories, Carlsbad, CA) according to the supplier’s protocol. Confluent HAEC were serum-starved by incubation in ECM media containing 0.5% serum for 24 hours prior to Ox-LDL treatment. 293 cells were maintained in DMEM media (GIBCO) containing 10%FBS and 1% Pen-Strip. 293 cells were transfected using Lipofectamine 2000 (Invitrogen) as per manufacturer’s protocol and HAEC were transfected using Amaxa transfection system (Lonza).

DNA Constructs
Human FLAG Arg2 constructs were created from existing cDNA (Origene) via PCR by using the following primers.
Forward: 5’ATG TCC CTA AGG GGC AGC CTC TCG CGT CTC CTC CAG ACG CGA G 3’;
Reverse: 5’ TTA CTT ATC GTC GTC ATC CTT GTA ATC TCT CAC ACG 3’.
Truncated mutants were created by PCR using full length Arg2-Flag as a template to create truncation mutants.

Primer sequences used to create the 1-Δ22 Arg2 truncation were
Forward: 5’ GTCCCTAAGGGGCGTCCACTCCGTGG 3’;
Reverse: 5’ CCACGGAGTGGACGCCCCTTGGGAC 3’.

Primer sequences for the 1-Δ40 Arg2 truncation were :
Forward: 5’ TGTCCCTAAGGGGCGTGGAGCATGGTCC 3’;
Reverse: 5’ GGACCATGCTCCACGCCCCTTGGGACA 3’.

Arg2 was tagged at the C-terminus with GFP by using PEGFN1 vector (Clonetech) and following primers that introduce XhoI and EcoRI restriction sites in frame with C-terminus GFP using following primers:
Forward: 5’ CTC TCG AGC AAT GTC CCT AAG GGG CAG C 3’
Reverse: 5’ CGC GAA TTC AAT TCT CAC ACG TTG ATG AT 3’

Animals and reagents
All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at The Johns Hopkins University School of Medicine. Transgenic mice with Tie2 promoter-driven endothelial-specific Arg2 overexpression were used in vessel staining experiments[33]. LOX-1-/- mice were obtained from the Dr. Tatsuya Swamura (National Cardiovascular Center Research Institute, Osaka, Japan) and all other control mice were purchased from Jackson Laboratory. ApoE-/- mice were bred on Arg2-/- background mice to generate Arg2-/-/ApoE-/- mice (Harlan lab). Mice that were homozygous for deletions at both alleles were identified, and the offspring were genotyped for apolipoprotein E and arginase 2 using polymerase chain reaction. Male mice, 8-12 weeks old with either the Arg2-/- or ApoE-/- deletion or both were
fed with high fat rodent diet containing 1.25% cholesterol (Research Diet, New Brunswick NJ, USA) for 4 months, while age-matched wild type controls (WT; C57BL/6) were fed a normal diet. After 4 months of feeding, mice were euthanized and aortic tissue was used in the experiments described. Ox-LDL was purchased from Intracel Co (Frederick, MD). Y-27632 and O-phenanthroline were purchased from Calbiochem (Darmstat, Germany). Unless otherwise stated, all other reagents were obtained from Sigma.

Preparation of Aortas
Heparin was administered 1 hour before mice were sacrificed. The animals were euthanized and the aorta was dissected from aortic root to the bifurcation of the iliac arteries and immersed in Krebs solution containing (in mM: 118 NaCl, 4.7 KCl, 1.2 KH2PO4, 2.5 CaCl2, 1.2 MgSO4, 25 NaHCO3, and 11.1 glucose). Vessels were carefully cleaned to remove connective tissue. For the arginase activity assay, aortic tissue was immediately frozen in liquid nitrogen and stored at -80°C until the time of the assay.

Gross Pathological Assessment of Plaque
Aortas were fixed with 4% paraformaldehyde overnight. The aorta was opened longitudinally and pinned onto a wax surface by microneedles and the images of the submerged vessels were captured with a digital camera. The lipid-rich intraluminal lesions were stained with Sudan IV. Digitized images were transferred to a PC and analyzed using NIH Java Image (Image J, version 1.42n). Thereafter, aortic roots were embedded in paraffin, and cross serial 1 mm sections were prepared and stained with hematoxylin-eosin to evaluate the atherosclerotic lesion area. Plaque thickness was measured by light microscopy. The burden of aortic atheroma in each animal was recorded as percentage of the total area for each sample aorta that was occupied by plaque.

Adenoviral shRNA constructs:
Ad-shNontargeted, Ad-MPPα, Ad-MPPβ, Ad-shArg1 and Ad-shArg2 encoded viruses were generated using a pAdBLOCK-iT kit (Life Sciences). Briefly, oligonucleotides that were nontargeted, and others targeting 2 different regions of Human MPPα, MPPβ, Arg1, and Arg2, Mice MPPα & MPPβ were designed with proprietary software from Life Sciences and cloned into pU6-ENTR. Sequences used were as follows.
Non targeted: Top, 5’-CAC CGA TGG ATT GCA CGC AGG TTC TCG AAA GAA CCT GCG TGC AAT CCA TC-3’; Bottom, 5’-AAA AGA TGG ATT GCA CGC AGG TTC TTT CGA GAA CCT GCG TGC AAT CCA TC-3’.
Arg1sh#A: Top, 5’-CAC CGG GAT TAT TGG AGC TCC TTT CCG AAG AAA GGA GCT CCA ATA ATC CC-3’; Bottom, 5’-AAA AGG GAT TAT TGG AGC TCC TTT CTT CGG AAA GGA GCT CCA ATA ATC CC3’;
Arg1sh#B: Top, 5’- CAC CGG AGA CAA AGC TAC CAC ATG TCG AAA CAT GTG GTA GCT TTG TCT CC-3’; Bottom, 5’- AAA AGG AGA CAA AGC TAC CAC ATG TTT CGA CAT GTG GTA GCT TTG TCT CC-3’;

Arg2sh#A: Top, 5’- CAC CGG TTC TTT AGC TGT CAC TTA GCG AAC TAA GTG ACA GCT AAA GAA CC-3’; Bottom, 5’- AAA AGG TTC TTT AGC TGT CAC TTA GTT CGC TAA GTG ACA GCT AAA GAA CC-3’;

Arg2sh#B: Top, 5’- CAC CGC ATT CCA TCC TGA AGA AAT CCG AAG ATT TCT TCA GGA TGG AAT GC-3’; Bottom, 5’- AAA AGC ATT CCA TCC TGA AGA AAT CTT CGG ATT TCT TCA GGA TGG AAT GC-3’.

Human MPPαsh#A: Top, 5’- CAC CGC GTG GCA TCT CAG AAT AAG TCG AAA CTT ATT CTG AGA TGC CAC GC-3’; Bottom, 5’- AAA AGC GTG GCA TCT CAG AAT AAG TTT CGA CTT ATT CTG AGA TGC CAC GC-3’;

Human MPPαsh#B: Top, 5’- CAC CGC CTG TGA TCT TCG AGG ATG TGA GAA CAT CCT CGA AGA TCA CAG GC-3’; Bottom, 5’- AAA AGC CTG TGA TCT TCG AGG ATG TTC TCA CAT CCT CGA AGA TCA CAG GC-3’;

MiceMPPαsh#A: 5’- CAC CGC ACC ACT GGA TGT ACA ATG CCG AAG CAT TGT ACATCC AGT GGT GC-3’; Bottom, 5’- AAA AGC ACC ACT GGA TGT ACA ATG CTT CGG CAT TGT ACA TCC AGT GGT GC-3’;

MiceMPPαsh#B: 5’- CAC CGC CTA AAG GTA TTT CAC AAT CCG AAG ATT GTG AAA TAC CTT TAG GC-3’; Bottom, 5’- AAA AGC CTA AAG GTA TTT CAC AAT CTT CGG ATT GTG AAA TAC CTT TAG GC-3’;

HumanMPPβsh#A: Top, 5’- CAC CGC TGC GGG ACG GTC ATT ATA TCG AAA TAT AAT GAC CGT CCC GCA GC-3’; Bottom, 5’- AAA AGC TGC GGG ACG GTC ATT ATA TTT CGA TAT AAT GAC CGT CCC GCA GC-3’;

HumanMPPβsh#B: Top, 5’- CAC CGC ACA CAC AAA GGA GAA ATA CCG AAG TAT TTC TCC TTG TGT GC-3’; Bottom, 5’- AAA AGC ACA CAC AAA GGA GAA ATA CTT CGG TAT TCT TTC TCC TTG TGT GC-3’;

MiceMPPβsh#A: Top, 5’- CAC CGC TCA TCT TAA CGC CTA TAC CCG AAG GTA TAG GCG TTA AGA TGA GC-3’; Bottom, 5’- AAA AGC TCA TCT TAA CGC CTA TAC CTT CGG GTA TAG GCG TTA AGA TGA GC-3’;

MiceMPPβsh#B: Top, 5’- CAC CGC AGA TGC TAT GCT ATA ATA GCG AAC TAT TAT AGC ATA GCA TCT GC-3’; Bottom, 5’- AAA AGC AGA TGC TAT GCT ATA ATA GTT CGC TAT TAT AGC ATA GCA TCT GC-3’.

The resulting pU6-sh-Nontargeted, pU6-Arg1shRNA and pU6-Arg2shRNA plasmids were tested for function in transient transfection experiments with 293A
cells. The constructs showing the greatest inhibition were LR recombined with pAD/BLOCK-iTDEST (Invitrogen) to generate pAd-Nontargeted, Ad-MPPα, Ad-MPPβ, pAd-shArg1 and pAd-shArg2. Viruses were amplified, purified, and concentrated using a Millipore Kit.

An adenoviral construct containing GFP Arg2 was constructed by subcloning C-terminally tagged Arg2-GFP into PENTR1a and finally into the PDEST destination vector.

Subcellular fractionation
Mitochondrial and cytosolic components were prepared by cell fractionation using a mitochondrial separation kit (Clonetech). Protein quantity was determined using the Biorad protein assay kit. Fractionated samples were boiled with SDS sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% Glycerol and 5% βME) and subjected to western blotting. Purity of the fractions was measured by western blotting for LDH and MnSOD.

Immunoprecipitation and Western blotting
After 48hrs of 293 HEK or HAEC transfection, cells were lysed in ice-cold modified lysis buffer consisting of 20 mM Tris–HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 1% sodium deoxycholate, 1 mM Na3VO4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 µg/mL leupeptin, and 1:1000 diluted protease inhibitor cocktail (Sigma). For immunoprecipitation studies, whole cell lysate lysates were centrifuged at 14,000 x g and supernatant were precleared by incubation with Protein A/G-agarose beads for 2 h at 4º C with rocking. Agarose beads were then pelleted by centrifugation at 1,000 x g. FLAG-Arg2 or endogenous Arg2 in precleared lysates were immunoprecipitated by incubation overnight at 4º C with rocking following addition of anti-FLAG (Agilent) or anti-Arg2 antibody (Santa Cruz) (10 µl). Immune complexes were eluted in 2x SDS sample buffer by boiling for 5 minutes before loading into SDS–PAGE. Western blotting analysis was performed by transferring the gels onto PVDF membranes, and visualized using primary antibodies to target proteins and secondary antibodies conjugated to alkaline phosphatases.

Endothelial Imaging in murine aortas
Mice aortic segments were permeabilized for 20 min with 0.5% Triton X-100 (Fisher Scientific) in 3% paraformaldehyde (Sigma) followed by fixation with 3% paraformaldehyde for 30 min at room temperature. Aortas were incubated in goat serum (1.5%, 1 hour) followed by Arg2 rabbit polyclonal primary antibody (as above) and goat anti rabbit Cy3 secondary antibody (Jackson ImmunoResearch Laboratories (West Grove, PA) incubation for 2 hour each. To visualize nuclei, aortas were incubated with DAPI for 15 min. Aortic segments were cut open to expose the intima and mounted on coverslips using Fluorosave. Images were captured with a Zeiss 710-NLO confocal unit mounted on an Axio Examiner body with 3 channel spectral module PMT (Oberkochen, Germany), a
Immunofluorescence
FLAG-Arg2-transfected HAEC were incubated with 100nM of Mitotracker Red CMXRos (Invitrogen) for 45 min to stain mitochondria followed by fixation with 3.7% formaldehyde for 15 minutes. After fixation cells were rinsed several times with PBS and permeabilized using 0.2% Triton X-100 for 10 minutes. Cells were rinsed several time with PBS and stained with appropriate antibodies. DAPI was used to stain nuclei. Cells were observed on an epifluorescence Nikon TE-200 microscope. Images were captured with a Rolera EMCCD camera (Q-Imaging, BC, Canada) with Volocity software (PerkinElmer, Lexington, MA).

For time lapse studies and movies, HAEC of passage between 3 and 5 were transfected with GFP-Arg2 using the Nucleofector 96-well shuttle system (Amaxa Biosystems, Gaithersburg, MD). One day after transfection, cells were trypsinized and seeded onto fibronectin-coated 35 mm glass bottom dishes (Plastek Cultureware, Ashland, MA) for one hour before cells exposed to new medium with or without OxLDL (50 µg/ml). Alternatively, HAEC were seeded on fibronectin-coated 35 mm glass bottom dishes and transduced with Ad-GFP-Arg2 overnight to achieve close to 100% transduction rate. Transduced cells were serum-starved for 24 hours and then treated with OxLDL. Epifluorescence images were acquired at 1-min intervals for up to one hour after OxLDL treatment using a Nikon TE200 microscope (Melville, NY), QImaging camera (Surrey, BC, Canada), and Velocity software (Improvision, Lexington, MA), and then converted to QuickTime movie format. An objective heater (Bioptechs, Eugene, OR) was used to maintain the media temperature at 37°C throughout the image acquisition.

Mass spectrometry analysis
Cytosol and mitochondria were fractionated and immunoprecipitated from EC (~6 x 10^6 cells per data point) using Arg2 polyclonal antibody (above). Samples were then resolved with 10% SDS-PAGE and stained with Coomassie brilliant blue. Gel bands corresponding to both control and Ox-LDL treated samples were excised, digested with Lys-C and subjected to mass spectrometry analysis. Protein identification by liquid chromatography tandem mass spectrometry (LCMS/MS) analysis of peptides was performed using an LTQ ion trap MS (Thermo Fisher Scientific) interfaced with a 2D nanoLC system (Eksigent, Dublin, CA). Peptides were fractionated by reverse-phase HPLC on a 75 um x 100 mm C18 column. Tandem mass spectra were extracted by Proteome Discoverer 1.2 (Thermo Scientific) and analyzed using Mascot 2.2 (Matrix Science, London) to search the NCBInr_20080819 database, selected for Mammalia (401346 entries). Mascot search criteria included the digestion enzyme lys-C allowing 1 missed cleavage, mass tolerances of 0.80 Da for fragment ions and 1.5 Da for parent ion, and oxidation of methionine as a variable modification. Mascot search results files were imported into Scaffold 3.6 (Proteome Software Inc., Portland) to validate MS/MS based peptide and protein identifications. Peptide identifications
were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [34]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm[35]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Fragmentation spectra containing HSVAVIGAPFSQGQK identified by Mascot were confirmed and annotated manually.

**Edman degradation analysis**

293 cells overexpressing FLAG-tagged Arg2 was immunoprecipitated with anti-FLAG antibody. The C-terminal FLAG-Arg2 complex was eluted in 2x SDS sample buffer by boiling for 5 minutes before loading into 10% SDS–PAGE. The gel were stained with Coomassie blue (Biorad) and the band corresponding to Arg2 molecular weight was excised and sent to New England Peptide (Gardner, MA) for N-terminal amino acid sequencing via Edman degradation for 6 cycles. Manual validation of the chromatograms was carried out to confirm amino acid sequences.

**Arginase activity assay**

Arginase activity was determined using the urea assay using α -isonitrosopropiophenone as described previously [36]. 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 x g at 4°C.

**Measurements of aortic NO production and superoxide O$_2^-$**

Aortic strips were isolated from 10-week-old male LOX-1-/- or wild type C57BI/6 mice, pinned down on silastic with the endothelial side up, and exposed in the dark to either 5µmol/L DAF2-DA for 2 h to measure NO, or to 0.1µM DHE for 30 m. Images were acquired using a NikonTE-200 epifluorescence microscope. To confirm that superoxide and NO were produced by eNOS, the NOS inhibitor L-NAME was used as a control. Rates of NO and superoxide production were calculated as the slope of the fluorescence measured over time. Where noted in the text endothelium was denuded from the aorta using a wire.

In additional experiments superoxide and NO production were determined using the Luminol analog L-012, and a Siever’s NO analyzer respectively. To measure superoxide, HAEC cells were plated into white TC treated 96-well plates (ThermoLabsystems) at a density of approximately 5x10$^4$ cells per well. The cells were incubated at 37°C in phenol free DMEM (Sigma) containing 400µM of the luminol analogue L-012 (Wako) for a minimum of 20 minutes prior to the addition of agonists [37]. Luminescence was quantified over time using a FlexStation 3 microplate reader (Molecular Devices). The specificity of L-012 for reactive oxygen species was confirmed by co-incubation with the superoxide scavenger SOD (5mM), and this yielded virtually undetectable levels of
luminescence under control or OxLDL-stimulated conditions. Thus the relative light units (RLU) quantified from the luminescence of L-012 were reflective of changes in production of superoxide. NO release was determined by a chemiluminescence assay using Siever NO analyzer equipment that determines NO byproduct: Nitrite accumulation in cell culture medium. Briefly, media containing Nitrite was refluxed into glacial acetic acid containing 65mM sodium iodide that reduced Nitrite into Nitric Oxide. NO is further purged onto a reaction vessel with Nitrogen and Ozone which produces chemiluminescence that is quantified by the Siever’s analyzer and expressed as relative light units (RLU).

**Measurement of L-Arginine concentrations**
L-Arginine concentrations in both cytosolic and mitochondrial fractions were measured using a Biochrom-20 amino acid analyzer (Cambridge, UK) according to the manufacture’s protocol.

**Measurement of mitochondrial potential**
HAEC were incubated with 150nM TMRM (Tetramethylrhodamine, methyl ester, Perchlorate) at 37°C for 5min prior to 50uM Ox-LDL exposure for 2hr with or without CCCP (Carbonyl cyanide m-chlorophenyl hydrazone, 10um) for 30m. Absorbance data (Excitation at 560nm, Emission at 595nm) were read at on a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA).

**Statistical analysis**
Each graph represents cumulative data from 3–5 independent experiments, and each individual assay was performed in triplicate. Statistical significance was determined by one-way ANOVA (mean ± SEM) with a post-hoc test or t-test (mean ± SEM) (Graphpad Prism software). P values are supplied in each figure legend, and significance was adjudged to be present for all data at p values less than 0.05.
Supplemental Figure I. A. HAEC were plated at ~70-80% of confluence one day before cells were transduced with adenoviruses encoding Arg1, Arg2 or MPPα. After 48 hours, cells were lysed and subjected to arginase activity (upper panel) and immunoblotting (bottom panel) with Arg2 and GAPDH antibody. B. Arg1shRNA-transduced-HAEC were incubated with IL4 for the final 24 hours and cell lysates were subjected to immunoblotting with Arg1 antibody. C. MPPα shRNA-transduced HAEC were incubated for 48 hours and cell lysates were subjected to immunoblotting with anti-MPPα antibody.
Supplemental Figure II. Release of GFP-Arg2 from mitochondria induced by OxLDL. HAEC were transfected with GFP-Arg2 for one day and then treated with OxLDL (50 µg/ml) (A) or transduced with Ad-GFP-Arg2 for 18 hours followed by serum starvation for 24 hours before treated with OxLDL 50 µg/ml (B). Transfected or transduced HAEC were seeded onto fibronectin-coated 35 mm glass bottom dishes and images were taken at 1 min intervals for up to one hour immediately (A) or 18 hours after (B) OxLDL was added. The yellow, boxed region was magnified (approximately 4X) and is shown in the insets at the lower right corners. Scale bar denotes 10 nm.

Please see Supplemental movie files I, II, and III for serial documentation of these dynamic events.
Supplemental Figure III. After 24 hours of serum starvation, HAEC were exposed to 50 µM OxLDL for 2 hours. OxLDL exposure was tested either alone or in the presence of either Oph or ABH, and the effects of each inhibitor alone were also tested. Cells treated with the mitochondrial uncoupler, CCCP (10µM) were used as a positive control for disruption of mitochondrial membrane potential (as detected by a drop in TMRM fluorescence), and this change was not seen in any of the experimental treatment groups. All groups were loaded with 150nM TMRM, and incubated at 37°C for 5 min just before fluorescence was measured using a plate reader (excitation 560nm and emission 595nm). *p < 0.05 vs Control.
Supplemental Figure IV. Following 24h of serum starvation, HAEC were exposed to 50 µg/mL of OxLDL for 2h, either alone or in presence of Oph. Mitochondria and cytoplasm were separated and L-arginine concentrations were measured using a Biochrom-20 amino acid analyzer. *p < 0.05 vs Control(Cy), #p < 0.05 vs OxLDL(Cy), $p < 0.05 vs Oph+OxLDL (Cy), $p < 0.05 vs Con (Mito and Cy)
Supplemental Figure V. HAECs were transduced with adenoviruses encoding shRNA for either A) MPPα or B) MPPβ. 48 hours later, post-transduction cell lysates were collected and subjected to immunoblotting with MPPα, MPPβ and GAPDH antibodies.
**Supplemental Figure VI.** A. HAEC were transfected with either FLAG-tagged PINK1 alone or co-transfected with GFP-tagged active Rho Kinase or dominant negative Rho kinase. Cell lysates were subjected to immunoblotting with FLAG, GFP and GAPDH antibodies 48 hours after transfection. B. HAEC were transfected with FLAG-tagged PINK1 and incubated with or without OxLDL (50 µg/mL) and in the presence or absence of the ROCK inhibitor Y27632 (10µM) for 24 hours. Cell lysates were then subjected to immunoblotting with FLAG and GAPDH antibodies.
Supplemental Figure VII. L-NAME ablated the dose-response effects of acetylcholine (Ach) on vascular relaxation in isolated aortas from ApoE-/- mice and Dbl-KO (Arg2-/-/ApoE-/-) mice that had been fed a high cholesterol diet. Measurements were obtained using wire myography. N=6.
Supplemental Figure VIII. Arginase 2 was tagged with GFP at the C-terminus and the chimeric Arg2-GFP was cloned into an adenoviral vector. Activity (A), Expression (B), and Localization (C) of the chimera were determined in HAEC that were transduced with Arg2-GFP.
Supplemental Figure IX. Microscopic grading of aortic production of ROS by DHE (dihydroethidium bromide) (5 nmol/L) (panel A), and NO by DAF-FM-DA (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate) (5nmol/L) (panel B). Slopes of basal NO and ROS production were normalized by initial fluorescence intensity. * indicates p < 0.05 vs Con, ** indicates p < 0.001