OxLDL Triggers Retrograde Translocation of Arginase2 in Aortic Endothelial Cells via ROCK and Mitochondrial Processing Peptidase

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Rationale: Increased arginase activity contributes to endothelial dysfunction by competition for l-arginine substrate and reciprocal regulation of nitric oxide synthase (NOS). The rapid increase in arginase activity in human aortic endothelial cells exposed to oxidized low-density lipoprotein (OxLDL) 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 on mitochondrial processing peptidase, lectin-like OxLDL receptor-1 receptor, and rho kinase.

Methods and Results: OxLDL-triggered translocation of Arg2 from mitochondria to cytosol in human aortic endothelial cells and in murine aortic intima with a concomitant rise in arginase activity. All of these changes were abolished by inhibition of mitochondrial processing peptidase or by its siRNA-mediated knockdown. Rho kinase inhibition and the absence of the lectin-like OxLDL receptor-1 in knockout mice also ablated translocation. Aminoterminal sequencing of Arg2 revealed 2 candidate mitochondrial targeting sequences, and deletion of either of these confined Arg2 to the cytoplasm. Inhibitors of mitochondrial processing peptidase or lectin-like OxLDL receptor-1 knockout 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 reactive oxygen species production, enhanced NO, and improved endothelial function when compared with ApoE−/− controls.

Conclusions: These data demonstrate dual distribution of Arg2, a protein with an unambiguous mitochondrial targeting sequence, in mammalian cells, and its reverse translocation to cytoplasm by alterations in the extracellular milieu. This novel molecular mechanism drives OxLDL-mediated arginase activation, endothelial NOS uncoupling, endothelial dysfunction, and atherogenesis. (Circ Res. 2014;115:450-459.)

Key Words: arginase atherosclerosis mitochondria mitochondrial processing peptidase nitric oxide synthase type III oxidized low density lipoprotein

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.1,2 A major target for OxLDL-mediated vascular injury in atherosclerosis is the aortic intima.3,4 Most of these effects occur through OxLDL interaction with its lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1).5 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.6

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An emerging paradigm in NO biology is the concept that arginase (Arg) reciprocally regulates NOS activity by competing for \(\text{l-arginine} \) substrate and effectively inhibiting NO-dependent processes by depleting the substrate pool available for NO biosynthesis. Furthermore, because \(\text{l-arginine} \) is spatially confined to a minimum of 3 distinct subcellular pools that are regulated by different transporters and enzymes, local concentrations of \(\text{l-arginine} \) 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 lipopolysaccharide (LPS), tumor necrosis factor (TNF-\(\alpha\)), and hypoxia. The role of Arg2 in endothelial dysfunction has 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 human aortic endothelial cells (HAEC) in a rapid, dose-dependent manner that leads to impaired endothelial NO synthesis. However, the signaling pathways that connect OxLDL exposure to Arg2 in EC remain incompletely defined. Thrombin increases Arg2 activity in EC via the Rho/\(\text{Rho kinase (ROCK)} \) signaling pathway, and RhoA may also be important in OxLDL-mediated Arg2 activation in EC. Another clue about its regulation is that Arg1 is expressed in ECs. Arg1 and Arg2 are known to be the predominant arginase isoforms in HAEC, some reports indicate that Arg1 is expressed in ECs. To determine the contribution of individual Arg 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 <25% of control levels, whereas Arg1 knockdown had little effect on total HAEC arginase activity (Online Figure 1A). 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

### Methods

Detailed Methods are found in the Online Data Supplement, which includes the following:

- **Nonstandard Abbreviations and Acronyms:**
  - Arg2: arginase 2
  - DKO: double knockout
  - eNOS: endothelial nitric oxide synthase
  - HAEC: human aortic endothelial cells
  - LOX-1: lectin-like oxidized low-density lipoprotein receptor-1
  - MPP: mitochondrial processing peptidase
  - MS: mass spectrometry
  - MTS: mitochondrial targeting sequences
  - OxLDL: oxidized low-density lipoprotein
  - ROCK: rho kinase
  - ROS: reactive oxygen species
  - WT: wild-type

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-mitochondrial processing peptidase (MPP) \(\alpha\), Ad-MPP\(\beta\), Ad-shArg1, and Ad-shArg2–encoded viruses were generated using a pAdBLOCK-iT kit (Life Sciences). An adoviral construct containing green fluorescent protein (GFP) Arg2 was constructed by subsequent subcloning of C-terminal GFP epitope-tagged Arg2 into the PENTR1a entry 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 OxLDL-treated samples were excised, digested with LysC, and subjected to mass spectrometry (MS) analysis. Protein identification by liquid chromatography tandem MS analysis of peptides was performed using an LTQ ion trap MS (Thermo Fisher Scientific) interfaced with a 2-dimensional nanoLC system (Eksigent, Dublin, CA).

Superoxide and NO measurements were determined using the Luminol analog L-012, and a Siever 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. This time course is consistent with translocation between subcellular compartments and post-translational modification(s). Serum-starved HAEC were incubated with 50 \(\mu\)g/mL OxLDL for 2 hours. OxLDL exposure promoted robust translocation of Arg2 from mitochondria to the cytosol (Figure 1A). To confirm that Arg2 is predominantly targeted to mitochondria, FLAG epitope-tagged Arg2 was expressed in HAEC cells (Figure 1C and 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 when 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 ECs.

To determine the contribution of individual Arg 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 <25% of control levels, whereas Arg1 knockdown had little effect on total HAEC arginase activity (Online Figure 1A). 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
OxLDL–Mediated Cytosolic Release and Activation of Endothelial Arg2 Are Dependent on the LOX-1 Receptor and ROCK Activity

We have previously demonstrated that OxLDL–mediated increases in EC Arg activity are dependent on the LOX-1 receptor. Here, we measured Arg2 quantity and activity in cytotoxic and mitochondrial fractions of primary cultures of aortic ECs isolated from wild-type (WT) and LOX-1 receptor knockout (LOX-1−/−) mice (Figure 2A and 2B). This supports our previous finding that LOX-1 mediates acute OxLDL–induced increases in the activity of Arg2.

Our previous data 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 preincubated HAEC with the specific ROCK inhibitor Y-27632 (10 μmol/L Y-27632; 2 hours), which obviated both OxLDL–induced cytotoxic translocation of Arg2 and the associated increase in arginase activity (Figure 2C and 2D).

Truncated Arg2 (Without the Mitochondrial Targeting Sequences) 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, the MTS for Arg2 is still undefined and bioinformatics-based predictions of MTS of dual-targeted genes have shown to be inaccurate. Identification and characterization of the Arg2 MTS were undertaken to understand the dual targeting of this protein and its regulation by the OxLDL stimulus. We used MS and N-terminal sequencing using Edman 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 immunoprecipitation 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. Because the N terminal 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 (SVHSVAVIGAPFSQGQK). To investigate the amino acid(s) that form the N terminus of full-length Arg2 further, Edman degradation analysis was performed on immunoenriched C-terminal Flag-Arg2 isolated from HEK-293 cells. The N-terminal amino acid sequences obtained were from 2 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 MPP because 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 2 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 to 22 or 1 to 40 resulted in almost exclusive cytoplasmic distribution of Arg2 (Figure 4B and 4C). 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 Arg activity.

OxLDL-Induced Translocation and Activation of Arg2 in HAEC Involves the MPP

The MTS and putative MPP cleavage site in the N terminus of human Arg2 that we identified by MS and Edman degradation analysis are regions of Arg2 that are highly conserved among species (Figure 5A). Given the redistribution of mitochondrial Arg2 after 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, 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, while inhibiting Arg activity, did not prevent OxLDL-induced Arg2 translocation to the cytosol (Figure 5B and 5C). In addition, MPPα knockdown blocked cytosolic redistribution of Arg2 with OxLDL exposure and prevented Arg2 activation in response to OxLDL (Figure 5D and 5E). However, 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 ≥50% in HAEC (Figure 5F).

Immunofluorescence localization experiments were then conducted in native vascular ECs in aortic strips from transgenic
mice with Tie2 promoter-driven endothelial-specific Arg2 overexpression. Arg2 was localized 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 O-phenanthroline (OPH). The same redistribution pattern was observed in Arg2-GFP–transduced HAEC (Figure 6B; Online Figure VIII). In addition, although 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 multiplicity of infection (MOI) of control shRNA (nontargeted) exhibited attenuated relaxation responses to acetylcholine (endothelial dysfunction). This was substantially improved in aortic rings that were transduced with 100 MOI of adenoviruses with shRNA for either MPPα or MPPβ when added 24 hours before OxLDL stimulation (Figure 6C and 6D). MPPα and MPPβ knockdown in these vessels were verified by immunoblotting with MPPα- and MPPβ-specific antibodies (Online Figure V).

The process termed eNOS uncoupling occurs under several pathological 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. 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...
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Nanoscale ROS generation in aortas from LOX-1− mice. In addition, endothelium-denuded (E-) strips demonstrated 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 NO analyzer to determine NO production. The specificity of L-012 for superoxide was examined by treating cells with the superoxide scavenger (superoxide dismutase; 20U/mL). Superoxide dismutase 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 superoxide dismutase. Adenoviral knockdown of either Arg2 or MPPα with human-specific shRNA significantly reduced OxLDL-dependent increases in ROS (Figure 7E), and MPPβ knockdown obviated the drop in NO that was caused by OxLDL (Figure 7F).

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 the 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 (Online Figure IV). Inhibition of MPP with O-phenanthroline before OxLDL exposure prevented this OxLDL-dependent decrease in cytosolic l-arginine.

**OxLDL Increases 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 or dominant negative mutants of ROCK were expressed in HAEC (Online Figure VI). Cleaved PINK1 increased in the presence of constitutively active-ROCK, whereas PINK cleavage did not change when control GFP cDNA or dominant negative-ROCK were coexpressed. The negligible effect observed with dominant negative-ROCK may be attributed to its lower expression level when compared with active mutant (third segment of Online Figure VIA). HAEC-expressing FLAG-PINK1 were also treated with OxLDL alone or in combination with the ROCK inhibitor Y-27632 (Online Figure VIB). OxLDL upregulated PINK1 cleavage (and thus MPP activation), and this was inhibited by Y-27632. Taken together, these findings suggest a signaling cascade that is initiated by OxLDL and extends through RhoA and ROCK to MPP.

**Arg2 Knockout Reduces Atheromatous Plaque Burden, Improves Endothelial Function, Enhances NO, and Reduces ROS in ApoE−/− Mice**

Endothelial dysfunction and inflammation are 2 critical events that drive atherosclerosis. To investigate the role of Arg2 in endothelial inflammation, we examined OxLDL-dependent nuclear factor kappa light chain enhancer of activated B cells (NFκB) activity in the presence or absence of amino-2-borono-6-hexanoic acid. 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 amino-2-borono-6-hexanoic acid (Figure 8A).

Aortic atheromatous plaque burden was then determined in ApoE−/− and Arg2−/−/ApoE−/− double knockout (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 when compared with ApoE/− mice (178.6±25.6 versus 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% versus 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 DKO mice that were fed a high cholesterol/high-fat diet. Endothelial-dependent vascular relaxation in response to acetylcholine was significantly improved in aortas from DKO mice that were fed a high cholesterol diet. Aortic strips from wild-type or LOX−/− mice were exposed to OxLDL alone, OxLDL with 5 mmol/L O-phenanthroline (Oph), or OxLDL plus L-Nω-nitroarginine methyl ester (L-NAM; 100 μmol/L). A, Reactive oxygen species (ROS) production was measured as the slope of dihydroethidium (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, Human aortic endothelial cells (HAECs) production of ROS was determined by L-012 chemiluminescence after incubation with or without superoxide dismutase (SOD; 20 U/mL) and expressed as relative light units (RLU). E, HAECs were incubated with 100 multiplicity of infection (MOI) of nontargeted (NT), MPPα, or MPPβ shRNA adenoviruses. Twenty-four-hours after transduction, media were replaced with fresh media with or without OxLDL (50 μg/mL). After 48 hours, the production of ROS was determined in the presence or absence of SOD (20 U/mL) via L-012 chemiluminescence. F, HAECs were incubated with 100 MOI of NT shRNA or MPPα shRNA adenoviruses. Cells were treated the next day with control medium or OxLDL (50 μg/mL). After 48 hours, the production of NO was determined by measuring nitrite levels with Siever NO analyzer, mean±SE are shown, n=6. *P<0.05 vs control (Con), #P<0.05 vs OxLDL.

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 when compared with those measured in samples from ApoE−/− mice (Online 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 on processing by MPP. Furthermore, using LOX−/− KO mice and ROCK 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 ROCK-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 electric potential that exists across the mitochondrial inner membrane. Some proteins, such as fumarase, may undergo bidirectional trafficking between cytosol and mitochondria based on processing by MPP.

Our data indicate that Arg2 is dual-targeted to mitochondria and the cytosol, thereby adding Arg2 to several other proteins that share this distinction—up to one quarter of the mitochondrial proteome in some species. There are ≥4 potential mechanisms by which a single translation product may be present in dual compartments. These include an ambiguous targeting sequence recognized by ≥1 organelle, multiple targeting sequences; differential targeting accessibility; and reverse translocation—a mechanism that may be dependent on 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 because the inhibition of MPP in HAEC by an MTS (24 amino acids at the N-terminal sequence) that contains its structural complex in an active conformation.46

Recent studies demonstrate that arginine succinate lyase supports the concept that specific subcellular pools of l-arginine do exist. We speculate that Arg2 in closer proximity to binding and regulatory proteins that enhance its function after its translocation to cytosol. Recent studies demonstrate that arginine succinate lyase enhances NO production by NOS through recycling citrulline to l-arginine and maintaining the integrity of the NOS complex. Similarly, cytosolic arginine succinate lyase could recycle l-citrulline to l-arginine and provide substrate for Arg2 and maintain its structural complex in an active conformation.

Activation of ROCK has been linked to the acceleration of atherosclerosis in a mouse model. Furthermore, we have demonstrated that RhoA and ROCK are critical to 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 Krebs cycle enzyme fumarase is distributed between the mitochondria and the cytoplasm based on metabolic cues from the glycolytic 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 was comparable. This finding suggests that cytosolic Arg2 is more active than mitochondrial Arg2. This could clarify some controversies on 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 2 separate compartments on cellular stress, and that Arg2 can constrain eNOS activity supports the concept that specific subcellular pools of l-arginine do exist.

Figure 8. Arginase 2 (Arg2) inhibition ameliorates atherogenesis in ApoE knockout mice. A. Human aortic endothelial cells expressing nuclear factor kappa light chain enhancer of activated B cells (NF-kB)-luciferase (LUC) were incubated with oxidized low-density lipoprotein (OxLDL; 50 μg/mL) alone or with amino-2-borono-6-hexanoic acid (ABH; 200 μmol/L) for 8 hours, and luciferase activity was determined using chemiluminescence. B. Quantitative graph (top) of plaque formation in histological sections of ascending aortas (bottom). C. Cumulative quantitative assessment (top) of the plaque area determined by pixel count of Sudan IV-stained areas of the lipid-rich intraluminal lesions in longitudinally opened aortas (bottom). D and E. Dose-response effects of acetylcholine ACh (D) and sodium nitroprusside (SNP; E) on vascular relaxation in isolated aortas from ApoE−/− mice and double knockout (Dbi-KO; Arg2−/−/ApoE−/−) mice fed with high cholesterol diet were determined.

*P<0.05 vs control, **P<0.001, #P<0.05 vs OxLDL without ABH.
OxLDL-dependent increases in EC Arg activity,26 In the current study, we present data demonstrating that inhibition of ROCK with Y-27632 prevented rises in both cytosolic Arg2 content and activity after OxLDL stimulation. These new data suggest that ROCK facilitates OxLDL-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 t-arginine availability, changes in t-arginine concentrations in response to OxLDL have not been measured in individual cellular compartments. Our data demonstrate that OxLDL significantly diminished cytosolic t-arginine concentrations (Online 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. Furthermore, inhibition of MPP both attenuated the increase in cytoplasmic arginase activity and prevented an OxLDL-dependent decrease in cytosolic Arg2 concentrations (Online 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. Furthermore, inhibition of MPP both attenuated the increase in cytoplasmic arginase activity and prevented an OxLDL-dependent decrease in cytosolic Arg2 concentrations (Online 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 HAE. 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.

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Disclosures

D.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. The other authors report no conflicts.

References


2. Yia-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witzum JL, Steinberg D. Evidence for the presence of oxida-


4. Galle J, Bengen J, Schollmeyer P, Wanner C. Impairment of endothelium-


9. Haraguchi Y, Takiguchi M, Araya Y, Kawamoto S, Matsuda I, Mori M. Molecular cloning and nucleotide sequence of cDNA for human liver ar-


15. Morris SM Jr, Kepka-Lenhart D, Chen LC. Differential regulation of argi-


17. Collado B, Sánchez-Chapado M, Prieto JC, Carmenina MJ. Hypoxia regu-
lation of expression and angiogenic effects of vasoactive intestinal peptide (VIP) and VIP receptors in LNCaP prostate cancer cells. Mol Cell Endocrinol. 2006;249:116–122.


19. Jung C, Gonon AT, Sjöquist PO, Lundberg JO, Pernow J. Arginase inhibi-


20. Zhang C, Hein TW, Wang W, Miller MW, Fossum TW, McDonald MM, Humphrey JD, Kuo L. Upregulation of vascular arginase in hyper-


21. Johnson FK, Johnson RA, Peyton KJ, Durante W. Arginase inhibition re-
stores arteriolar endothelial function in Dahl rats with salt-induced hyper-


42. 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.


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**Novelty and Significance**

**What Is Known?**

- Arginase 2 (Arg2) inhibits nitric oxide (NO) production by endothelial NOS synthase (eNOS) by competing for the common substrate l-arginine.
- In quiescent endothelial cells (ECs), Arg2 is confined predominantly to the mitochondria.
- Activation of ECs 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.
- Pharmacological inhibition of arginase improves NO production and endothelial function and reduces plaque burden in atheroprotective 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 on 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.
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SUPPLEMENTAL MATERIALS

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

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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’ GTCCCTAAGGGGCCTCACCTCCGTG 3’;
Reverse: 5’ CCACGGAGTGGACGCCCCTTAGGGACA 3’.

Primer sequences for the 1-Δ40 Arg2 truncation were:
Forward: 5’ TGTCCCTAAGGGGCCTGAGCATGGTCC 3’;
Reverse: 5’ GGACCATGCTCCACGCCCCTTAGGGACA 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 TGC TTG 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 GGG 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#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 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 TTT GTG TGT GC-3'; Bottom, 5'-AAA AGC ACA CAC AAA GGA GAA ATA CTT CGG TAT TCC TTT GTG TGT GC-3';

MiceMPPβsh#A: Top, 5'-CAC CGC TCA TCT TAA CGC CTA TAC CGG AAC GGA TAA TAG GCG TTA AGA TGA GC-3'; Bottom, 5'-AAA AGC TCA TCT TAA CGC CTA TAC TTG CGG TAA 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-MPP\(\alpha\), Ad-MPP\(\beta\) 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%\(\beta\)ME) 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, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1%NP40, 1% sodium deoxycholate, 1 mM Na\(_3\)VO\(_4\), 2.5mM sodium pyrophosphate, 1mM \(\beta\)-glycerophosphate, 1µg/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 \(\mu\)l). 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 (Santa Cruz) (10 \(\mu\)l). 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.

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Chameleon Vision II multiphoton system (Coherent, Inc., Santa Clara, CA), and Zeiss Zen software.

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 \( \text{-}\)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 \( \text{O}_2^- \)**

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 manufacturer’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) (5 nmol/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