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' GTCCCTAAGGGGCGTCCACTCCGTGG 3';
Reverse: 5' CCACGGAGTGGACGCCCCTTAGGGAC 3'.

Primer sequences for the 1-Δ40 Arg2 truncation were :
Forward: 5' TGTCCCTAAGGGGCGTGGAGCATGGTCC 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 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 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 AAGTAT TTC TCC TTT GTG TGT GC-3’; Bottom, 5’- AAA AGC ACA CAC AAA GGA GAA ATA CTT CGG TAT TTC TCC TTT GTG 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, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1%NP40, 1% sodium deoxycholate, 1 mM Na_3VO_4, 2.5mM sodium pyrophosphate, 1mM β-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 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

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 EMC2 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^{o}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 C57Bl/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 37ºC 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.

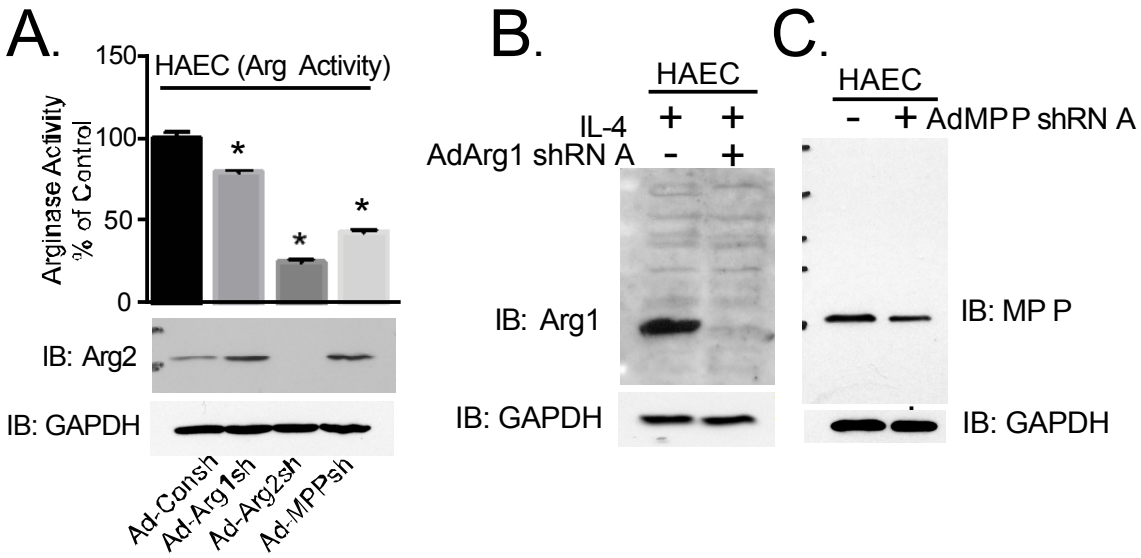
Measurement of mitochondrial potential

HAEC were incubated with 150nM TMRM (Tetramethylrhodamine, methyl ester, Perchlorate) at 37^{o}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).

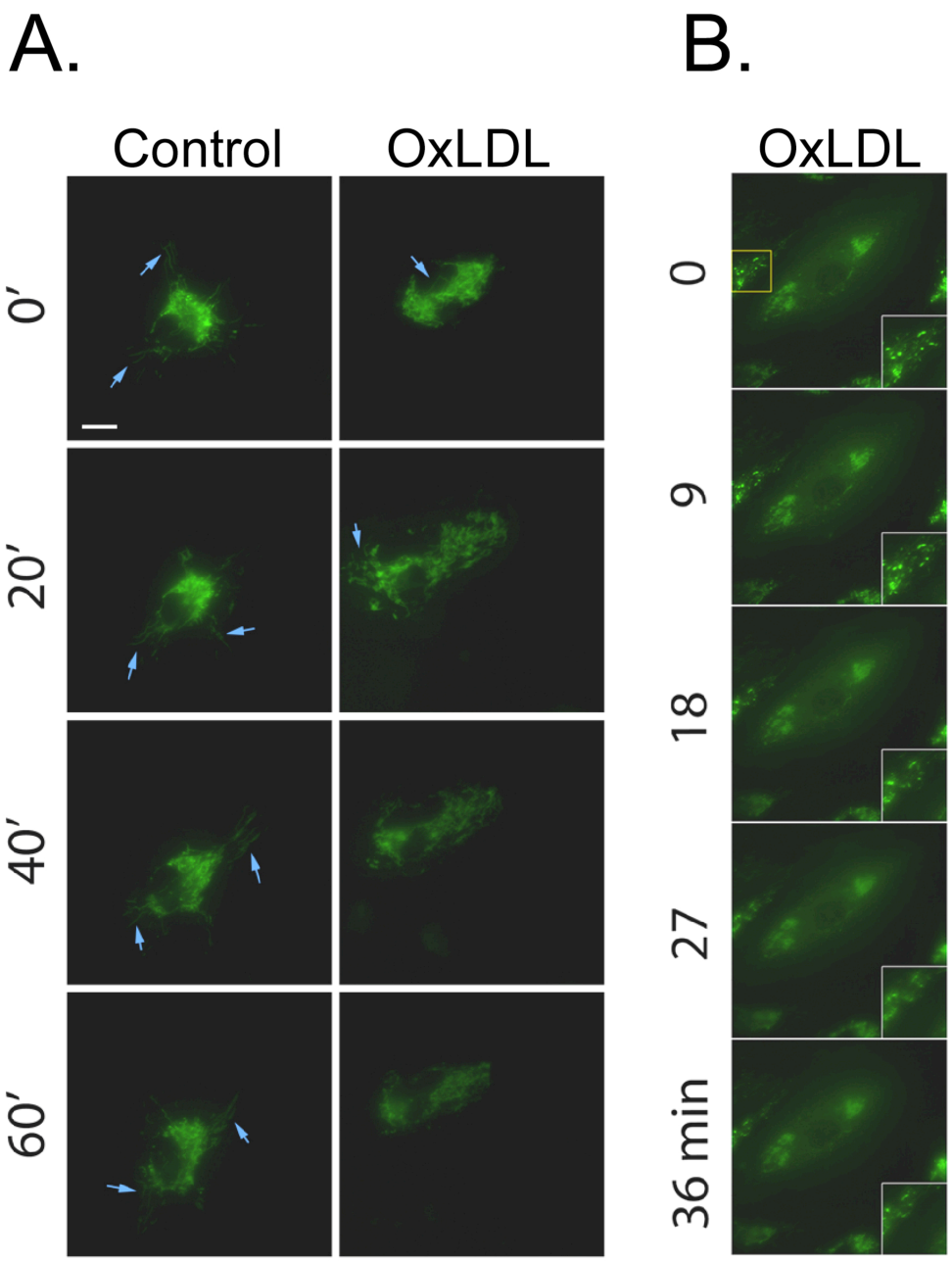
Statistical analysis

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.

ONLINE SUPPLEMENTAL FIGURES:

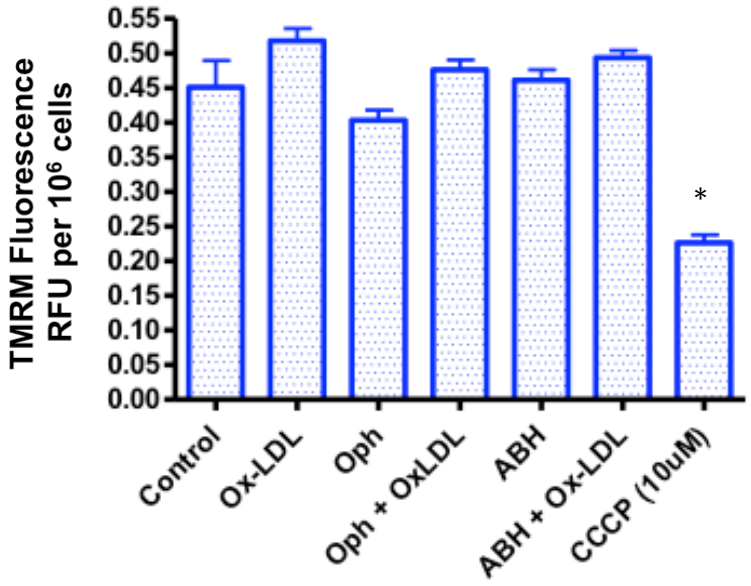


Supplemental Figure I. A. HAEC were plated at ~70-80% of confluence one day before cells were transduced with adenoviruses encoding Arg1, Arg2 or MPPα. After 48 hours, cells were lysed and subjected to arginase activity (upper panel) and immunoblotting (bottom panel) with Arg2 and GAPDH antibody. B. 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.

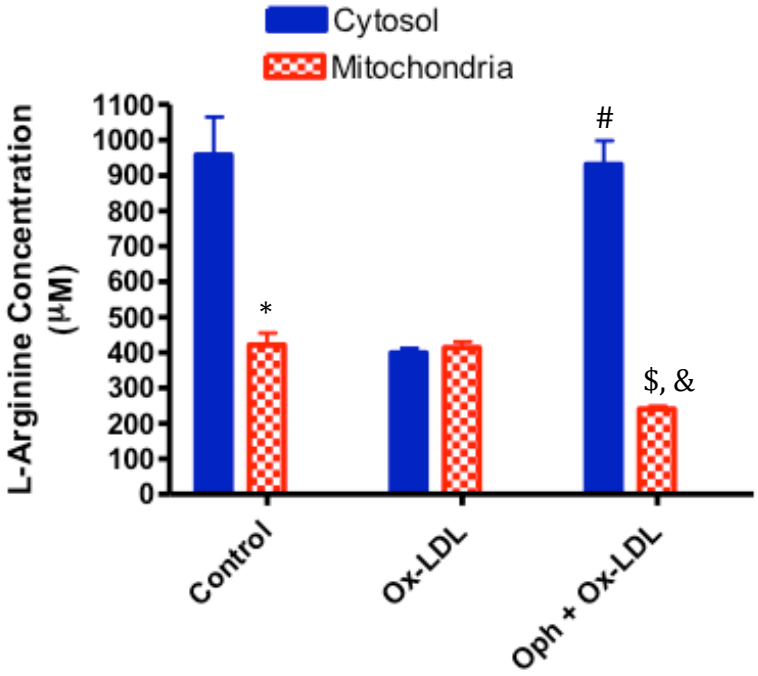


Supplemental Figure II. Release of GFP-Arg2 from mitochondria induced by OxLDL. HAEC were transfected with GFP-Arg2 for one day and then treated with OxLDL (50 μg/ml) (A) or transduced with Ad-GFP-Arg2 for 18 hours followed by serum starvation for 24 hours before treated with OxLDL 50 μg/ml (B). Transfected 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.

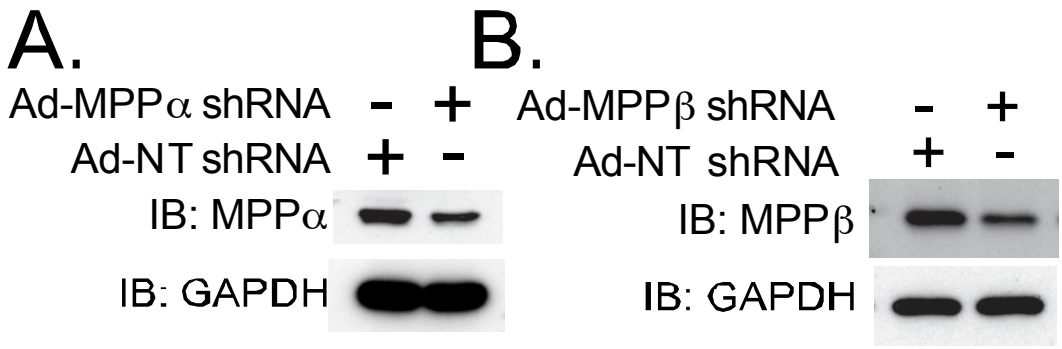
Please see Supplemental movie files I, II, and III for serial documentation of these dynamic events.



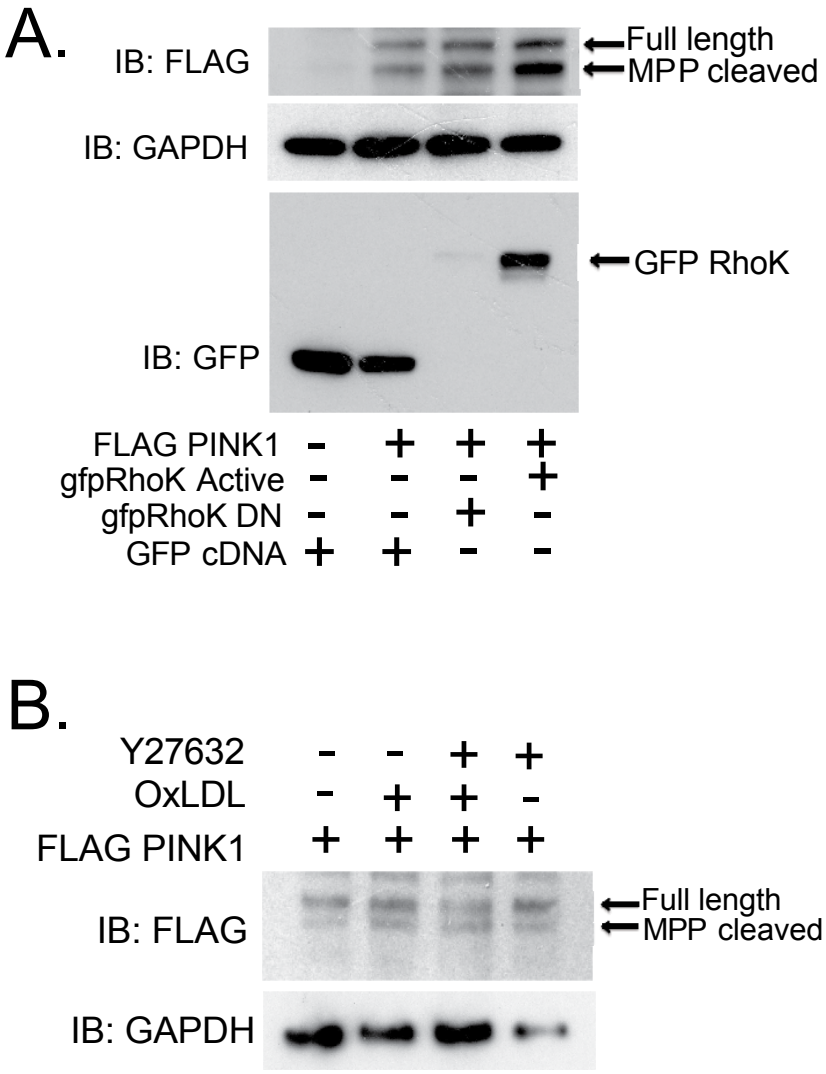
Supplemental Figure III. After 24 hours of serum starvation, HAEC were exposed to 50 μM OxLDL for 2 hours. OxLDL exposure was tested either alone or in the presence of either Oph or ABH, and the effects of each inhibitor alone were also tested. Cells treated with the mitochondrial uncoupler, CCCP (10μM) 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^{o} C for 5 min just before fluorescence was measured using a plate reader (excitation 560nm and emission 595nm). *$p < 0.05$ vs Control.



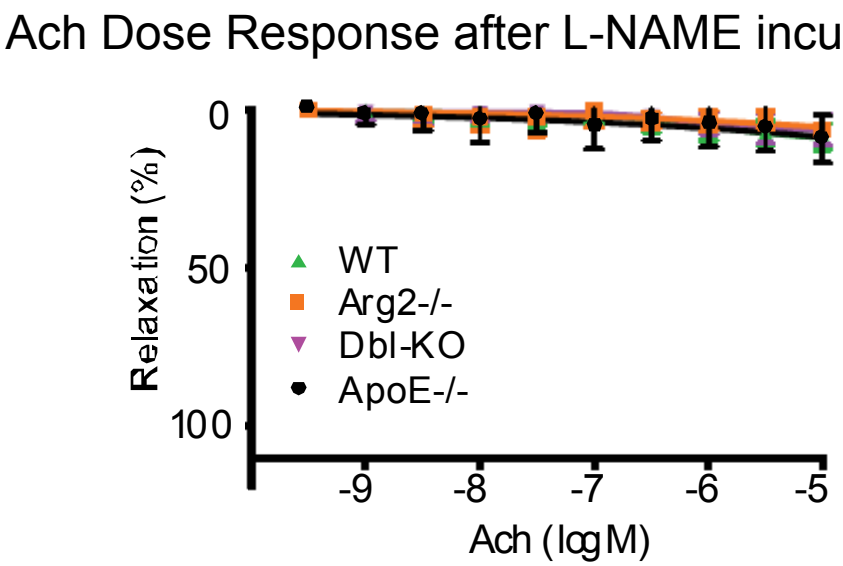
Supplemental Figure IV. Following 24h of serum starvation, HAEC were exposed to 50 µg/mL of OxLDL for 2h, either alone or in presence of Oph. Mitochondria and cytoplasm were separated and L-arginine concentrations were measured using a Biochrom-20 amino acid analyzer. *$p < 0.05$ vs Control(Cy), $^{\#}p < 0.05$ vs OxLDL(Cy), $^{\$}p < 0.05$ vs Oph+OxLDL (Cy), $^{\&}p < 0.05$ vs Con (Mito and Cy)



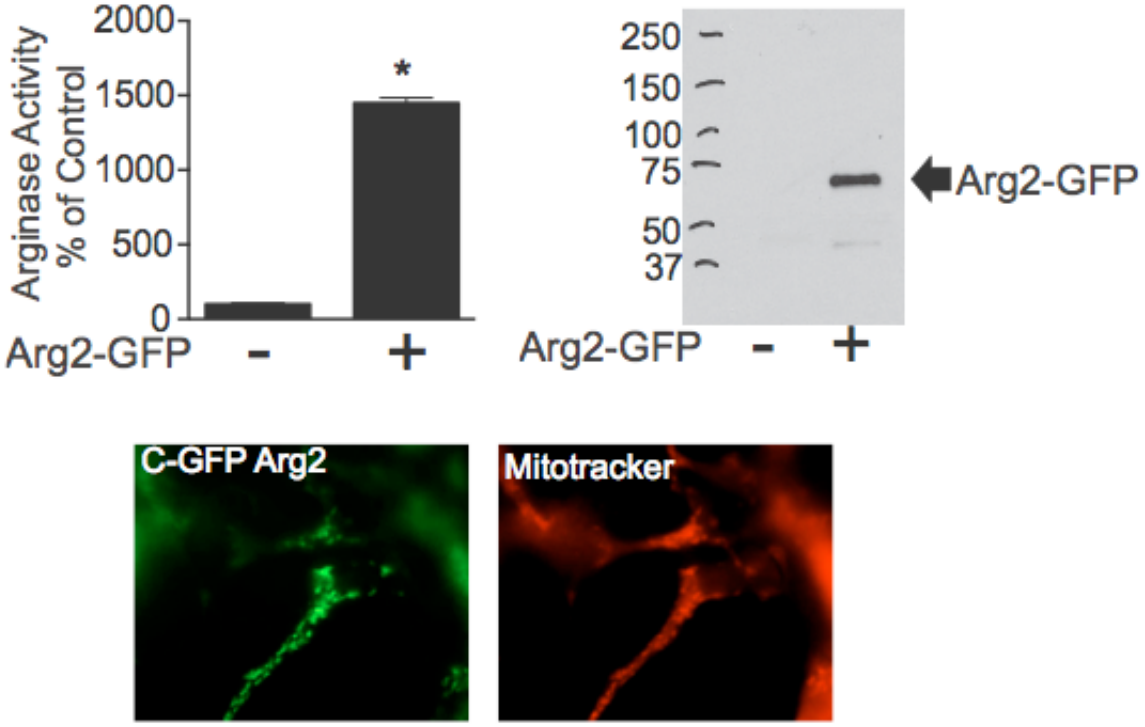
Supplemental Figure V. HAECs were transduced with adenoviruses encoding shRNA for either A) MPPα or B) MPPβ. 48 hours later, post-transduction cell lysates were collected and subjected to immunoblotting with MPPα, MPPβ and GAPDH antibodies.



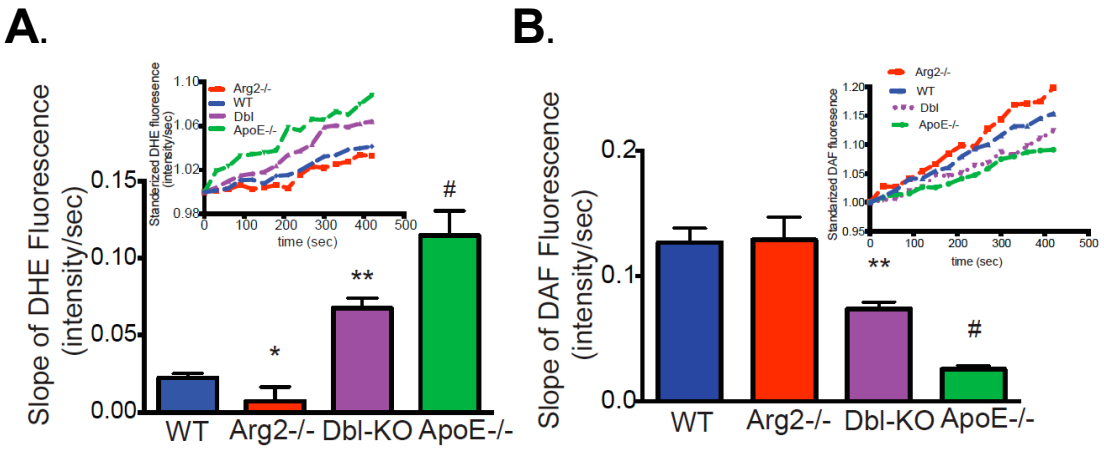
Supplemental Figure VI. A. HAEC were transfected with either FLAG-tagged PINK1 alone or co-transfected with GFP-tagged active Rho Kinase or dominant negative Rho kinase. Cell lysates were subjected to immunoblotting with FLAG, GFP and GAPDH antibodies 48 hours after transfection. B. HAEC were transfected 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 $p < 0.001$