Materials and Methods

Chemicals and Antibodies

All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Three different types of lysophosphatidylcholine (16:0, 18:0, 18:1) were purchased from Avanti Polar Lipids, Inc (Alabaster, Alabama). For mtROS measurement, MitoSOX Red Mitochondrial Superoxide Indicator (Life technologies, Carlsbad, CA) and mito-TEMPO-H Spin Probe (Enzo, Farmingdale, NY) were used. For cytosolic ROS measurement, CPH Spin Trapping reagent (Enzo) was used. VAS-2870 was purchased from EMD Millipore (Billerica, MA). Fluo-4 and Rhod-2 (Life technologies) were purchased for the measurement of cytosolic and mitochondrial calcium, respectively. IRDye 700 AP-1 and NF-κB oligonucleotides were purchased from LI-COR (Lincoln, Nebraska) for detecting the transcription factor bindings to the gene promoter of interest. Calcium signaling inhibitor, ruthenium red, was purchased from Calbiochem (Billerica, MA). Mitochondrial ROS inhibitor, MitoTEMPO, was purchased from Enzo. The primers (5'-3') used for the real-time PCR are: human ICAM1: TCTACGCTGACAATGAATCCTG and AGGGCCACTCAAATGAATCTC; and human ACTB: ACCTTCTACAATGAGCTGCG and CCTGGATAGCAACGTACATGG. Anti-ICAM-1 antibody (#4915) and anti-SOD2 antibody (#13141P) were purchased from Cell Signaling (Danvers, MA). Anti-UCP3 antibody (sc-7756) and anti-ANT1/2 antibody (sc-9299) were purchased from Santa Cruz Biotechnology (Dallas, Texas).

Animals

All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) Guidelines and Authorization for the use of Laboratory Animals and were approved by the IACUC of Temple University School of Medicine. All mice used were on a C57BL/6 background. ApoE mutant mice, commonly known as $ApoE^{-/-}$ mice (strain name: B6.129P2-Apoetm1Unc/J), and wild-type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Both WT and $ApoE^{-/-}$ mice were weaned at 4 weeks of age and maintained on a chow diet for another 4 weeks. At 8 weeks old, they were given 0.2% (w/w) cholesterol and 21.2% (w/w) fat high fat diet (TD.88137, Harlan, Hayward, CA) for 3 weeks. At 11 week-old, mice were sacrificed and tissues were collected.

For mini-pump implantation, mini-pumps containing saline or MitoTEMPO (1500 μg/kg/day) were implanted in $ApoE^{-/-}$ mice at eight weeks of age before they were fed with a high fat diet for three weeks. Afterwards, intravital microscopy and flow cytometry analyses were carried out to determine the endothelial activation status.

Metabolomics Analysis

Aortas were collected from wild-type (WT) mice and $ApoE^{-/-}$ mice after 3 weeks of high-fat diet feeding. Global and non-targeted metabolic profiling was performed by Metabolon (Durham, NC).[1] Briefly, after the extraction of metabolites from tissues, the extracts were analyzed by ultra-high performance liquid chromatography-tandem mass spectrometry (positive and negative ion modes) and gas chromatography-mass spectrometry (GC-MS). Compounds were identified by library search, and statistical analysis was performed.

Microarray Analysis

RNA of the aorta from five male WT and ApoE$^{-/-}$ mice that were fed a Western diet for 3 weeks starting from 8 week-old were isolated with the RNeasy Kit (Qiagen). RNA quantity was determined by the NanoDrop ND-2000 (Thermo Scientific). RNA samples were sent to the Fox Chase Cancer Center Genomic Facility. The RNA integrity was determined by the RNA 28S/18S ratio using the Agilent 2100 Bioanalyzer (Agilent Technologies). Samples were then labeled and hybridized to the Affymetrix Genechip Mouse Gene 2.0ST Arrays, following the manufacturer's instructions. Scanned microarray images were analyzed using the Affymetrix Gene Expression Console with Robust Multi-array Average normalization algorithm. Microarray data are available through the GEO database (GSE72248).

Cell Culture

Human aortic endothelial cells (HAECs) (Lonza, CC2535; Walkersville, MD) were cultured in medium M199 (Hyclone laboratories, Logan, UT) supplemented with 15% fetal bovine serum (FBS; HyClone); endothelial cell growth supplement (ECGS, 50μg /mL); BD Biosciences, San Jose, CA); heparin (50μg/mL); and 1% penicillin, streptomycin, and amphotericin (PSA; Invitrogen, Carlsbad, CA). HAECs were grown on 0.2% gelatin-coated flasks, plates, or dishes and experiments were performed at passage 9.

Fluorescence Activated Cell Sorting (FACS)

For mtROS measurement: After staining with MitoSOX (5μM), HAECs were incubated at 37°C for 10min and washed with PBS twice afterwards. After LPC treatment, cells were washed once with ice-cold PBS and Trypsin-EDTA was added to detach cells. Trypsinization was terminated by adding FACS buffer (2% FBS in PBS) and cells were collected by centrifugation. After re-suspension in 0.2ml FACS buffer, samples were subjected to flow cytometry analysis, where fluorescence emissions were measured at FL2 channel (excitation: 490 /emission: 520) by FACSCalibur machine (BD).

For mitochondrial membrane potential measurement: After LPC treatment, cells were stained with TMRM (30nM) for 20min and washed with PBS twice afterwards. Cells were then trypsinized and collected by centrifugation. After re-suspension in 0.2 ml FACS buffer, samples were subjected to flow cytometry analysis, where fluorescence emissions were measured at FL2 channel by FACSCalibur flow cytometer (BD).

Fluorescent Microscopy

Fluorescent microscopy was performed to visualize LPC-induced mtROS in HAECs. After staining with MitoSOX (5μM), HAECs were incubated at 37°C for 10min and washed with PBS twice afterwards. After treatment with drugs, cells were washed twice with PBS and used for measurement by fluorescent microscopy. Images were captured by the Zeiss Axioscope microscope (Thornwood, NY).

Electron Spin Resonance (ESR)

ESR was used to measure mtROS and cytosolic ROS in HAECs. After LPC treatment, HAECs were washed with PBS twice and collected by cell scraper. HAECs were then centrifuged and collected in 100μl of deoxygenated, pH 7.35 Krebs HEPES buffer (KHB) (99mM NaCl, 4.69mM KCl, 25mM $NaHCO_3$, 1.03mM KH_2PO_4, D-glucose 5.6mM, Na-HEPES 20mM, $CaCl_2(x2H_2O)$ 2.5mM, $MgSO_4$ 1.2mM). Cells were then vortexed briefly and aliquoted into 10μl suspension in Eppendorf tubes. To each aliquot of cells, 80 μl of modified KHB buffer (plus 25 μM Deferoxamine and 5 μM DETC) and 10μl of MitoTEMPO-H/CP-H buffer (10mM) were added to measure mitochondrial/cytosolic ROS. The remaining cells were centrifuged and the cell pellets were frozen for protein concentration determination. MtROS and cytosolic ROS were quantified by normalizing MitoTEMPO-H and CPH ESR signals with protein concentrations.

Toxicology Assay

In vitro Toxicology Assay Kit, Lactic Dehydrogenase based (SIGMA-Aldrich, TOX7; St. Louis, MO) was used according to the manufacturer's suggestion. Briefly, cytoplasmic lactate dehydrogenase (LDH) released into the medium was measured based on the reduction of NAD^+ to NADH by LDH. The resulting reduced NADH is converted to a colored compound and measured by spectrophotometer plate reader. LDH activity was expressed by subtracting the 630nm absorbance value (background signal from instrument) from the 490nm absorbance value.

Seahorse XF96 Analyzer

Seahorse XF96 analyzer was used to assess 6 mitochondrial parameters in HAECs, including mitochondrial O_2 reduction, proton leak, ATP production, maximal respiration, spare respiratory capacity, and non-mitochondrial O_2 reduction. Firstly, we seeded different densities of HAECs, ranging from 5,000 cells/well to 30,000 cells/well, to determine the optimal cell seeding density for the experiments. The results showed that oxygen consumption rate (OCR) increased linearly as the seeding density increased, and 30,000 cells/well was picked for subsequent experiments (**Supplemental Figure 2A**). Secondly, since different cell type responds differently to the mitochondrial membrane uncoupler FCCP, which is used to induce maximal mitochondrial respiration, FCCP titration experiment was also performed, based on which 1 μM FCCP concentration was used for subsequent experiment (**Supplemental Figure 2B**). Thirdly, HAECs were seeded at 30K per well in 96-well plates and cultured overnight. Culturing media was changed to modified DMEM media (2mM sodium pyruvate, 25mM glucose) and placed into a 37ºC non-CO_2 incubator for 1 hour. After preparation of drugs and XF Cell Mito Stress Test Kit (Seahorse Bioscience, North Billerica, MA) into cartridge ports, the cartridge and cell culture plates were loaded into XF96 analyzer (Seahorse Bioscience). After 3 basal measurements of oxygen consumption rates, vehicle control, LPC, and LPC plus Ruthenium Red were added, and basal respirations were measured every 15min for 1 hour. XF Cell Mito Stress Test Assay was performed afterwards to determine the six different mitochondria parameters (**Figure 4B and 4C**).

Confocal Microscopy

Confocal microscopy was used to measure mitochondrial Ca^{2+} and cytosolic Ca^{2+} simultaneously in HAECs. Briefly, HAEC cells were grown on 25-mm glass coverslips and loaded with 2μM Rhod-2 AM (50min) (for mitochondrial Ca^{2+} detection) and 5μM Fluo-4 AM (30min) (for cytosolic Ca^{2+} detection). After 1 min of baseline recording, LPC (10μM) was added, and confocal images were recorded every 3sec (510 Meta; Carl Zeiss) at 488- (green fluorescence, for Fluo-4) and 561-nm (red fluorescence, for Rhod-2) excitation using a 63× oil objective to simultaneously monitor cytosolic and mitochondrial Ca^{2+} dynamics. Images were analyzed and quantified using custom-made software (Spectralyzer, Elmsford, NY).

Western Blot Analysis

Protein extracts were collected from HAECs. Protein concentrations were determined by the bicinchoninic acid (BCA) assay with BSA standards. Proteins were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% BSA in Tris buffered saline containing 0.01% Tween 20 [TBST, 50mM Tris (pH 7.5), 150mM NaCl, and 0.1% Tween 20 (v/v)]. Membranes were incubated with primary antibodies overnight at 4°C. Membranes were then washed extensively with TBST and incubated with the appropriate horseradish peroxidase-labeled secondary antibodies for 1 hour at room temperature. Afterward, membranes were incubated with enhanced chemiluminescence (ECL) substrate for horseradish peroxidase (Pierce/Thermo, Rockford, IL) and the ECL intensity was detected by X-ray film exposure in a dark room. The X-ray films were developed by the SRX-101A medical film processor. The expression levels of proteins as indicated by the ECL intensity were measured with ImageJ software (NIH, Bethesda, MD, USA).

Real-Time PCR

Total RNA was isolated from HAECs with RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's suggestion. The RNA was solubilized in 30μl nuclease-free water. RNA quality and concentration were determined by Nanodrop 2000 (Thermo Scientific, Wilmington, DE). 2μg of total RNA were reverse transcribed to generate complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Invitrogen). The mRNA expression levels of genes were determined by quantitative real-time PCR (qRT-PCR) with the SYBR-green dye (Invitrogen) on the StepOnePlus PCR system (Applied Biosystems, Foster City, CA).

Human EC Biology PCR Array

RNA collected from HAEC was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was converted to cDNA with the RT^2 First Strand Kit (SABiosciences) and was used to screen for 84 EC biology-related gene expression changes following the direction of the Human Endothelial Cell Biology PCR Array (SABiosciences). Data was analyzed with the SABiosciences PCR Array Data Analysis Software.

Monocyte adhesion assay

HAECs were seeded in 24-well plates and stimulated by LPC with or without MitoTEMPO for 4 hours. Afterwards, primary human peripheral mononuclear cells

(PBMCs) were stained with 2 μM calcein green AM for 30 min at 37ºC. Then 1×10^6 labeled PBMCs were added to HAEC monolayer in each well. After 1 hour incubation at 37ºC, the unattached PBMCs were removed and the plates were read in a fluorescence microplate reader.

Electrophoretic Mobility Shift Assay (EMSA)

Transcription factor interactions with DNA response elements were assessed using EMSA. AP-1 and NF-κB consensus oligonucleotides end-labeled with IR700 were purchased from LI-COR (Lincoln, Nebraska). The sequences of the probes are as follows: AP-1, 5'-CGCTTGATGACTCAGCCGGAA-3'; and NF-κB, 5'-AGTTGAGGGGACTTTCCCAGGC-3'. EMSA was carried out using an Odyssey Infrared EMSA kit (LI-COR) according to the manufacturer's instructions. 2-5μg of nuclear extracts were added to each binding reaction. The probe and nuclear proteins were incubated for 20min at room temperature and DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide gel afterwards. Images of gel were then obtained in an Odyssey scanner (LI-COR).

Intravital Microscopy

Intravital microscopy was used to assess *in vivo* leukocyte rolling and adheshion as previously described[2]. Briefly, Male mice were anesthetized and the cremaster muscle was exteriorized. The muscle was allowed to equilibrate for 15 minutes prior to observing leukocyte rolling and adhesion. Venules and arterioles that are 40~50μm in diameter as measured by the CellSens Dimension software (Olympus, Tokyo, Japan) were observed on a microscope (Olympus BX51WI) with a digital camera mounted (Olympus DP80). Leukocyte rolling was determined as the number of cells that rolled past an imaginary line that is perpendicular to the vessels in 1 minute. Adhesion was determined as cells that adhered to the vessel wall for 1 minute on a 100 μm length observed. Rolling and adhesion in five venules and two arterioles were observed in each mouse.

Aortic Cell Isolation and Staining

High fat diet fed *ApoE*$^{-/-}$ mice were deep-anesthetized at the end of experiment and their vasculature were perfused by cardiac puncture with PBS containing 20 U/ml of heparin to remove blood cells from all vessels. Aortas, free of adipose tissue, were collected and weighed to control the total amount of collected aortic tissues. The harvested aortas were minced with scissors and digested with 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I, 60 U/ml DNase I, and 450 U/ml collagenase type I-S in PBS containing 20 mM HEPES at 37 °C for 30 mins. Aortic cell suspensions were obtained by mashing the aorta through a 70 μm cell strainer. For flow cytometry analysis, cells were first stained with Live/Dead violet (Invitrogen, USA) at room temperature for 30 minutes to exclude dead cells, then washed and co-incubated with three mouse antibodies: CD11b-BV421 (M1/70, BD Biosciences, monocyte marker), Ly6C-APC (AL-21, BD Biosciences, inflammatory monocyte marker), and CD45.2-Alexa Fluor 488 (104, Biolegend, leukocyte marker) at 4 °C for 30 min. Flow cytometry analysis was performed on a LSRII (BD Biosciences, San Jose, CA). Data were analyzed using the FlowJo software (Tree Star Inc., Ashland, OR).

Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) throughout the manuscript. For comparisons between two groups, two-tailed Student *t* test was used for evaluation of statistical significance or, when the data were not normally distributed, a nonparametric Mann-Whitney U test was used. For comparisons across multiple groups, one-way ANOVA with Bonferroni post-test adjustment was used or, when the data were not normally distributed, the data were analyzed using one-way ANOVA with the Kruskal-Wallis test, followed by pairwise comparison using the Dunn test. Data shown are representative of two to three independent experiments, including analyses from FACS, ESR, Seahorse XF96 analyzer, fluorescent and confocal microscopy, Western Blot, PCR, adhesion assay, and EMSA. NS, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

References

1. Du F, Virtue A, Wang H, Yang XF. Metabolomic analyses for atherosclerosis, diabetes, and obesity. *Biomarker research*. 2013;1:17
2. Sha X, Meng S, Li X, Xi H, Maddaloni M, Pascual DW, Shan H, Jiang X, Wang H, Yang XF. Interleukin-35 inhibits endothelial cell activation by suppressing mapk-ap-1 pathway. *The Journal of biological chemistry*. 2015;290:19307-19318