Cell culture

HCAECs and endothelial growth medium-2 (EGM-2) were purchased from Cambrex BioWhittaker Inc. (Walkersville, MD). Recombinant human sCD40L (monomer) was obtained from PeproTech (Rocky Hill, NJ). The endotoxin level in sCD40L is less than 0.1 ng per μg (1 EU/μg). When HCAECs grew to 80%-90% confluence in 6-well plates, they were divided into six major groups. Group I, cells were treated with different concentrations (1 and 5 μg/mL) of sCD40L or heat-inactivated (HI-sCD40L, 5 μg/mL) for different times (6, 12, 24 or 48 hours). In separate experiments, HCAECs were treated with actinomycin D (2.5 μg/mL, Sigma, St. Louis, MO) in the presence or absence of sCD40L (5 μg/mL). Total cellular RNA was isolated at multiple time points (0.5, 1, 3, 6 and 12 hours) and analyzed for eNOS mRNA levels by real-time PCR to study the eNOS mRNA degradation rate. sCD40L trimer (R&D System, Inc., Minneapolis, MN) were also used to treat HCAECs for eNOS levels. Group II, cells were pre-treated with anti-CD40L antibody (0.5, 2 or 5 μg/mL), anti-CD40 antibody (1, 4 or 10 μg/mL), or isotype control antibody (rabbit IgG, 5 or 10 μg/mL) for 30 minutes before adding sCD40L (5 μg/mL) for 24 hours. Group III, cells were co-cultured with seleno-L-methionine (SeMet, 20 μM) and sCD40L (5 μg/mL) for 24 hours. Group IV, cells were treated with sCD40L (5 μg/mL) for 5, 10, 20, 30, 45, 60 or 90 minutes. Phosphorylation of MAPKs and IκB-α were studied. Group V, cells were pre-treated with p38 inhibitor (SB239063), ERK1/2 inhibitor (PD98059) or mitochondrial complex II inhibitor (4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione, TTFA, Sigma-Aldrich Inc., St. Louis, MO) for 30 minutes and then co-cultured with sCD40L (5 μg/mL) for 24 hour. Group VI, cells were infected with various recombinant adenoviruses for indicated times and then treated with sCD40L for different times. eNOS levels and NF-κB nuclear translocation were studied. In addition, cells were prepared for cell proliferation assay, eNOS mRNA 3'UTR gel shift assay, and miRNA profiling. In all groups, cells cultured in EGM-2 alone were used as negative controls. The cells did not undergone serum starvation before any treatments because the serum starvation alone affected endothelial functions. Antibodies against human CD40L and CD40 were obtained from Abcam (Cambridge, MA).

MTS assay.

Cell proliferation was determined by CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) Kit (Promega, Madison, WI). HCAECs were seeded on the 96 well plate (1000 cells/well) for 24 hours and then treated with sCD40L (5 μg/mL) or SeMet (20 μM) in the growth medium for 1, 2, 3, and 4 days. At each time point, 20 μl MTS reagent mixed with 100 μl growth medium was added to each well, and incubated in 37^{o}C for 2 hours. Absorbance was recorded at 490 nm using an EL-800 universal microplate reader. Data were standardized with initial seeding density and presented as % of initial seeding density.

Porcine coronary artery cultures

Fresh porcine right coronary arteries were carefully dissected and cut into multiple 5-mm rings. The rings were then incubated in DMEM (Invitrogen, Carlsbad, CA) with 5 μg/mL of sCD40L at 37ºC and 5% CO_2 for 24 hours. The myograph system used in our laboratory has been previously described.[1-3] Briefly, the rings were suspended between the wires of the organ bath myograph chamber (Danish Myo Technology Organ Bath 700 MO, Aarhus, Denmark) in 6 mL of Kreb's solution, maintained at 37ºC and oxygenated with pure oxygen gas. Rings were slowly subjected stepwise to a predetermined optimal tension of 30 millinewton (mN) and allowed to equilibrate for at least 30 minutes. Following equilibration, each ring was precontracted with 20 μL of thromboxane A2 analogue U46619 ($3X10^{-8}$ M). Following 60-90 minutes of contraction, a relaxation concentration-response curve was generated by adding 60 μL of four cumulative additions of the endothelium-dependent vasodilator bradykinin (10^{-9} to 10^{-6} M) every 3 minutes. In addition, 60 μL of sodium nitroprusside (10^{-6} M) was added into the organ bath, and endothelium-independent vasorelaxation was recorded.

Levels of superoxide anion (O_2^-) produced by porcine coronary artery endothelial cells were detected by using the lucigenin-enhanced chemiluminescence method.[1-3] Six sets of vessel rings in each group were used. The rings were cut open longitudinally and trimmed into approximately 5 x 5 mm pieces. Assay tube (12 X 75 mm) was filled with 500 μL of Kreb's HEPES buffer solution and 25 μL of lucigenin (final concentration 5 μM). After gently vortexing, the vessel segments were placed endothelium-side-down in the tubes. Time-based readings of the luminometer were recorded by FB12 software (Berthold Detection System GmbH, Pforzheim, Germany). The data, in relative light units per second (RLU/sec) for each sample, were averaged between 5 and 10 minutes. Values of blank tubes containing the same reagents as the vessel ring samples were subtracted from their corresponding

vessel samples. The area of each vessel segment was measured and used to normalize the data for each sample. Final data represent mean ± SEM (RLU/sec/mm^2).

Real-time PCR

Total RNA from HCAECs and porcine coronary artery endothelial cells was isolated using RNAqueous®-4PCRkit (Ambion Inc.). Equal amount of mRNA (0.5 μg) was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad laboratories, Hercules, CA) according to the manufacturer's instructions. The iQ SYBR Green SuperMix Kit was then used for real-time PCR reaction, which was performed in an iCycler iQ real-time PCR detection system (Bio-Rad). SYBR Green, a dsDNA fluorescence dye, binds to all dsDNA PCR products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to account for variations in mRNA loading. Human and porcine eNOS and GAPDH primers as well as human NOX4, SOD and CAT primers (Table S1) were designed by Beacon Designer (Premier Biosoft, Palo Alto, Ca), and synthesized by Sigma Genosys (The Woodlands, TX). Thermal cycle condition used for RT was as follows: 5 minutes at 25ºC, 30 minutes at 42ºC, and 5 minutes at 85ºC. The condition used for real-time PCR was as follows: 3 minutes at 95ºC, 40 repeats of 20 seconds at 95ºC, and 1 minute at 60ºC. Controls were performed with no RT (mRNA sample only) or no mRNA (water only) to demonstrate the specificity of the primers and the lack of DNA contamination in samples. Specific reaction was also confirmed by a single melting curve for each molecule. Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units (RFU) versus PCR cycle number during exponential amplification so that sample measurement comparisons were possible. The eNOS mRNA levels in each sample was calculated as $2^{(40 - Ct)}$ and further normalized to GAPDH mRNA levels as $[2^{(Ct[GAPDH] - Ct[gene\ of\ interest])}]$.

MicroRNA (miRNA) profiling assay

HCAECs were treated with sCD40L (5 μg/mL) or TNF-α (2 ng/mL) for 24 hours. miRNAs were extracted and purified using mirVana miRNA Isolation kit (Applied Biosystems/Ambion, Austin, TX) following the manufacturer's instructions. RNA (5 μL) was directly converted to cDNA with the QuantiMir™ RT System (SBI System Biosciences, Mountain View, CA). Commercial 95 miRNA array kit (SBI System Biosciences) was used. All 95 miRNAs are selected based on their potential roles in cell regulations. The array plate also includes the U6 transcript as a normalization signal. The mature miRNA sequences and primer sequences used in real time-PCR were listed in Table S2. For real time PCR analysis, cDNAs from different cells were mixed with a SYBR Green Mastermix (Bio-

Rad) plus the Universal reverse primer. SYBR Green Mastermix was aliquoted into qPCR optical plate. Primers in the Primer plate were resuspended with 10 μL RNase-free water. Each (1 μL) of the miRNA-specific primers from the Primer plate was added into the corresponding well of the qPCR plate. Real-time PCR was performed and the expression levels of mature miRNAs were analyzed by Ct values and normalization to U6 miRNA levels ($2^{-\Delta CT}$). Treated and control cells were separately calculated. Fold changes of each miRNA in treated cells were calculated in relation to that in untreated control cells.

eNOS mRNA 3'UTR gel shift assay

Human eNOS mRNA 3'UTR probe sequence (5'-UUAGAUUCCUCUUGCCUCUCUC-3') was chosen from gene bank accession number L10709. This sequence was previously reported to be able to bind to unknown cytoplasmic proteins in endothelial cells treated with TNF-α, which induced eNOS mRNA downregulation.[4-6] The biotin-labeled and unlabeled RNA oligonucleotides were synthesized and purified by Bio-Synthesis Inc. (Lewisville, TX). HCAECs were treated with sCD40L (5 μg/mL) for 24 hours. Cytoplasmic extracts were prepared from cells in a lysis buffer consisting of 10 mM HEPES (pH 7.6), 40 mM KCl, 3 mM MgCl2, 1 mM DTT, 5% glycerol, 0.2% Nonidet P-40 and protease inhibitor cocktail tablets and mechanically homogenized. The nuclei were removed by centrifugation (4500 g, 15 minutes, 4°C), and the cytoplasmic fraction (supernatant) was frozen at -80°C. Protein content was determined by BCA method (Pierce). For RNA band shift assay, 20 μg of cytoplasmic proteins were mixed with 50 μg of total yeast RNA (non-specific competitor) at 4°C in a binding buffer of the following composition: 10 mM HEPES (pH 7.6), 40 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 5% glycerol and protease inhibitor cocktail tablets. Then, 10 ng of biotin-labeled eNOS RNA probe was added to make a total volume of 30 μL for further incubation at 25°C for 10 minutes. The binding reactions were electrophoresed on a 6% native polyacrylamide gel in 0.5X TBE (Tris-borate-EDTA) as running buffer. After electrophoresis, the samples were transferred to nylon membrane and immobilized by UV cross-linking. The biotin-labeled RNA was detected by lightshift chemiluminescent EMSA kit (Pierce). For the competition experiment, 100 fold molar excess of identical unlabelled RNA probe was preincubated for 10 minutes with cytoplasmic proteins prior to the addition of biotin-labeled RNA probe.

Recombinant adenoviruses

Recombinant adenoviruses expressing human Cu/Zn SOD (Ad-SOD), human catalase (Ad-CAT) were generous gifts of Dr. Toren Finkel (National Institutes of Health) and have been previously described.[7,8] Adenoviruses expressing a dominant negative mutant form of human NADPH oxidase NOX4 (Ad-NOX4 DN) and the *Escherichia coli* β-galactosidase gene (Ad-LacZ) were generous gifts of Dr. Barry Goldstein (Thomas Jefferson University).[9] Adenoviruses expressing a dominant negative mutant form of human IκBα (Ad-IκB DN) and green florescence protein (Ad-GFP) were purchased from Vectors Biolabs, Philadelphia, PA. The viruses were amplified in HEK-293 cells and purified on double cesium gradients. HCAECs grown to 90% confluence were infected with recombinant adenoviruses with the indicated multiplicity of infection (MOI) for indicated times (48 or 72 hours). High transfection rate (over 70%) was confirmed by observing GFP fluorescence cells and real time PCR analysis for each specific gene delivered. After adenovirus infection, the medium was aspirated and replaced with fresh medium and sCD40L (5 μg/ml) for indicated times (10 minutes, 45 minutes, or 24 hours). The eNOS expression at both mRNA and protein levels were determined by real time PCR and western blot, respectively. The protein levels of nuclear NF-κB (p65) were also determined by western blot.

Western blot

Total proteins were isolated from HCAECs using cell lysis buffer (Cell Signaling Technology, Danvers, MA). The same amount of endothelial proteins (6 μg) was resolved electrophoretically by one-dimensional SDS-PAGE (10% polyacrylamide) for approximately 1 hour at 150 V. Subsequently, the gel was equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.03% SDS, and 20% methanol) and the proteins were electrophoretically transferred to the nitrocellulose filter for 2 hour at 100 V. Filter was blocked using 5% nonfat dried milk in Tris buffer saline (TBS) with 0.05% Tween 20 (TBS-T) for 1 hour at room temperature. eNOS protein was detected using a mouse anti-human eNOS monoclonal antibody diluted 1:1000 (BD Biosciences) and β-actin protein was detected using a mouse anti-human β-actin monoclonal antibody diluted 1:10000 (Chemicon). The eNOS and β-actin primary antibodies were detected with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody diluted 1:2000. Blots were developed using ECL-plus kit and analyzed with gel documentation system and analysis software (Alpha Innotech Co., San Leandro, CA). For assay of nuclear translocation of NF-κB, nuclear proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) following manufacturer's instructions. Equal amounts of

nuclear proteins (40 μg) were loaded onto 8% SDS-PAGE, fractionated by electrophoresis, and transferred to nitrocellulose membrane. The membrane was incubated with NF-κB p65 antibody (Santa Cruz Biotech) at 4°C overnight. Loading efficiency for nuclear proteins was determined by blotting the membrane with an antibody against nuclear membrane protein laminin A (Abcam).

Flow cytometry

Cells were harvested with 0.02% Trypsin/EDTA and adjusted to $1X10^6$ cells per FACS tube. For O_2^- and NO staining, dihydroethidium (DHE, 3 μM, Molecular Probes) and 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF–FM DA, 10 μM, Molecular Probes) were added, respectively, and incubated at 37°C for 30 minutes. The staining reaction was stopped by washing with staining buffer (0.01 M PBS, 3% FBS, 0.9% NaN_3). Final samples were collected in 500 μl staining buffer and stored in 4°C. Samples were analyzed using FACScan and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ) within 24 hours of preparation. In each experiment, at least 10,000 events were analyzed.

eNOS enzyme activity assay

HCAECs were treated with sCD40L (5 μg/mL) for 24 hours and eNOS enzyme activity was determined by a special fluorimetric assay kit (Sigma). Briefly, it measures intracellular production of NO by a non-radiometric method. A cell-permeable diacetate derivative of 4,5-diaminofluorescein (DAF-2 DA) can penetrate cells rapidly where it is hydrolyzed by intracellular esterase to DAF-2 that, in turn, reacts with NO produced by eNOS to form a fluorescent triazolo-fluorescein. The fluorescent product can be quantitated using an excitation filter at 492 nm and an emission filter at 515 nm. The eNOS enzyme activities in CD40L-treated HCAECs were normalized with untreated cells, and data were shown as mean ± SEM (% of controls).

Assessment of mitochondrial membrane potential ($\Delta\psi$m)

Loss of $\Delta\psi$m was assessed using flow cytometry analysis of cells stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole-carbocyanide iodine (JC-1, MitoScreen kit). Mitochondria with a normal $\Delta\psi$m concentrate JC-1 into aggregates (red fluorescence), but with a de-energized or depolarized $\Delta\psi$m, JC-1 forms monomers (green fluorescence). Cells ($5x10^5$) were incubated with 10 μg/mL JC-1 for 12 minutes at 37°C and analyzed by flow cytometry using the FACScan and Cell Quest software. The analyzer threshold was adjusted on the FSC channel to exclude most of the

subcellular debris. Photomultiplier settings were adjusted to detect JC-1 monomer fluorescence signals on the FL1 detector (green fluorescence, centered at 390 nm) and JC-1 aggregate fluorescence signals on the FL2 detector (red fluorescence, centered at 340 nm). Data analyses were performed with Paint-a-Gate Pro Software (Becton Dickinson). In each experiment, at least 10,000 events were analyzed. Mitochondrial complex II inhibitor (TTFA, 10 μM) was used to detect the specificity and functional role of mitochondrial dysfunction in sCD40L-treated cells.

Measurement of ATP levels

ATP levels were measured with an ATPLite kit. The ATPLite assay system (PerkinElmer) is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. The emitted light is proportional to the ATP concentration within certain limits. Cells were seeded on 96-well black plates (5,000 cells/well) and cultured with or without sCD40L (5 μg/mL) for 24 hours. The lysis solution and substrate solution (PerkinElmer) were added to each well of the plate, respectively. The luminescence was measured by TopCount Microplate Scintillation and Luminescence Counter (PerkinElmer).

Assessment of NADPH oxidase activity

The activity of NADPH oxidase (NOX) was measured using the methods previously described.[10,11] HCAECs were seeded in the 96-well plate (CulturPlate, PerkinElmer) at the density of 10,000/well. When cells grew to 80%-90% confluence, they were treated with EMG-2 containing sCD40L (5 μg/mL) and/or SeMet (20 μM) for 24 hours. After washed in ice-cold PBS, cells were incubated in Krebs-HEPES buffer (KHB, pH 7.4) for 10 minutes. Dark-adapted lucigenin (5 μM) was added and the plate was sealed with an adhesive sealing film and loaded into a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer). Chemiluminescence was recorded for 10 seconds with the counter running in Single-Photon-Count mode. Responses of the cells to the β-NADPH (100 μM) with or without the presence of Tiron (cell-permeable O_2^- scavenger, 20 mM), DPI (flavoprotein inhibitor, 5 μM) were also tested. A buffer blank background was subtracted from each respective reading. Final data were presented as RLU/sec.

Measurement of CAT and SOD activities

Treated HCAECs were homogenized and centrifuged in HEPES buffer (pH 7.4) containing 1 mM EDTA. CAT and SOD enzyme activities were measured with commercial enzyme assay kits

(Cayman Chemical, Ann Arbor, MI) following manufacturer's protocols. CAT and SOD enzyme activities were calculated from the average absorbance of each sample using the equations provided in the kit manuals. Final data were represented as mean ± SEM (nmol/min/mL) for CAT activity, and mean ± SEM (U/mL) for SOD activity.

Bio-Plex luminex immunoassay

Bio-Plex system (Bio-Rad) combines the Luminex multianalyte profiling technology with unique antigen-coated fluoromagnetic bead chemistry and versatile software. HCAECs were cultured with sCD40L (5 μg/mL) for 0, 5, 10, 20, 30, 45, 60 or 90 minutes. Cell lysate was prepared using the kit obtained from Bio-Rad. Detection of phospho- and total ERK2, JNK, p38 and IκBα was performed using manufacturer's protocols. Each test included four positive controls obtained from Bio-Rad which were designed to monitor detector stability, specimen and sample integrity. Final data were analyzed and presented as a ratio of phosphoprotein/total protein for each MAPK and IκBα (average of triplicates).

Immunohistochemistry of human CD40L and CD40

Full-thickness arterial wall specimens of aortas were obtained from 5 autopsy patients with or without atherosclerosis (National Disease Research Interchange, Philadelphia, PA). All samples were fixed in formalin and embedded in paraffin. Slides for immunohistochemistry were incubated in 0.3% hydrogen peroxide solution to quench endogenous peroxidase activity for 15 minutes and were subsequently washed with PBS. The slides were then incubated at room temperature in blocking serum for 30 minutes. The anti-human CD40 (Santa Cruz Biotechnology, Santa Cruz, California) or CD40L (Chemicon, Billerica, MA) antibody was applied for 30 minutes at room temperature. After washing with PBS, the sections were incubated with a biotinylated secondary antibody for 30 minutes. An avidin-biotin reaction using peroxidase enzyme was used for protein detection (ABC kit; Vector Laboratories, Burlingham, CA). Immune complexes were detected with diaminobenzidine under the microscope before counterstaining with hematoxylin for 1.5 minutes.

Statistical analysis

All non-parametric data (percentage or ratio of the control) were analyzed by using a non-parametric test Mann-Whitney *U* test (two-tailed) for comparison of the experimental group versus the control group. For all other parametric data, significant comparison was performed by using the paired

Student's *t* test (two-tailed). Significance was considered if $P < 0.05$. Data were reported as mean ± SEM. All statistical analyses were performed by using Minitab software (Sigma Breakthrough Technologies, Inc., San Marcos, TX).

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