Online Supplementary Materials

Detail Materials and Methods

Cell Culture

HCAECs and endothelial growth medium-2 (EGM-2) were purchased from Cambrex BioWhittaker Inc. (Walkersville, MD). Cells were used at passage 4 to 6. When HCAECs grew to 80%-90% confluence in 6-well plates, they were divided into six groups (I-VI). Group I cells were treated with different concentrations (10, 40 or 80 ng/ml) of human recombinant resistin (Phoenix Pharmaceuticals Inc., Belmont, CA) for various periods of time (6, 12 or 24 h). Some of the cells were then treated with actinomycin D (2.5 μg/ml, Sigma, St. Louis, MO) for additional 30 min, 1, 3, 6 and 12 h to test the eNOS stability. Group II cells were pre-treated with an antibody against resistin (Phoenix Pharmaceuticals Inc., 1 or 4 μg/ml), isotype control antibody (rabbit IgG, 4 μg/ml) or irrelevant antibody (anti-bromodeoxyuridine antibody, 4 μg/ml) for 30 min before adding resistin (40 ng/ml) for 24 h. Group III cells were co-cultured with antioxidants seleno-L-methionine (SeMet, 20 μM) or ginsenoside-Rb1 (Rb1, 10 μM) or superoxide dismutase (SOD) mimetic Mn (III) tetrakis (4-benzoic acid)porphyrin (MnTBAP, 2 μM, A.G. Scientific, San Diego, CA) with resistin (40 ng/ml) for 24 h. Group IV cells were treated with superoxide generator 6-anilino-5,8-quinolinequinone (LY83583, 3 μM, Cayman Chemical, Ann Arbor, MI), with or without SeMet (20 μM) for 24 h. Group V cells were treated with resistin (40 ng/ml) for 5, 10, 20, 30, 45, 60 or 90 min. Group VI cells were pre-treated with 1 μM p38 MAPK inhibitor (SB239063) for 1 h and then co-cultured with resistin (40 ng/ml) for 24 h. In all groups, cells cultured in EGM-2 alone were used as negative controls. p38 inhibitor (SB239063), bradykinine, N (G)-nitro-L- arginine methyl ester (L-NAME), an eNOS inhibitor, thenoyltrifluoroacetone (TTFA), an inhibitor of mitochondria electron transport chain complex II, were obtained from Sigma Chemical (St. Louis, Mo).

In separate experiments, HCAECs, human aorta smooth muscle cells (AoSMCs, Walkersville, MD) and human THP-1 monocytes (human acute monocytic leukaemia) were used for analysis of resistin mRNA expression in response to tumor necrosis factor-alpha (TNF-α, Sigma). AoSMCs were cultured in Smooth Muscle Medium-2 (SmGM-2) with growth factors and antibiotic (SmGM-2 Bullet Kit) supplemented with 10% fetal bovine serum (FBS). THP-1 monocytes (human acute monocytic leukaemia) were obtained from ATCC (Manassas, Va), and cultured in RPMI 1640 medium containing glutamine (2 mM), penicillin (20 IU/ml), streptomycin (20 IU/ml), 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and FBS (10%, v/v). Cells were seeded onto 96-well plates at

2×10^5 cells per well and differentiated into macrophages by the addition of phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) for 4 days.

Real-Time PCR

Total RNA from HCAECs was isolated using the tri-reagent kit (Sigma) following the manufacturer's instructions. RNA from each well was resuspended in 20 μl of RNase-free water and the concentration was determined by absorbance at 260-nm wavelength using a spectrophotometer. cDNA was generated by reverse transcription (RT) from mRNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Then the iQ SYBR Green SueperMix Kit (Bio-Rad) was used for real-time PCR reaction. Master mixture was used to reduce variability in primer and reagent concentrations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to account for variations in mRNA loading. The same total RNA, 1 μg, was loaded for all samples. Human eNOS and GAPDH primers were designed by Beacon Designer (Bio-Rad), and synthesized by Sigma Genosys (Woodlands, TX). The human eNOS (GenBank # NM_000603) primer sequences are (forward) 5'-AGGAACCTGTGTGACCCTCA-3' and (reversed) 5'-CGAGGTGGTCCGGGTATCC-3'. The human GAPDH (GenBank # NM_002046) primer sequences are (forward) 5'-TGCACCACCAACTGCTTAGC-3' and (reversed) 5'-GGCATGGACTGTGGTCATGAG-3'. Real-time PCR was performed in an iCycler iQ real-time PCR detection system (Bio-Rad). Thermal cycle condition used for RT was as follows: 5 min at 25ºC, 30 min at 42ºC, and 5 min at 85ºC. The condition used for real-time PCR was as follows: 3 min at 95ºC, 40 repeats of 20 s each at 95ºC, and 1 min 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. To assess the mRNA stability or half life ($t_{1/2}$) of eNOS mRNA, HCAECs were treated with 5 μg/ml actinomycin D (a direct inhibitor of RNA polymerase II) in the presence or absence of resistin (40 ng/ml). Total cellular RNA was isolated at multiple time points (0, 0.5, 1, 3, 6 and 12 h) and analyzed for mRNA levels by real-time PCR using eNOS primers.

HCAECs, AoSMCs and THP-1 macrophages were treated with or without TNF-α (10 ng/ml) for 24 h, and resistin mRNA levels were determined by real time PCR. The human resistin (GenBank # NM-020415) primer sequences are (forward) 5'-CCATGGAAGAAGCCATCAAT-3' and (reversed) 5'-ACTGGCAGTGACATGTGGTC-3'.

Western Blot

Total protein was isolated from HCAECs using the tri-reagent kit, and resuspended in 20 μl of 10 M urea. Protein concentration was determined using the Bradford protein assay with bovine serum albumin as standard. The same amount of endothelial protein (6 μg) was resolved electrophoretically by one-dimensional SDS-PAGE (10% polyacrylamide) for approximately 1 h 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 overnight at 30 V. The filter was blocked using 5% nonfat dried milk in Tris buffer saline (TBS) with 0.05% Tween 20 (TBS-T) for 1 h at room temperature. eNOS was detected using a mouse anti-human eNOS monoclonal antibody (BD Biosciences, San Jose, CA) diluted 1:1000, and β-actin was detected using a mouse anti-human β-actin monoclonal antibody (Sigma) diluted 1:10,000. The eNOS and β-actin primary antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibodies diluted 1:2000. Blots were developed using ECL-plus kit (Amersham Biosciences, Piscataway, NJ) and analyzed with gel documentation system and analysis software (Alpha Innotech Co., San Leandro, CA). All other chemicals, unless specified, were obtained from Sigma Chemical Co.

Nitric Oxide Synthase Activity Assay

A fluorometric cell-associated NOS detection system (Sigma) was used to measure intracellular production of NO from supplemented L-arginine by a non-radiometric method. A cell-permeable diacetate derivative of 4,5-diaminofluorescein (DAF-2 DA) was hydrolyzed by intracellular esterase to DAF-2 that, in turn, reacts with NO produced by NOS to form a fluorescent triazolo-fluorescein. The fluorescent product was quantitated using an excitation filter at 492 nm and an emission filter at 515 nm (fluorescence microplate reader). NOS activities in resistin-treatment groups were normalized with untreated cells. Data were showed as mean ± SEM (% of controls).

Nitrite Detection.

The level of NO released from HCAECs was determined by measuring the accumulation of its stable degradation products, nitrite and nitrate (Griess reaction NO assay kit, Calbiochem). Nitrate is reduced to nitrite by nitrate reductase. Thus, total nitrite levels represent total NO levels. HCAECs were cultured with resistin and/or other molecules for 24 h. A group of HACECs were stimulated with bradykinine (10^{-5}M) for 5 min. The supernatant was collected and total nitrite levels were measured.

Absorbance of the samples was determined at 540 nm wavelength and compared with standard solutions. The amount of nitrite detected was normalized to total proteins of HCAECs (pmol/mg protein).

Measurement of Cellular ROS and NO Levels

Cells were harvested with 0.025% Trypsin/EDTA and adjusted to 1 X 10^6 cells per FACS tube. For reactive oxygen species (ROS) and NO staining, respectively, dihydroethidium (DHE, 3 μM, Molecular Probes) and 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF–FM DA, 10 μM, Molecular Probes) were added and incubated in 37°C for 30 min. Final samples were collected in 500 μl staining buffer (BD Biosciences) and stored in 4°C. Samples were analyzed using a FACScan and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ) within 24 h of preparation. In each experiment at least 10,000 events were analyzed.

DHE is often used for the detection of ROS production because it can be oxidized by ROS such as O_2^- into two red fluorescent molecules 2-hydroxyethidium (2-EOH, excitation wavelengh 490 and emission wavelength 590 nm) and ethidium (excitation wavelength 480 and emission wavelength 580 nm), respectively (1). Thus, we can use a FACS Caliber flow cytometer to detect 2-EOH and ethidium by using an Argon laser (excitation at 488 nm) and a emission filter (FL-2 channel), which detects a pick emission wavelength 585 nm (ranking from 564 to 606 nm). In this way, DHE staining with current FACS analysis is correlated to the ROS production, while it can not identify individual ROS such as O_2^- because FACS can not separate 2-EOH and ethidium. 2-EOH is generated specifically by O_2^- oxidation of DHE, whereas ethidium is associated mainly with pathways involving H_2O_2 and metal-based oxidizing systems, including heme proteins and peroxidases (2-4).

HPLC Analysis for Superoxide Anion (O^{2-})

Recently, high performance liquid chromatography (HPLC) analysis of DHE-derived fluorescent compounds (2-EOH and ethidium) has been developed in order to achieve separation and individual analysis of 2-EOH and ethidium (2-4). Thus, we confirmed the increased production of O_2^- in the resistin-treated HCAECs by HPLC analysis (2-4). HCAECs were grew in 10 cm petri dishes with EGM plus 10% FBS upto 90% confluence, and changed the medium with EGM plus 2% FBS and different treatments for 3 h: 1). no treatment; 2). resistin (40 ng/ml) treatment; 3). SOD mimetic MnTBAP (2 μM), and 4). resistin and MnTBAP. DHE was added into the cell culture medium with final concentration of 25 μM and incubated for 30 min, and then stopped by washing the cells two

times with 1X cold PBS. The cells were harvested by scrapping, and pelleted by centrifuging at 4000rpm for 5 min at cold room; 200 µl methanol was added and the cells were sonicated for 10 seconds. The fresh samples were avoided from light and subjected to HPLC analysis within 2 h. HPLC (Bio-Rad BioLogic DuoFlow, Hercules, CA) was equipped with a 250 × 4.6 mm, 5 micron Phenomenex C-18 (2) Luna column. The mobile phase was CH_3CN/H_2O and the eluent was monitored by an on-line UV-vis detector (Bio-Rad, BioLogic QuadTec) at 210, 285, 350 and 470 nm. Ethidium and 2-EOH were separated by a linear increase in acetonitrile concentration from 37 to 47% over 23 min at a flow rate of 0.5 ml/min. A positive control of 2-EOH was prepared by the method described by Zielonka et al using nitrosodisulfonate radical dianion (NDS, Sigma-Aldrich) with DHE in the aqueous phosphate buffer pH 7.4 containing diethylene triamine pentaacetic acid (DTPA, Sigma-Aldrich) (4).

Cellular GSH Assay.

GSH-Glo Glutathione assay (Promega, Madison, WI) measures a change in the redox state of the cell due to oxidants, which are downstream metabolites of O_2^-. HCAECs were treated with resistin and/or other molecules for different time periods. Cellular GSH levels were measured following the manufacturer's instructions.

Assessment of Mitochondrial Membrane Potential *(Δψm)*

Changes in Δψm 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, BD Biosciences). Mitochondria with a normal Δψm concentrate JC-1 into aggregates (red fluorescence), but with a de-energized or depolarised Δψm, JC-1 forms monomers (green fluorescence). Cells ($5x10^5$) were incubated with 10 µg/mL JC-1 for 12 min at 37°C and analyzed by flow cytometry using a FACScan and Cell Quest software (Becton Dickinson). 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 20,000 events were analyzed.

Measurement of ATP Levels

ATP levels were measured with an ATPLite kit (PerkinElmer, Wellesley, MA). The ATPLite assay system 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 resistin (40 ng/ml) for 24 h. 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).

Measurement of CAT and SOD Activity

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 for the CAT and SOD activity was represented as nmol/min/ml and U/ml. respectively.

BioPlex Luminex Immunoassay

BioPlex 2200 (Bio-Rad) is a fully automated, floor-standing random access platform with multiplex capabilities. The instrument combines the Luminex multianalyte profiling technology with unique antigen-coated fluoromagnetic bead chemistry and versatile software. HCAECs were cultured with 40 ng/ml of resistin for 0, 5, 10, 20, 30, 45, 60 or 90 min. Cell lysate was prepared using the kit obtained from Bio-Rad. Detection of phospho- and total ERK1/2, JNK and p38 was performed based on the manufacturer's protocol. Each test included three 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 (average of triplicates).

Cell Viability.

HCAEC viability was measured with CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega, USA). Briefly, HCAECs (2000 cells/well in 96-well plate) were treated with resistin (40 and 80 ng/ml) for 24 and 48 h. Then the cells were incubated with 20 μL MTS reagent in 100 μL of fresh culture medium for 2 h at 37ºC in a humidified and 5% CO_2 atmosphere.

The absorbance at 490 nm was recorded using an ELISA plate reader (EL800, Bio-Tek Instruments, Inc., Vermont, USA).

Immunohistochemical Analysis

Full-thickness arterial wall specimens of aorta and carotid arteries were obtained from 5 patients with or without atherosclerosis undergoing autopsy [National Disease Research Interchange (NDRI), Philadelphia, PA]. All samples were fixed in formalin and embedded in paraffin. Immunohistochemistry was done with anti-resistin antibody (1:200) (Phoenix Pharmaceuticals, Burligame, CA), biotinylated secondary antibody, and avidin-biotin reaction using peroxidase enzyme (ABC kit; Vector Laboratories, Burlingham, CA). The protocol for use of human tissues obtained from NDRI was approved by the Institutional Review Board (IRB) at the Baylor College of Medicine. The investigation conformed to the principles outlined in the Declaration of Helsinki. In addition, eNOS expression of HCAECs was analyzed by immunohistochemistry staining. The cells were seeded on the chamber slides for 24 and 72 h to achieve low density and high density of cell growth, respectively. eNOS was detected using a mouse anti-human eNOS monoclonal antibody (BD Biosciences, San Jose, CA) diluted 1:200, biotinylated secondary antibody, and avidin-biotin reaction using peroxidase enzyme.

Statistical Analysis

Data from the different treatment groups and control groups were compared with a paired Student's *t* test (two tails). Significance was considered if $P < 0.05$. Data are reported as mean ± SEM.

References

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2. **Fink B, Laude K, McCann L, Doughan A, Harrison DG, Dikalov S.** Detection of intracellular superoxide formation in endothelial cells and intact tissues using dihydroethidium and an HPLC-based assay. *Am J Physiol Cell Physiol* 287: C895-902, 2004

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4. **Zielonka J, Vasquez-Vivar J, Kalyanaraman B.** Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. *Nat Protoc* 3: 8-21, 2008.

Supplemental Figures:

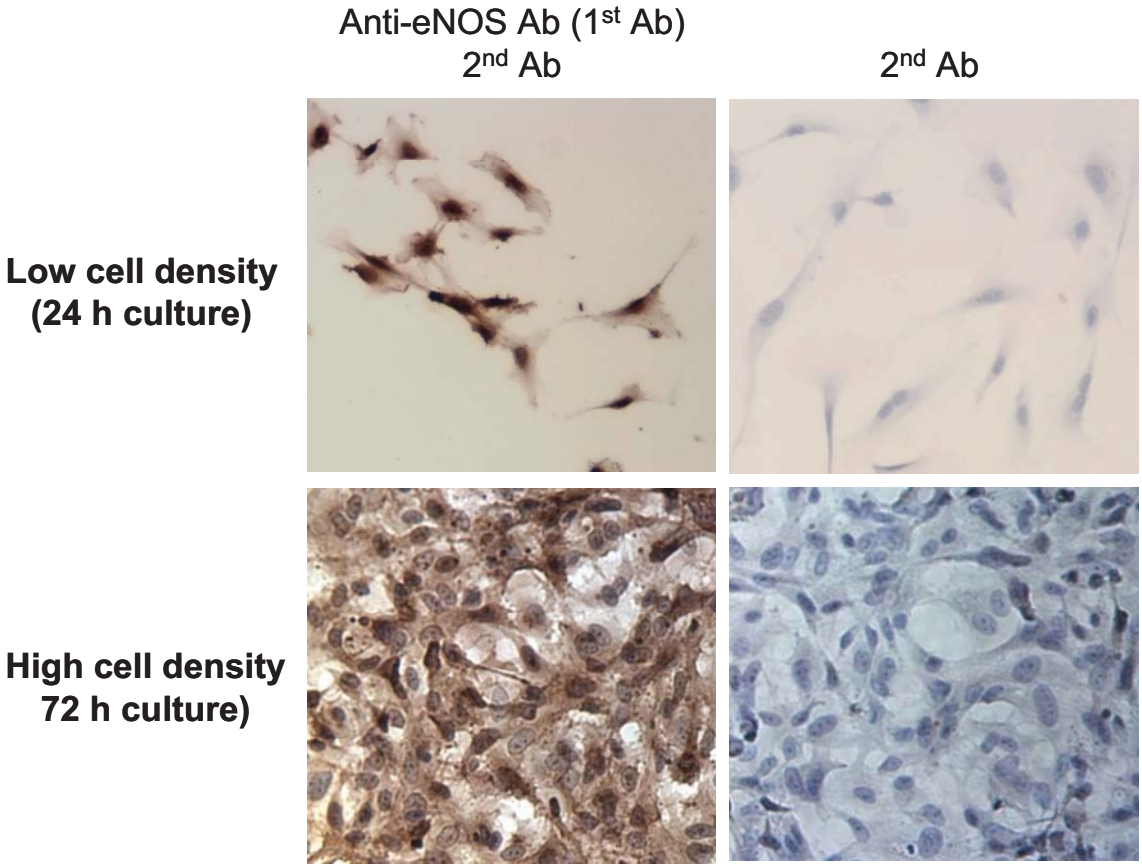

Fig. S1. eNOS immunohistochemistry of HCAECs. Cells were cultured for 24 h (low density) and 72 h (high density), respectively. Dark brown color represents positive staining of eNOS. Negative control was set with 2nd antibody staining and without anti-eNOS primary antibody.

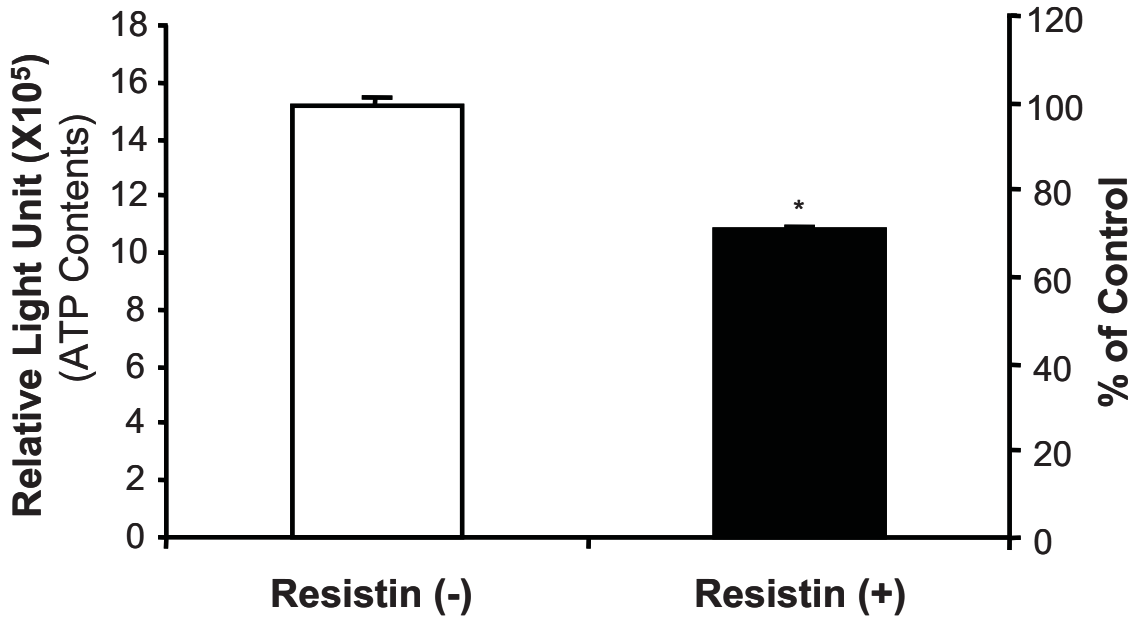

Fig. S2. ATP levels. HCAECs were treated with or without resistin (40 ng/ml) for 24 h. Cellular ATP levels were measured with an ATPLite kit. **P* < 0.05, n=3.

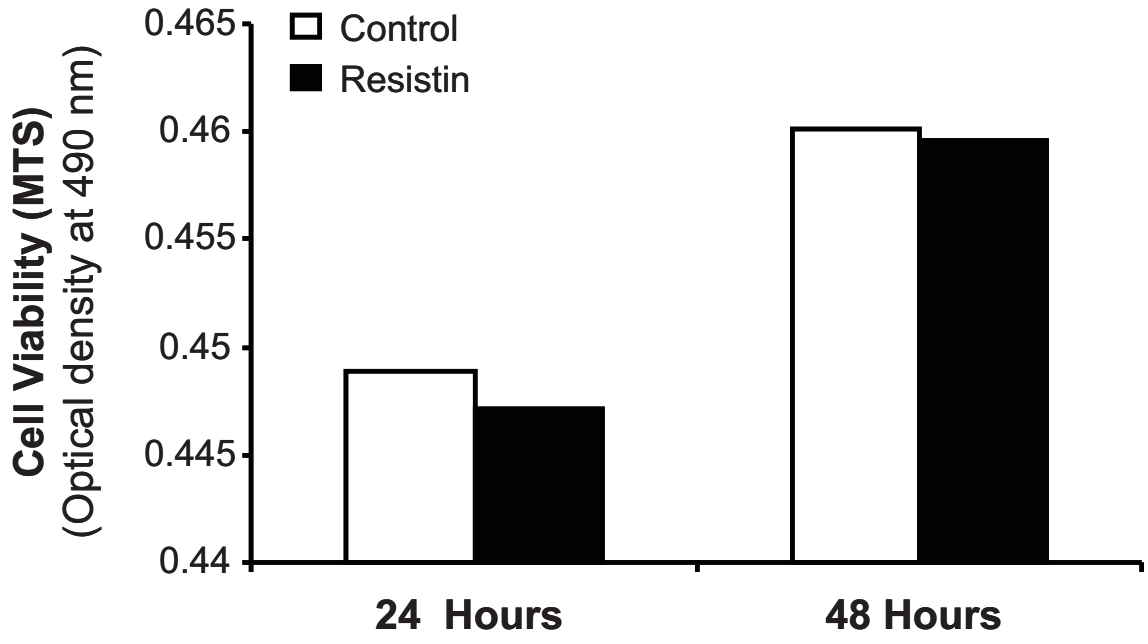

Fig. S3. Cell viability (MTS assay). HCAECs were treated with resistin (40 and 80 ng/ml) for 24 and 48 h.

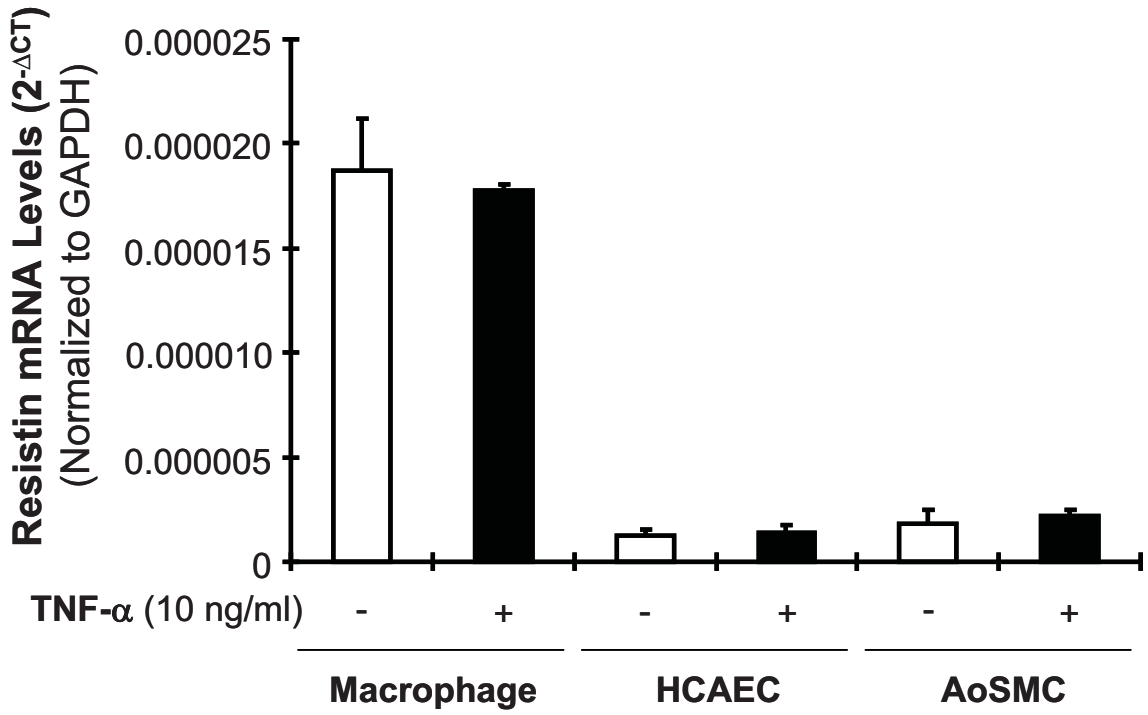

Fig. S4. Resistin mRNA levels in HCAECs, human aorta smooth muscle cells (AoSMCs) and human THP-1-derived mancrophages. The cells were treated with or without TNF-α (10 ng/ml) for 24 h, and resistin mRNA levels were determined by real time PCR. GAPDH was used as an internal control.