Supplemental Material

Caspase-1 inflammasome activation mediates homocysteine-induced pyrop-apoptosis in endothelial cells

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Supplement Materials and Methods

Chemicals and reagents:

Dulbecco's modified Eagle's medium, M199, penicillin, streptomycin, L-glutamate, and heparin were purchased from Invitrogen (Carlsbad, CA). Basic fibroblast growth factor was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Dichlorofluorescein diacetate (DHE) was purchased from Molecular Probes (Eugene, OR). Ac-YVAD-CHO (Caspase-1 inhibitor), Ac-IETD-CHO (caspase-8 inhibitor), Ac-LEHD-CHO (caspase-9 inhibitor), Ac-DEVD-CHO (caspase-3 inhibitor), and Z-VAD-fmk (broad spectrum caspase inhibitor) were obtained from Alexis (San Diego, CA). Cathepsin B inhibitor IV was from Calbiochem (Gibbstown, NJ). Antibodies against cytochromec, and actin were purchased from Transduction Laboratories (Lexington, KY). Antibodies against caspase-1, 9, Bax, Bcl-2 were purchased from Santa Cruz Biotech (Santa Cruz, CA). L-Homocysteine (L-Hcy) was freshly prepared by reducing L-homocysteine with a 2-fold molar excess of DTT for 30min at 37°C, pH 8.0, as described previously.[1] All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless indicated otherwise.

Cell culture:

Human umbilical vein endothelial cells (HUVECs) were cultured as we described previously.[1] The cells were grown on a gelatin-coated 75-cm^2 flask in M199 with 20% fetal bovine serum, 66 μg/ml endothelial cell growth supplement (ECGS, BD Bioscience), 10 units/ml heparin, and 1% streptomycin/amphotericin B/penicillin (Thermo Fisher) at 37°C under 5% CO_2, 95% air. The cells used in this study were between passages 4~8. For cell death and cell death-related functional analysis, cells were cultured to 80% confluence, synchronized in serum free M199 for 6hr, and then switched to 0.5%FBS M199 medium containing L-homocycteine (L-Hcy), L-cysteine (L-Cys), lipopolysaccharide (LPS) and hydrogen peroxide (H_2O_2) for 24 hr. Inhibitors were added 30 min prior to these treatment.

Mouse aortic endothelial cell (MAEC) isolation and primary culture:

Mice was anesthetized, followed by exposure of the thoracic cavity. Mouse aorta was isolated and removed aseptically into 6 cm dish, and rinsed with DMEM. Both ends of the aorta were cannulated with PE-20 tubes (Intramedic, BD Diagnostic System, Sparks, MD) and tied. The aorta was flushed with PBS for twice. Then, 100 μL of collagenase type II (2mg/mL in DMEM; Worthington Biochem. Lakewood, NJ) were instilled into the aorta, allowed to dwell by closing on both ends of tubes. Whole aorta was incubated in serum free DMEM for 20min at 37°C. The aorta was opened and flushed with 20% FBS/DMEM for twice. The digestion fluid of each aorta was drained into a 15mL tube containing 10mL of endothelial growth media [EGM = 50% DMEM; 50% F-12 (Invitrogen-Gibco 11765); 10% FBS; 66 μg/ml ECGS (Invitrogen-Gibco 11765); 10 unit/mL Heparin; 1% streptomycin/amphotericin B/penicillin (Thermo Fisher)]. After spin down at 1,500 rpm for 3 min, all the cells were collected and re-suspended with fresh EGM. Cells then were transferred to collagen-coated 12 well-plate (per aorta/well) and incubated till 80% confluence.[2]

Mouse Lung endothelial cell (MLEC) single cell suspension and FCM analysis:

Mice was anesthetized, followed by exposure of the thoracic cavity. Cold DMEM (5mL) was injected via the right ventricle to flush the lung of blood cells. Lung tissues were removed aseptically, rinsed in DMEM, minced into about 1 mm^3 pieces, and then incubated with 2.5mL collagenase (Type 1a, 1mg/mL, Sigma) for 45 min in a 37°C water bath in a 15 mL tube. Every 15 min during incubation, the tube was gently agitated to further dissolve the tissues. After the incubation, 2.5 ml 10%FBS/DMEM was added to the tube. The resulting tissue/cell suspension was filtered through a 100-μm strainer into a 50 mL tube. The filtered cell suspension was centrifuged for 5 min at 1500 rpm. After removal of the supernatant, the cell pellet was washed and then re-suspended in serum free DMEM. Next, cells were incubated with monoclonal antibodies to CD-31 (anti-CD31–PE, BD Pharmingen) for 20min. After wash, cell was incubated in 37°C with caspase-1 activity staining peptide FAM-peptide-FMK (Cat# FAM-600, Cell Technology, Inc., CA) for 90 min as described below in Caspase activity assay followed manufacturer's instruction. Finally, cells was suspended in washing buffer, and caspase-1 activity in MLECs was quantified by flow-cytometry (FACSCalibur, BD Biosciences).

Flow cytometry (FCM) analysis:

FCM was utilized to detect programmed cell death (pyroptosis and apoptosis) by Annexin V/PI/7-AAD staining, caspase activities using caspase activity kit, intracellular reactive oxygen species (ROS) by Dihydroethidium (DHE) staining, and mitochondrial membrane potential (Δψm) by JC-1 staining as detailed below. Approximately 50,000 gated cells were analyzed per sample for each study. Data were analyzed using the FlowJo software (Tree Star Inc., Ashland, OR).

Programed cell death (Pyroptosis & apoptosis) characterization (FCM):

We employed 8 methods to characterize programed cell death, including 1) FCM with Annexin/PI or Annexin/7-AAD staining, 2) TUNEL staining for DNA fragmentation, 3) flow cytometry for caspase-1/9/8/3 activities, 4) Co-IP to characterize inflammasome complex and activities, 5) IL-1b activity by WB, 6) ROS gradient analysis for different cell population, 7) cell fraction apoptotic protein characterization, and 8) mitochondrial function and collapse analysis. We have most of these methods detailed below separately.

For FCM analysis, programed cell death were characterized by Annexin V/7-aminoactinomycin (7AAD), or Annexin V/propidium iodide (PI) staining with fluorescein isothiocyanate (FITC) conjugated Annexin V from Apoptosis Detection Kit I (BD Biosciences, San Jose, California) following manufacturer's instruction. Cells were trypsinized, washed with PBS twice, incubated with binding buffer for 15min at RT, and then stained with Annexin V and 7AAD or PI for 15 min in 37°C, and subjected for FCM. 7AAD and PI stain for DNA and are interchangeable for the convenience of color combination in flow cytometric analysis. As shown in Fig. 1B, non-programmed cell death were excluded from intact cells based on small size (FSC) and minimal DNA florescence, which justify debris and residuals of necrotic cells. Annexin V^+/PI^+ or Annexin

$V^+/7AAD^+$ cells were defined as apoptosis cells. Annexin V^-/PI^+ or Annexin $V^-/7AAD^+$ cells were defined as pyroptosis population.

Caspase activity assays (FCM, Fluorescence spectrometry, WB, microscopy):

Caspase activities were determined by 4 methods, including FCM, fluorescence spectrometry, WB and fluorescent micrpscopy using caspase activity kit (APO LOGIX kit, Cat# FAM-600, 300, 400, 200 for caspase-1, -8, -9, -3, respectively, Cell Technology, Inc., Mountain View, CA), which captains proper carboxyfluorescein labeled fluoromethyl ketone-peptide (FAM-peptide-FMK). Once inside the cell, these permeable peptides could bind covalently to the active caspase.[3] All process was performed according to manufacturer's instruction. Briefly, cultured HUVECs were cultured to 80% confluency or 96-well plate, treated as described above for cell viability assay.

For FCM assay, cells were cultured in 35mm dish, digested with trypsin, incubated with FAM-peptide-FMK (2.5 µL 150X to $1X10^5$ cells/200 µL suspension) in 37°C for 90 min and spanned at 1,000 rpm for 5 min at 4ºC. Cells were suspended in 500 µL washing buffer. FMK positive cells was quantified by flow-cytometry (FACSCalibur, BD Biosciences). Data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

For fluorescence spectrometry analysis, cells were cultured in 96-well plate and treated. FAM-peptide-FMK (2 µL 15X/well) were added at the end of the experiment and incubated for additional 90 min at 37°C. Caspase activity was immediately measured in a fluorescence spectrometry microplate reader (BIO-TEK Instruments, Inc., Flx800).

For WB analysis, we used caspase-1 domain recognizing antibody (SC-622, Santa Cruz) to detect the levels of activated caspase-1. As shown in Fig. 2E, pro-caspase-1 protein zymogen has 404 amino acid (45KD), and can be cleaved to generate a P20 and a P10 subunits, which form a hetero-tetrameric active enzymatic center. The levels of P20 band of caspase-1 in WB gel represent caspase-1 activity.

For fluorescent microscopy analysis, cells were cultured in 12-well plates to 80% confluence, treated, in same case pretreated with caspase-1 inhibitor (Ac-YVAD-CHO) or cathepasin B inhibitor for 30m. FAM-peptide-FMK (2 µL 15X/well) were added at the end of the experiment and incubated for additional 90 min at 37°C, washed with PBS, fixed and imaged using an fluorescence microscope (Carl Zeiss, Germany). Green cells were recognized as caspase activated cells and counted per field (40X) for quantification. Ten images were taken for each group. The average caspase activated cells in the control group were considered as 100%.

Rescue efficacy of caspase inhibitors:

HUVECs at was pretreated by specific caspase-1, -8, -9, -3 inhibitors (Cell Technology, Inc., Mountain View, CA) respectively for 2hr, and then was incubated with Hcy and/or LPS. Hcy/LPS induced pyroptosis/apoptosis were evaluated by FCM in vehicle and each pretreatment group. And then, mean

Rescue efficacy score of each caspase inhibitor was calculated according to following formula. Rescue efficacy = A/B x 100. Meanwhile, A = ($^{\text{Vehicle Pretreat}}$Treat - $^{\text{Inhibitor Pretreat}}$Treat); B = ($^{\text{Vehicle Pretreat}}$Treat - $^{\text{Vehicle Pretreat}}$Ctrl). The Rescue efficacy for caspase-1, -8, -9, -3 inhibitors represented their inhibitory capability for Hcy/LPS-induced pyroptosis and apoptosis.

ROS level detection (FCM):

Dihydroethidium (DHE), freely permeable to cells, is oxidized by superoxide anion to become DNA-binding fluorophore. DHE was applied to detect reactive oxygen species (ROS) levels in HUVECs. Aliquots of 1 X10^6 cells were incubated in 200ul PBS containing 100μM DHE for 15 min at 37°C in the dark; then the samples were washed with serum free medium and immediately analyzed. All samples were analyzed with by FCM (BD Bioscience, FACSCalibur). Forward and side scatter gates were used to select single cell population from clumps and debris.

Mitochondrial potential measurement (Immunochemistry staining & FCM):

We examined mitochondrial membrane potential (Δψm) by JC-1 immunochemistry staining and by FCM using tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining, a cationic dye from APO LOGIX (Cat# S-9074, Cell Technology). JC-1 is a lipophilic, cationic dye that can selectively enter mitochondria. In healthy cell with normal Δψm, JC-1 forms the J-aggregates showing red fluorescence. But, in the cells with collapsed Δψm, JC-1 remains in the monomeric form with green fluorescence. The responsive distribution of the red fluorescence and green fluorescence of JC-1 reflects the alteration of Δψm. Briefly, after Hcy, Hcy+LPS treatment, ECs were incubated with JC-1 for 15 min and washed by 1%FBS/PBS (FACS buffer).

For immunochemistry staining, the fluorescence FCM analysis, cells were cultured on 12 well plate to 80% confluence, treated, washed with PBS, stained with 5μL JC-I (10μg/mL) for 15 m at room temperature and washed with PBS before microscopy observation.[4, 5] Increased green fluorescence and decreased red florescence levels indicate the levels of Δψm collapse.

Mitochondrial potential dissipation led to mitochondrial membrane permeability increasing and its small contents leakage. Cytochrome *c* releasing to cytosol was detected by subcellular fractionation followed by Western blotting analysis as described previously.[6] HUVECs were resuspended in cell lysis buffer and homogenized 20~40 times on the ice. The homogenants were centrifuged at 1000*g* for 3min to remove unlysed cells and nuclei, and the supernatant containing was centrifuged again at 10,000*g* for 10min at 4°C. The supernatant from this spin was removed and saved as the cytosolic portion. The pellet containing the mitochondria was resuspended in protein lysis buffer and became mitochondrial fraction after 10second sonication. Protein measurements were performed and ready for Western Blotting assay. Protein in each fraction was equal loaded, and then labeled with anti-cytochorme *c* antibody. Antibodies of mitochondrial membrane protein VACD1 were used as mitochondrion fractionation quality control and Coommassie Blue staining was applied as loading control.

Cell viability assay (Chemiluminescence Spectrometer):

Cell viability was determined by the crystal violet staining as described previously (14).[7] HUVECs in 96-well plate (2 X 10^4 cells/well) were cultured in M199 containing 20%FBS to 80% confluence, synchronized in serum free M199 for 6hr, and then switched to 0.5%FBS M199 medium containing L-Hcy, L-Cys and LPS for 24 hr. Plates were then fixed with 50 µl/well of paraformaldehyde (PFA)/methanol (3%/30%) mixture and washed four times with distilled water. After air drying, cells were lysed in 100 µl/well of 33% acetic acid solution. Absorbance of dye uptake was measured at 595 nm in a Chemiluminescence microplate spectrometer (BIO-TEK Instruments, Inc., Elx800). Cell viability was calculated from relative dye intensity compared with untreated controls.

TUNEL assay:

HUVECs or isolated mouse aortic ECs were cultured on fibronectin-coated glass coverslips. Cell DNA fragmentation was detected in situ using terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL) assay.[8] DNA fragments were labeled with FITC-conjugated dUTP using TdT (BD Pharmingen). The total numbers of nuclei was determined by manual counting of DAPI-stained nuclei in eight random fields per section (original magnification, X200). All TUNEL-positive nuclei from HUVECs were counted as percentage from total cells in each section.

Cultured cell protein extraction, immunoblot analysis and fragmentation:

HUVECs were cultured in 6 cm-dishes to 80% confluence, treated, washed with 10 ml ice-cold PBS, scraped and collected. For immunoblot analysis, cell pellet was lysed in protein lysis buffer and sonicated for 10 sec, and clarified by centrifugation at 12,000 rpm. The supernatants (50 µg/lane) were subjected to SDS-PAGE followed by Western blot analysis as described.[2]

We used a mitochondrial fraction kit (Cat#40015, Active Motif) to separate cytosolic and mitochondrial fraction. This is because that Δψm collapse/dissipation leads to mitochondrial membrane permeability increasing and its small contents leakage. Cytochrome-*c* releasing to cytosol can be detected by WB Analysis as described previously.[6] Cells were washed by 10 ml ice-cold PBS, scraped and spun at 600g for 5 minutes at 4°C. Cell pellet was gently re-suspended in 1 ml ice-cold Cytosolic Buffer, incubated on ice for 15 minutes, and transferred an Eppendorf tube for homogenization on ice. After spin at 600g for 5 minutes at 4°C, supernatant was transferred a pre-chilled and sun at 800g for 20 minutes at 4°C to collect the supernatant. To collect the mitochondria fraction, the supernatant was spun at 10,000g for 20 minutes at 4°C to separate the cytosol (ready to use supernatant) from mitochondrial (pellet) fractions. The mitochondrial pellet was lysed in 100 µl Complete Mitochondria Buffer, incubated on ice for 15 minutes, and vortexed for 10 seconds to mix thoroughly.

Tissue section immunochemistry, protein extraction and immunoblot analysis:

The fragment of thoracic mouse aorta (5mm) was isolated and immersed into the Optimal Tissue Compound (OTC) and quickly frozen into liquid nitrogen. Cryostat sections (7μm/section) were achieved by using cryostats microtome (Leica Microsystems, Buffalo Grove, IL), and stained with antibodies against caspase-1 and CD31. After incubated with fluorescent-conjugated second antibodies, slides were mounted and examined under fluorescent microscope. Caspase-1 channel was converted to 8-bit image, the mean luminance in endothelium area was analyzed depending its grayscale by using NIH software Image-J (Bethesda, MD).

Mouse aorta was isolated, carefully cleaned by removing connective tissue under microscope, rinsed with PBS, minced and homogenized using an electrical tissue homogenizer in protein lysis buffer. Protein extracts were clarified by centrifugation at 12,000 rpm for 10 m. The supernatants were then equally subjected to SDS-PAGE followed by Western blot analysis.[9]

Co-Immunoprecipitation:

Method was described previously.[10] In brief, HUVECs were cultured in 10cm dish till 80% confluence. After 24hr treatment, the cells were washed with ice chill PBS and lysed in 500 μL EBC-IP buffer (Tris-HCl, 50mM (pH 8.0); NaCl,120mM; NP-40, 0.5%) per dish for 30min on ice. Cell lysates were collected and spin down at 14,000 rpm X 10 min at 4ºC. All supernatant were transferred to fresh tube, added with 10 μL protease inhibitor cocktail (*c*Omplete Protease Inhibitor Cocktail Tablets, Roche, 1 tablet per/mL in PBS) and 5μL protein G sepharose beads (Sigma) for Pre-clear, and incubated for 2 hr in roller at 4ºC followed by spinning down at 12,000 rpm for 5min at 4°C. The supernatants were transferred into fresh Eppendorf tubes, added with 4 μL anti-caspase-1 polyclonal antibody (sc-515, Santa Cruz) and incubated for overnight at 4ºC on roller. The sample was added with 30 μL protein G sepharose beads, and incubated for additional 4 hr at 4ºC on roller followed by spinning down at 12,000 rpm for 5min at 4°C. After carefully removed supernatant, 500 μL of EBC-IP buffer were added for washing and followed by spinning down at 12,000 rpm for 5min at 4°C. Finally, after discarding the supernatant, 45 μL Lammili buffer (20% glycerol; 0.004% bromophenol blue; 0.125 M Tris-HCl with pH 6.8) were added to each tube and incubated at 37ºC for 5min. The sample were vortexed and centrifuge for 2min at 14,000 rpm at 4°C. The supernatant was aspirated and placed in a fresh tube for immuno-blotting assay to identify proteins associated with casapsae-1.

Mice and diets:

All mice are in a C57B/L6 strain background. Mouse with cystathionine β-synthase (CBS) gene deficiency and an inducible human CBS transgene under the control of a zinc-inducible metallothionein promoter (*Tg-hCBS-Cbs*$^{-/-}$) was used as a hyperhomocysteinemia model as we described previously.[11] Pups were genotyped at day 10. These animals were all born to mothers drinking zinc water to induce human transgene expression to circumvent the neonatal lethality problem in the *Cbs*$^{-/-}$ mice. $ZnCl_2$ was withdrawn after weaning at 1 month of age. Caspase-1, NLRP3 knock out mice (*Casp-1*$^{-/-}$, *NLRP3*$^{-/-}$) were

generously provided by Dr. Richard Flavell's laboratory (Yale University School of Medicine, CT). *Casp-1*$^{-/-}$ were crossed with *Tg-hCBS-Cbs*$^{-/-}$ *to generate Tg-hCBS-Cbs*$^{-/-}$*/Casp-1*$^{-/-}$ mice. Animals were fed standard rodent chow diet (0.43% methionine; TD 2018SX; Harlan Teklad, Madison, WI). Age-matched littermates (12~16 weeks) with even sex distribution were selected for each experiment. Experiments were performed in wild-type, *Cbs*$^{+/-}$, *Cbs* $^{-/-}$, *Casp-1*$^{-/-}$, *NLRP3*$^{-/-}$, *Cbs*$^{-/-}$*/Casp-1*$^{-/-}$ mice. All the mouse protocols were approved by the Temple University Institutional Animal Care and Use Committee.

Vascular relaxation responses:

Vessel relaxation was monitored and recorded by a DMT, Multi Wire Myograph System (DMT-USA, MI, USA) as described previously.[1, 2, 12] The presence of intact endothelium in the vascular preparations was confirmed by observing the relaxation response to 1μM acetylcholine (ACH) in rings precontracted with 1μM phenylephrine (PE). Endothelium-dependent relaxation responses to cumulative concentrations of acetylcholine (ACH, 10nM-33μM) and endothelium-independent relaxation responses to sodium nitroprusside (SNP; 1nM-10μM) in rings pre-contracted with PE (1μM) were determined.

Plasma Hcy measurement:

Plasma Hcy level was analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as we previous described.[13]

Data Analysis:

All the experiments were performed at least three times independently, and results were expressed as the mean ± standard error (S.E.). The Kruskal-Wallis one-way ANOVA was used to compare the means of multiple treatments in groups. If there is significance, statistical comparison of single parameters between 2 treatments in same group or same treatment in different groups was performed by paired Student t test. Data were considered statistically significant if p was <0.05. As well as, Numbers above each bar is the percentage normalized by the mean of control, while control is as 100%.

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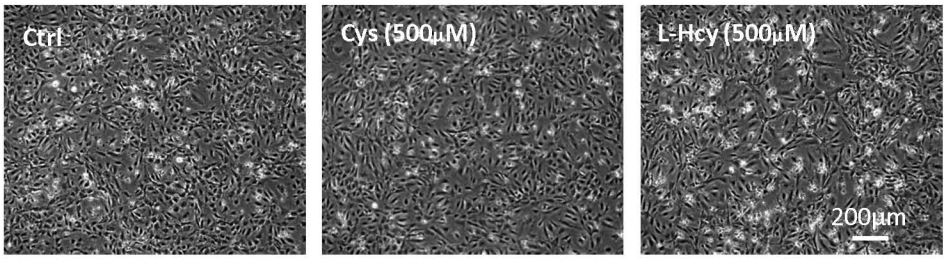
Supplemental Figures

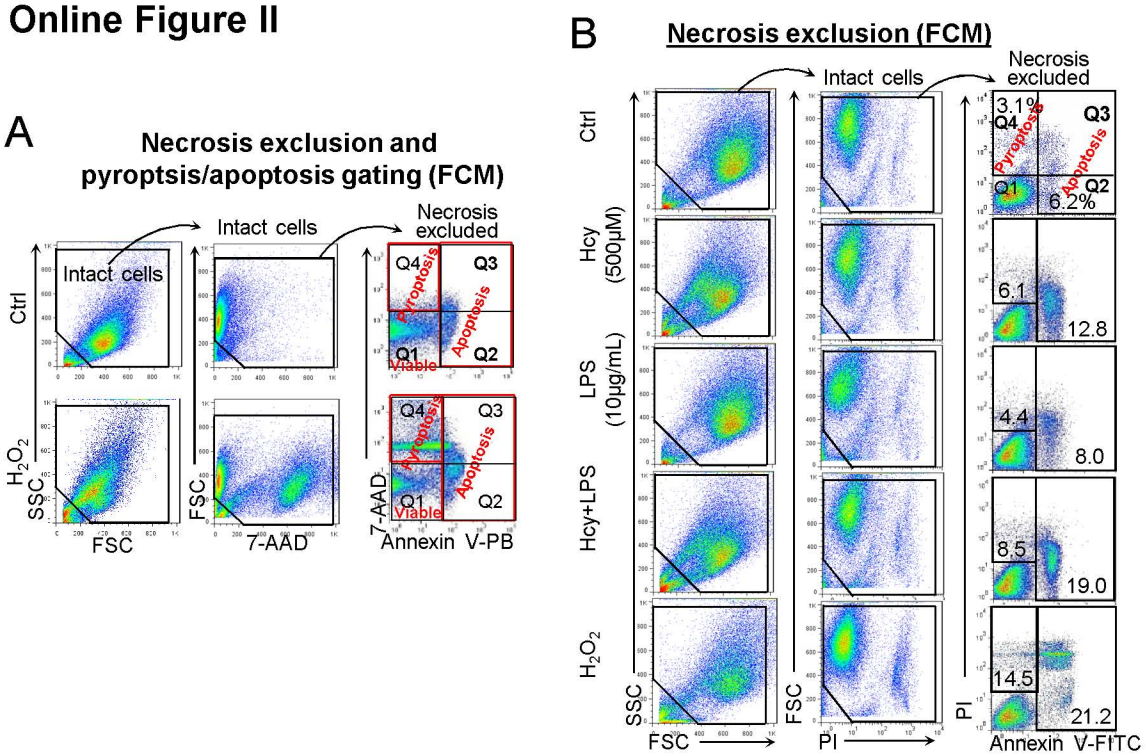
Online Figure I

Hcy reduces viable HUVECs (Microscopy)



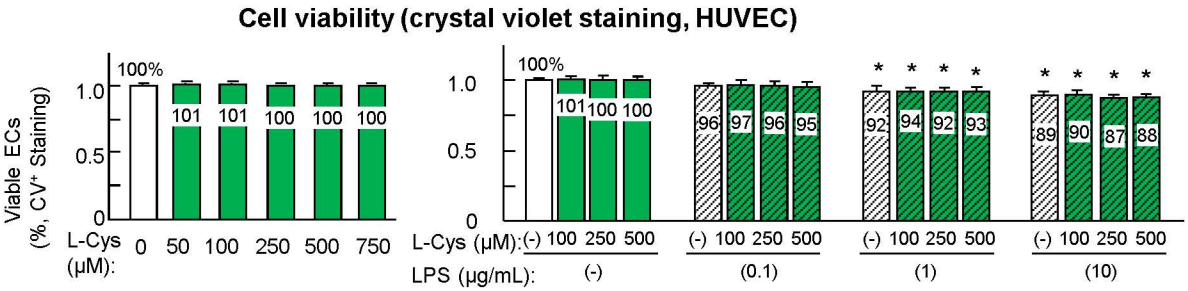
Online Figure I, Hcy suppresses cell viability in endothelial cells (EC). HUVECs were cultured in 6cm dish to 80% confluence, synchronized (serum free, 6hr), and then switched to 0.5% FBS medium containing L-Hcy (500μM), L-Cys (500μM) for 24hr and imaged.

Online Figure II



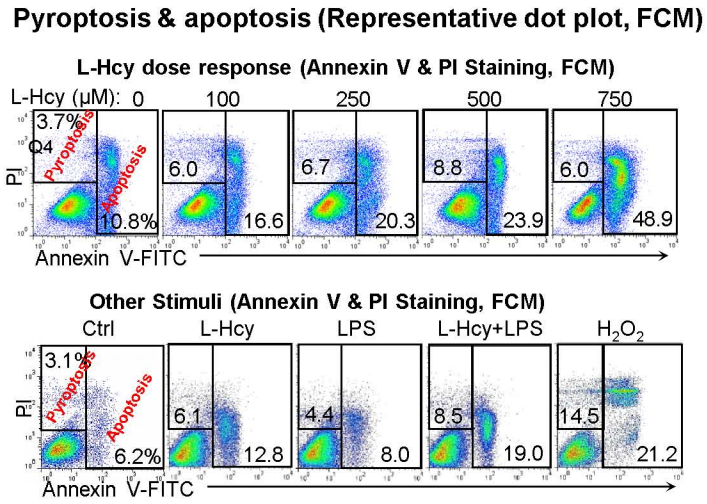
Online Figure II, Necrosis exclusion and pyroptsis/apoptosis gating in EC. HUVECs were cultured as described in Online Figure I, treated as indicated for 24Hr and harvested for flow cytometry (FCM) analysis as described in **Fig. 1**. **A, Pyroptosis and apoptosis gating (FCM, AV/7AAD staining).** Cells were stained by Annexin V-Pacific Blue (stains for phophatidylserine) and 7AAD (stains for DNA) for FCM analysis. Necrotic cells were excluded from intact cells by their content of nuclear debris. Based on Fig. 1A justification, Q2+Q3 cells were define as apoptosis. Q4 cells were defined as pyroptosis. **B. Necrosis exclusion (FCM, AV/PI staining).** Cells were stained by Annexin V-FITC and PI (stains for DNA, equivalent to 7AAD but more sensitive). Necrotic cells were excluded. Abbreviation: FCM, flow cytometry; AV, Annexin V; 7-AAD, 7-amino-actinomycin D; PI, propidium iodide; Hcy, homocysteine; LPS, lipopolysaccharides.

Online Figure III



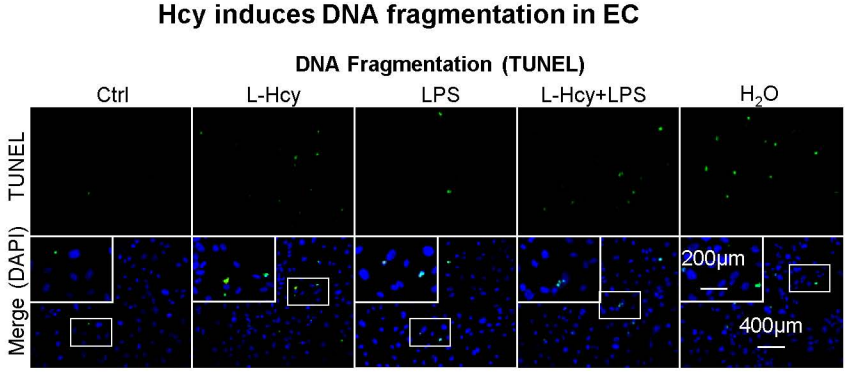
Online Figure III, Cell viability (crystal violet staining). HUVECs were cultured in 96-well plate indicated as described in Online Figure I, treated as indicated, fixed and stained by crystal violet for cell viability.

Online Figure IV



Online Figure IV, Hcy induces pyroptosis/apoptosis in EC. HUVECs were treated as indicated for 24Hr and harvested for FCM analysis as described in Online Figure II. Cell death forms were identified by FCM using Annexin V-FITC/PI staining. Annexin V^-/PI^+ are defined as pyroptosis, Annexin V^+ are defined as apoptosis. Abbreviation: FCM, flow cytometry.

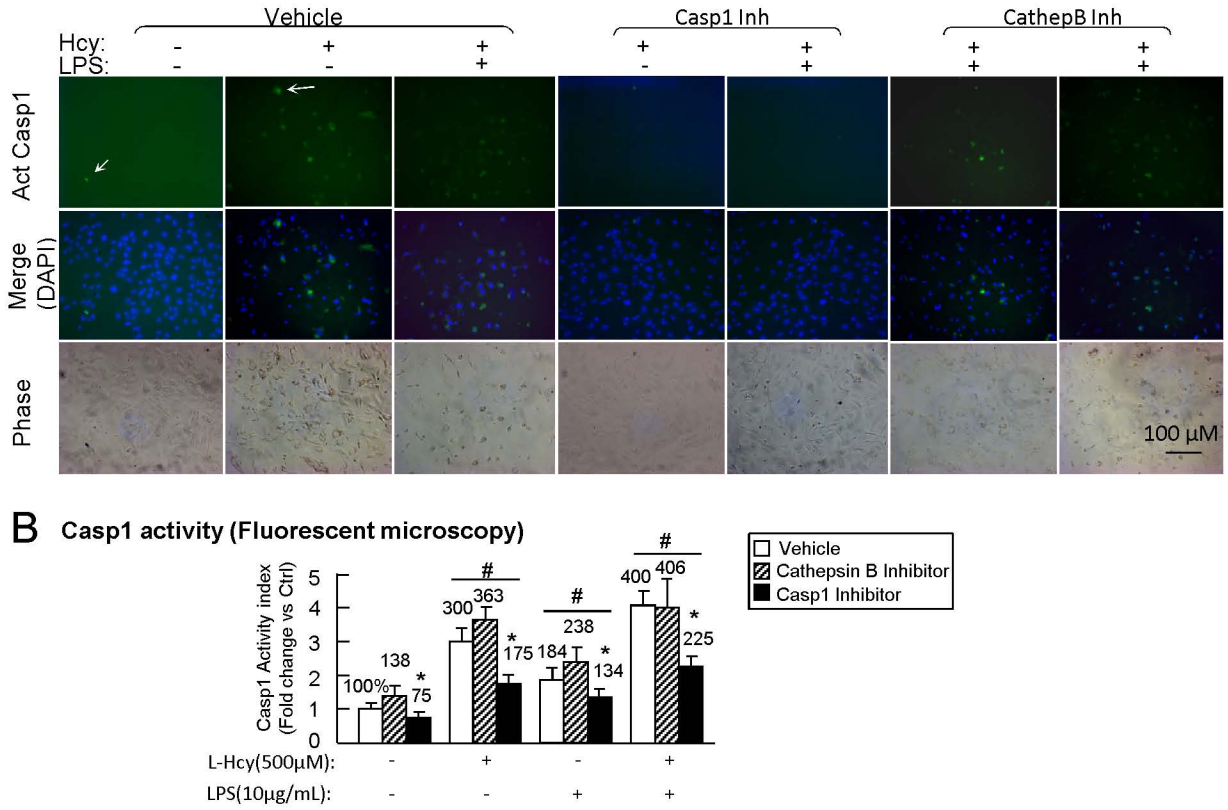
Online Figure V



Online Figure V, Hcy induces DNA fragmentation in EC. HUVECs were cultured and treated with L-Hcy (500μM), LPS (10μg/mL), and H_2O_2 (500μM) as described in Online Figure I. DNA fragmentation was determined by TUNEL staining (green), cell nucleolus were labeled by DAPI (blue).

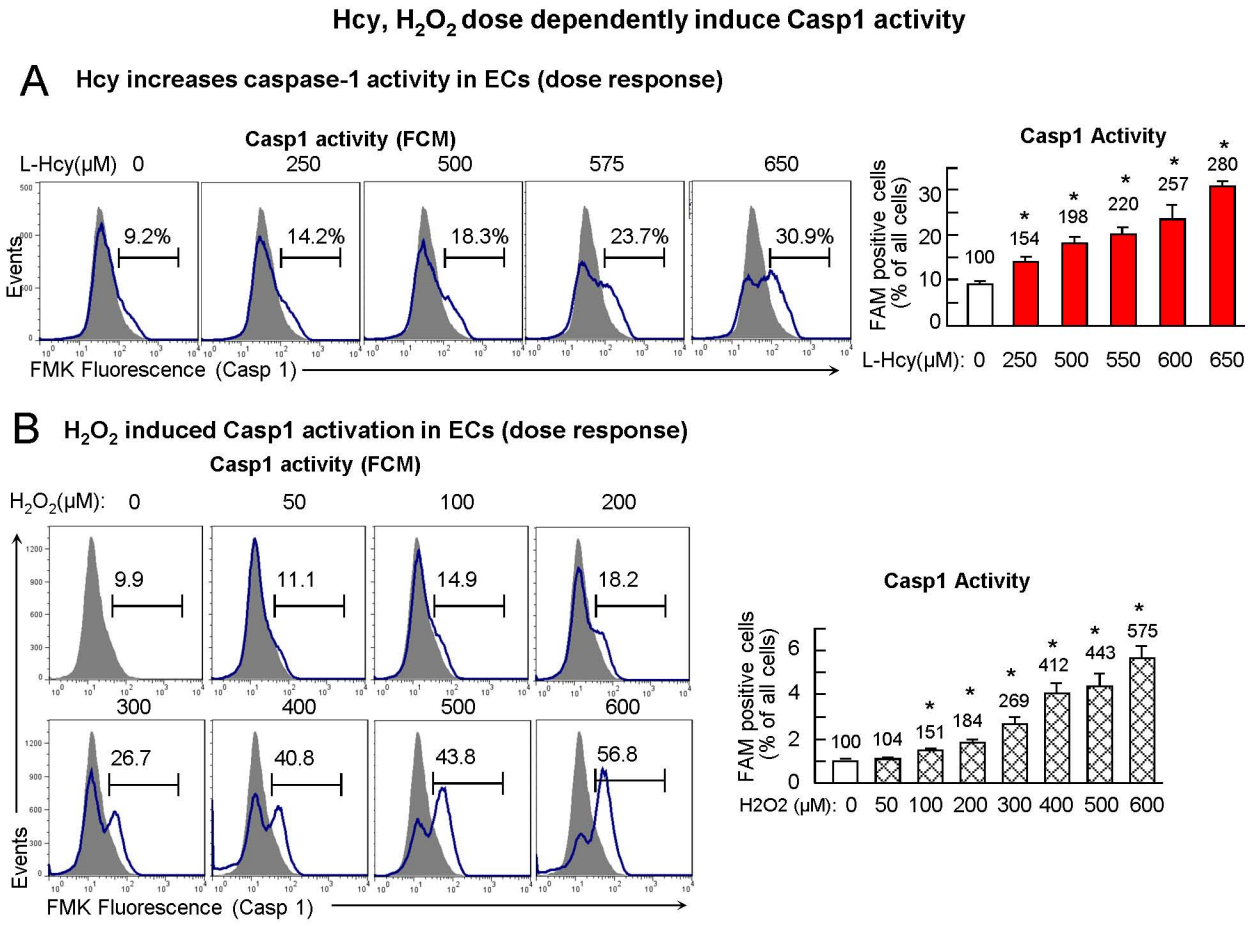
Online Figure VI

Hcy/LPS induced Casp1 activity is bluneted by Casp1 inhibitor



Online Figure VI, Hcy and/or LPS-induced Casp1 activity are suppressed by Casp1 inhibitor. HUVECs were cultured in 12-well plate till 80% confluence, pretreated with Casp1 or cathepesin B inhibitor 30min prior to L-Hcy and/or LPS treatment. Activated Casp1 was irreversibly labeled by FAM-peptide-FMK which was provided from a commercial kit. FMK positive cell (green fluorescence) represented caspase-1 activated cells. **A, Representative images of fluorescent microscopy.** Casp1 activity (green) were overlapped with DAPI staining to show EC nuclear (blue). Bar=100μm. **B, Casp1 activity (fluorescent microscopy).** Casp1 activity was quantified. *, $p<0.05$ vs vehicle; **#**, $p<0.05$ vs same treatment in control group. Abbreviation: FAM, carboxyfluorescein. FMK, fluoromethyl ketone; Casp1, caspase-1.

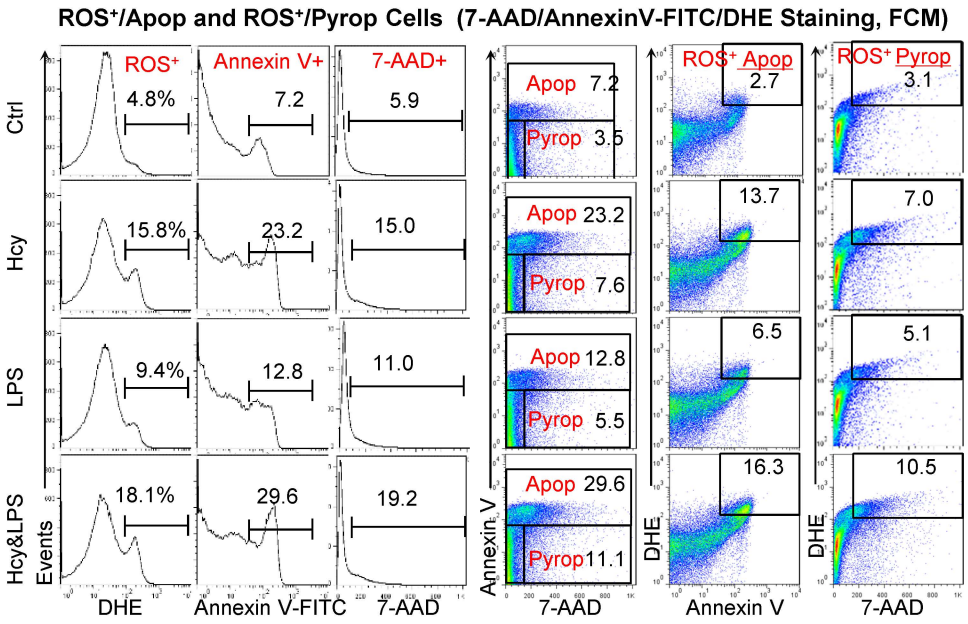
Online Figure VII



Online Figure VII, Hcy and H_2O_2 activated Casp1 dose dependently. HUVECs were cultured in 6cm dishes as described in Online Figure I, and treated as indicated. **A. Hcy increases Casp1 activity in EC (dose response).** Cells were treated with L-Hcy for 24hr at indicated doses, and subjected for Casp1 activity analysis by FCM. *, $P<0.05$ vs control. **B. H_2O_2 induced Casp1 activation in ECs (dose response).** Cells were treated with H_2O_2 for 24hr at indicated doses and subjected for Casp1 activity analysis. *, $P<0.05$ vs control. FCM, flow cytometry.

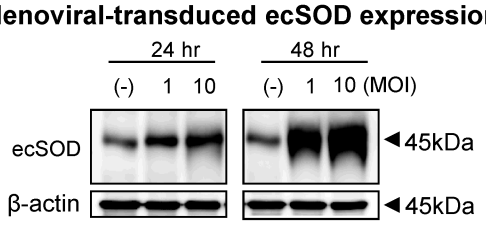
Online Figure VIII

Hcy/LPS induced intracellular ROS levels and pyroptosis/apoptosis in EC



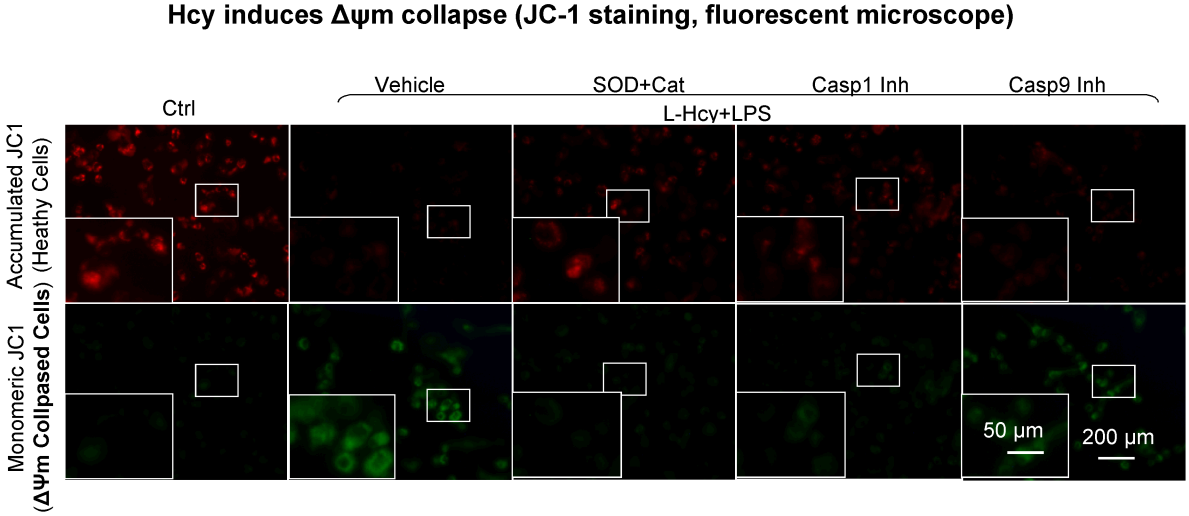
Online Figure VIII. Hcy/LPS induced intracellular ROS levels and programmed cell death (pyroptosis/apoptosis) simultaneously in EC. HUVECs were cultured and treated with L-Hcy (500μM), LPS (10 μg/mL) or H_2O_2 (500μM) for 24hr as described in Online Figure I. Triple staining was applied to detect intracellular ROS (DHE) and apoptosis/pyroptosis simultaneously (Annexin-V-FITC and 7-AAD). ROS^+/apoptosis and ROS^+/pyroptosis cells (triple staining, FCM). The ROS^+/apoptosis and ROS^+/pyroptosis cells were defined and quantified. Abbreviation: FCM, flow cytometry.

Online Figure IX



Online Figure IX. Adenoviral-transduced ecSOD expression in Ecs. HUVECs were cultured in 6cm dishes with 20%FBS medium till 80% confluence. Cells were then transduced by adenoviral ec-SOD (1, 10 MOI) for 24hr and 48hr, and harvested for Western Blotting analysis to measure SOD expression.

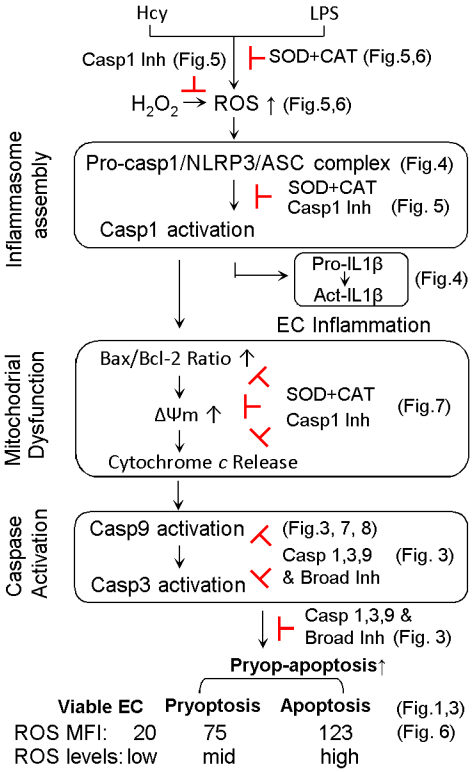
Online Figure X



Online Figure X. Hcy induces Δψm collapse and cytochrome-*c* release, and increase Bax/Bcl-2 Ratio via oxidative stress, Casp1 activation in ECs. HUVECs were cultured in 6cm dish and treated with L-Hcy (500μM) and/or LPS (10 μg/mL) as described **in Fig.1**. Cells were trasduced with antioxidants-adenoviral ec-SOD(1MOI) for 48hr and PEG-catalase (25mg/mL) for 30min, or treated with Casp1, 9 inhibitors for 30min, prior to Hcy/LPS treatment. Mitochondrial function was accessed by JC-1 staining to determine Δψm. JC-1 aggregates in mitochondrial in healthy cells appeared as red fluorescence, and disassociated to become monomer when Δψm collapses and appear as green fluorescence. **B, Δψm detection (JC-1 staining by fluorescent microscope).** Δψm, mitochondrial potential.

Online Figure XI

Hcy/LPS induced pyroptosis/apoptosis (Working model)



Online Figure XI, Working model. Flow chat illustrates our study in characterizing Hcy/LPS-induced pyrop-apoptosis and the underlying mechanism. We demonstrated that Hcy/LPS increase cellular ROS level, inflammasome assembly to activate casp1. Activated casp1 resulted in pyroptosis/apoptosis via mitochondrial dysfunction, caspase cascade, and inflammation via activated-IL1β in EC. We also present a model that oxidative gradient determines EC death destiny to undergo pyroptosis, apoptosis or viable cell. Abbreviation: RE, rescue efficacy; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry. TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; EC, endothelial cells; Act, activated; Casp, caspase; Ach, acetylcholine.

Supplemental Table

Online Table I

Hcy/LPS indecued Casp activities (24hr time course, FCM)

Online Table 1. Caspase activity (Arbitrary Unit)

	Treatment time (hr)					
	0	1	2	6	12	24
Caspase-1	1.00±0.12	1.22±0.36	1.72±0.17 ‡	1.75±0.26 ‡	1.83±0.12 ‡	1.94±0.15 ‡
Caspase-8	1.00±0.05	1.14±0.15	1.20±0.08	1.53±0.06 ‖	2.19±0.12 ‖	1.69±0.32 ‖
Caspase-9	1.00±0.03	1.00±0.08	1.28±0.02	1.49±0.15 ╫	1.95±0.23 ╫	1.56±0.12 ╫
Caspase-3	1.00±0.07	1.11±0.07	1.28±0.18	1.28±0.24	1.57±0.14 §	2.23±0.23 §

Online Table I, Time course of caspase activation in EC. HUVECs were cultured in 96-well plate and treated with L-Hcy in the presence of LPS for the indicated times as described in Online Figure I. Caspase-1,8,9,3 activities were measured as fluorescent intensity by using a manufacture's kit and detected by fluorescence spectrometry. Activity of each caspase determined at 0 hr was defined as 100% and used to normalize that recorded over the time period. Values represent mean ± SEM, n=3. ‡, $p<0.05$ vs Casp1 activity at 0hr; ‖, $p<0.05$ vs Casp8 activity at 0hr; ╫, $p<0.05$ vs Casp9 activity at 0hr; §, $p<0.05$ vs Casp3 activity at 0hr.