

## **SUPPLEMENT**

### **Expanded Material and Methods**

#### **Cell culture**

Human aortic endothelial cells (HAEC) were purchased from Cell Applications (San Diego, CA) and cultured on gelatin coated dish with endothelial growth medium (Cell Applications) with 4% heat-inactivated fetal bovine serum (FBS, GIBCO, Carlsbad, CA) at 37°C in a 5% CO<sub>2</sub> atmosphere. All experiments were performed within nine passages. Prior to LDL treatment, cells were washed by PBS and cultured in high glucose (4.5g/L) Dulbecco's Modified Eagle's Medium (DMEM, Mediatech Inc., Herndon, VA) supplemented with 0.5% FBS and 100U/mL penicillin G sodium and 100µg/mL streptomycin (GIBCO) for 16 hours.

#### **LDL preparation and oxidation**

Oxidized LDL was prepared by incubating LDL with CuSO<sub>4</sub> in PBS for 48-72 hours at room temperature. The extent of LDL oxidation was measured at an absorbance wavelength of 234nm.<sup>1</sup> Oxidized LDL was dialyzed with PBS/0.01% EDTA at 4°C overnight. Oxidized LDL was then filtered through a 0.22µm filter. Lipopolysaccharide (LPS) assay was performed by high performance liquid chromatography (HPLC) to ensure an endotoxin free preparation. The sample was stored at 4°C.

### **Adenoviral vectors**

The sub-confluent monolayer of HAEC were infected with Ad-Mn-SOD at Multiplicity of Infection (MOI) 15, and incubated at 37°C for 48 hours. A mock vector (Ad-Control) was used as a control.

### **Western blot analysis**

Proteins were separated by 4–20% polyacrylamide gel with SDS and electroblotted onto the polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK) and were blocked overnight at 4°C in Tris buffered saline-Tween20 (TBS-T) containing 5% BSA (SIGMA, St. Louis, MO). Next, membranes were incubated with primary antibodies (anti-phospho-JNK and anti-Mn-SOD from Upstate, anti-JNK from Cell Signaling Technology, Danvers, MA) for 1 hour at room temperature. To verify equal loading, we stained the membranes with anti- $\beta$ -tubulin antibody (Upstate). After treatment with peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature, labeled bands were detected by the ECL plus Western Blotting Detection System (GE Healthcare). The primary antibodies were diluted at 1:1000 and the secondary antibodies at 1:5000 with the exception of total JNK detection (1:1000). The intensities of bands were measured using densitometry (FlourChem<sup>®</sup> FC2, Alpha Innotech Corp., San Leandro, CA), and the values were normalized with those of the  $\beta$ -tubulin bands.

### **SiRNA transfection**

The siRNA target sequence for JNK1 was 5'- AAG CCG ACC ATT TCA GAA TCA -3', JNK2 was 5'- AAG CCG TCC TTT TCA GAA CCA -3' and Mn-SOD (*SOD2*) was 5'-

CAG GCC TGA TTA TCT AAA AGC -3'. SiRNA (60nmol/L) was transfected to HAEC with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) as described previously.<sup>2</sup> Cells were used for confirmation of gene knockdown or function assay 48 hours after transfection. Negative control siRNA (Qiagen, Valencia, CA) was used as the scramble siRNA. There was no observable damage due to the transfection procedure.

### **Mitochondrial membrane potential ( $\Delta\Psi_m$ )**

Mitochondrial membrane potential ( $\Delta\Psi_m$ ) was measured using the cationic fluorescent dye, tetramethylrhodamine methyl ester (TMRM<sup>+</sup>, Molecular probes, Carlsbad, CA).<sup>3,4</sup> After treatment with oxLDL, HAEC were washed twice with PBS and incubated with TMRM<sup>+</sup> at 30nmol/L. TMRM<sup>+</sup> was excited at 488nm, and the data were collected at 588/26 m (FL2) channel by Fluorescence activated cell sorting (FACS) (FACS Caliber system, BD Biosciences, San Jose, CA).

### **Conversion of TMRM<sup>+</sup> fluorescent intensity to voltage**

Mitochondrial membrane potential ( $\Delta\Psi_m$ ) was established by probing the fluorescent intensity, followed by applying the Nernst equation<sup>5</sup>:

$$E = E_0 - \frac{RT}{nF} \cdot \log\left(\frac{[TMRM^+]_{in}}{[TMRM^+]_{out}}\right)$$

where  $R$  denotes the universal gas constant, " $T$ " the temperature in Kelvin, " $F$ " the Faraday constant and " $n$ " the number of electrons transferred in the half reaction.  $[TMRM^+]_{in}$  denoted TMRM<sup>+</sup> concentration inside and  $[TMRM^+]_{out}$  outside the mitochondria. The value of " $RT/nF$ " was calculated to be 61mV at 37°C.<sup>6,7</sup>  $[TMRM^+]_{out}$  was constant due to the relatively higher concentration than  $[TMRM^+]_{in}$ , assuming that  $E_0 = \Delta\Psi_m = -150mV$  at

equilibrium under non-stimulation condition.<sup>5,6,8</sup> The changes in  $\Delta\Psi_m$  [mV] was determined as follows<sup>5,7,9</sup>:

$$\Delta\Psi_{m_{stimulation}} = -150 - 61 \cdot \log\left(\frac{TMRM^+ \text{ intensity}_{stimulation}}{TMRM^+ \text{ intensity}_{non-stimulation}}\right) \text{ [mV]}$$

### **Flow cytometry analysis to quantify mitochondrial superoxide production**

Mitochondrial superoxide ( $mtO_2^-$ ) production was measured using MitoSOX Red (Molecular probes). HAEC were incubated with 5 $\mu$ mol/L of MitoSOX Red. After 10 minutes, the cells were washed three times with PBS and treated with 50 $\mu$ g/mL of native LDL or oxLDL. After 1 hour, the cells were trypsinized, fixed and resuspended in PBS containing 1% paraformaldehyde. Cells were analyzed by the FACS Caliber system.

### **Quantitative real-time PCR analysis**

The expression levels were normalized by those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The forward primer sequence for Mn-SOD (*SOD2*) was 5'-GGA AGC CAT CAA ACG TGA CT-3' and the reverse primer was 5'-CCT TGC AGT GGA TCC TGA TT-3'; the forward primer sequences for GAPDH was 5'-CCT CAA GAT CAT CAG CAA TGC CTC CT -3', and the reverse primer was 5'-GGT CAT GAG TCC TTC CAC GAT ACC AA -3'. Fidelity of the PCR reactions was determined by melting temperature analysis. The differences in  $C_T$  values for various intervals versus 0 minutes with scramble siRNA's were used to determine the relative difference in the levels of Mn-SOD mRNA expression.

### **Immunoprecipitation assay**

HAEC were transfected with siRNA. Forty-eight hours later, cells were pre-incubated with

10 $\mu$ mol/L of proteasome inhibitor, Z-Leu-Leu-Leu-al (MG-132 for 1 hour, followed by 50 $\mu$ g/mL of oxLDL for 6 hours. Five hundred  $\mu$ g of entire cell protein were incubated with monoclonal Ubiquitin antibody (Upstate) conjugated with Dynabeads protein G-Ig (Invitrogen) for 1 hour at 4°C. Two percent of input (10 $\mu$ g of entire cell protein) was used as the loading control.

### **Cleaved caspase-3 activity assay**

HAEC were treated with siRNA for 48 hours, followed by 100 $\mu$ g/mL of oxLDL for 24 hours. The entire cell lysates were used and measured at a wavelength of 450nm by the microtiter plate reader (Molecular Devices, Sunnyvale, CA).

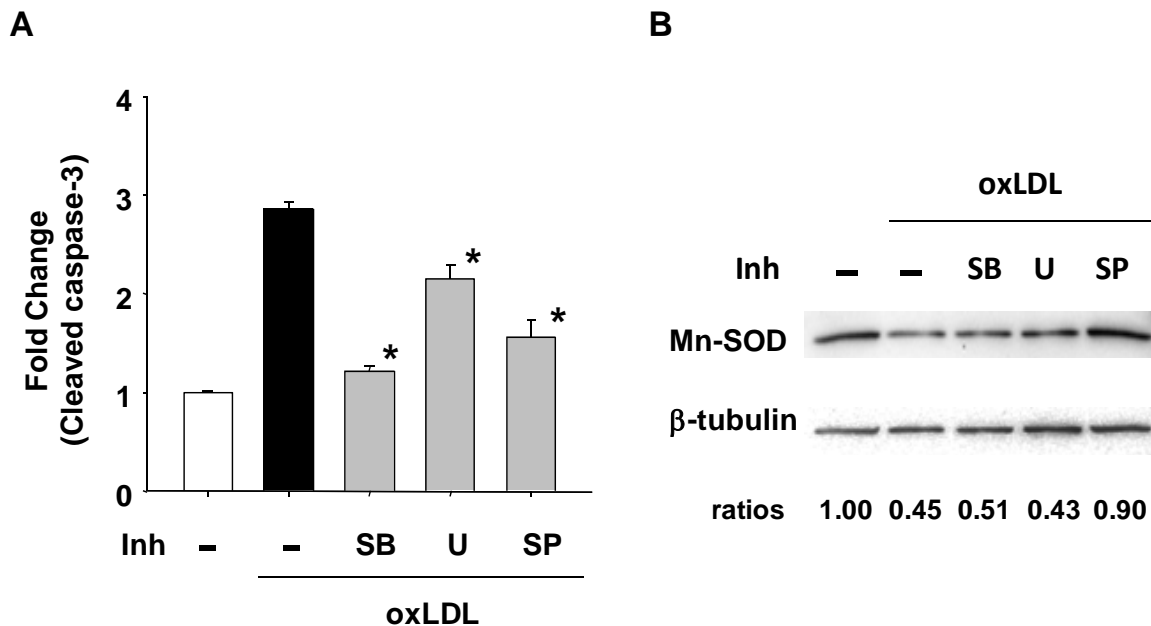
## References

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## Expanded figures



**Figure S1. Effect of Kinases inhibitors on cell dysfunctions by oxLDL.** HAEC were pre-incubated with 10 $\mu$ mol/L of chemical inhibitors for 1 hour. (A) Cells were treated with 100 $\mu$ g/ml of oxLDL for 24 hours. The cell lysates were collected and the caspase-3 activities were measured by ELISA kit. These data represented the means from triplicates of two independent experiments. \* $P$ <0.05 vs. in the presence of oxLDL. (B) Cells were incubated in the presence of 50 $\mu$ g/mL of oxLDL for 6 hours. Ten  $\mu$ g of entire cell protein was prepared and Mn-SOD levels were assessed. The blots were representative of two independent experiments with identical results. SB denoted SB203580 (p38 inhibitor); U U0126 (MEK/ERK inhibitor); and SP SP600125 (JNK inhibitor).