Activation of activator protein 2 alpha by aspirin alleviates atherosclerotic plaque growth and instability in vivo

Supplementary information

Methods

Reagents
Aspirin was purchased from Sigma Company (Cat: V900185). Polyclonal or monoclonal antibodies against p47, Nox4, GAPDH, α-SMA, p65, IκBα, AP-2α, 3-NT, and 4-HNE were obtained from Santa Cruz Biotechnology, Abcam, or Cell Signaling Company. All secondary antibodies were obtained Cell Signaling Technology. Antibody of pAP-2α Serine 219 was generated by Genscript Company. Dihydroethidium (DHE) was purchased from Molecular Probes. All PCR primers were purchased from Invitrogen. Other chemicals, if not indicated, were from Sigma-Aldrich (St. Louis, MO). All drug concentrations are expressed as the final molar concentration in the working buffer.

Patients and sample processing
For detecting the effects of aspirin in humans, we recruited 16 patients with coronary atherosclerotic heart disease taking aspirin or not. The profiles of these patients were shown in Supplementary Table S2 and S3. The blood was collected from these patients. Leucocytes were isolated from blood and subjected to biochemical analysis. These protocols complied with the Management Rules of the Chinese Ministry of Health and were approved by the Ethical Committee of Shandong University Qilu Hospital.

Animals and protocols of in vivo experiments
Apoe−/− mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in temperature-controlled cages with a 12-hrs light-dark cycle. The animal protocol was reviewed and approved by the University of Shandong Animal Care and Use Committee.

Our animal model consisted of three parts including high-fat diet induced aortic atherosclerosis, carotid collar induction of vulnerable plaques, and lentiviral knock down of AP-2α. The model of vulnerable plaque formation is shown in Supplementary Figure S1A. Apoe−/− mouse had a collar placed
around the left common carotid artery. In brief, a constrictive silastic tube (0.30-mm inner diameter, 0.50-mm outer diameter, and 2.5-mm long; Shandong Key Laboratory of Medical Polymer Materials, Jinan, China) was placed around the left common carotid artery near its bifurcation in male Apoe−/− mice at age of 8-12 weeks. Mice were then fed with a high fat diet (0.25% cholesterol and 15% cocoa butter) plus or minus aspirin administration (5, 20, 50 mg/kg/day) in drinking water for 8 weeks. At the end of experiment, all mice were sacrificed under anesthesia. The thoracic and abdominal aortas were collected for histological analysis of atherosclerotic lesion growth. The left common carotid arteries were collected for pathological analysis of atherosclerotic plaque stability.

In the second part of the animal study, as shown in Supplementary Figure S2A, male Apoe−/− mice at age of 8-12 weeks were infected with lentivirus containing negative control shRNA or AP-2α shRNA (once 4 weeks) via tail vein as described previously with light modifications1 and received a high fat diet (0.25% cholesterol and 15% cocoa butter). 4 weeks later, aspirin (50 mg/kg/day per mouse) was added into drinking water and maintained for 8 weeks. At the end of experiment, mice were euthanized with an intraperitoneal injection of 0.8% pentobarbital sodium (60 mg/kg), followed by cervical dislocation. The aortas were collected for histological and molecular biological analysis.

In the third part of in vivo study, as shown in Supplementary Figure S2C, male Apoe−/− mice at age of 8-12 weeks had left carotid collar placement plus local lentivirus infection as described previously2. After surgery, mice were fed a high fat diet (0.25% cholesterol and 15% cocoa butter). 4 weeks later, mice received aspirin administration in drinking water (50 mg/kg/day) and injection of lentivirus (once every 4 weeks) and kept for 8 weeks. The left common carotid arteries were collected for pathological and molecular biological analysis.

**Generation of shRNA construct and lentivirus production**

Based on the protocol from Signaling Gateway, the shRNA cassette containing target sequence of AP-2α (GGAGAGCGAAGTCTAAGAATG) was designed. The cassette was subcloned into pEN-hH1c vector as described previously3. The pEN-hH1c vector containing the AP-2α shRNA cassette was combined
with an attR-containing vector pDSL-hpUP in an LR recombination reaction. The recombinant constructs pDSL-hpUP-AP-2α-shRNA were confirmed by DNA sequence analysis. The sequence of negative control shRNA is TTCTCGAACGTGTCACGT. The lentivirus was produced by transiently transfecting HEK293T cells using SuperFect transfection reagent (Qiagen, USA) with three packing plasmid system (pGag/Pol, pRev, and pVSV-G). The virus-containing supernatant was collected 72 hours after transfection, and filtered through 0.45 mm filters (Millipore, USA), and stored at -80 °C. The titer of the viral vectors was determined by TCID50 (Tissue culture infective dose) method.

**Cell cultures**

Human vascular smooth muscle cells (VSMCs) from ATCC were grown in basal medium (Clonetics Inc. Walkersville, MD) supplemented with 2% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). In all experiments, cells were between passages 3 and 8. All cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were grown to 70-80% confluency before being treated with different agents.

**Generation and infection of adenoviral infection to cells**

To generate adenoviral vector containing Ad-S219A-AP-2α cDNA, we subcloned a human cDNA encoding full-length of AP-2α, which contained a substituted amino acid of serine 219 to alanine (S219A), into a shuttle vector (pShuttle CMV [cytomegalovirus]) as described previously. VSMCs were infected with adenovirus overnight in medium supplemented with 2% FBS. The cells were then washed and incubated in fresh medium for an additional 24 hours before experimentation. These conditions typically produced an infection efficiency of at least 80%.

**Western blot analysis**

Cell lysates or tissue homogenates were subjected to western blot analysis, as described previously. The protein content was assayed by BCA protein assay reagent (Pierce, USA). Protein of 20 µg was loaded to SDS-PAGE and then transferred to membrane. Membrane was incubated with a 1:1000 dilution of primary antibody, followed by a 1:5000 dilution of horseradish peroxidase–conjugated secondary antibody. Protein bands were visualized by ECL (GE Healthcare). The intensity (area × density) of the individual bands on
Western blots was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). The background was subtracted from the calculated area. We used control as 100%.

**Detection of ROS**

ROS productions in cultured cells were assayed by measuring the DHE fluorescence by HPLC with minor modification. Briefly, cells were incubated with DHE (10 µM) for 30 min, washed, harvested and homogenized, and subjected to methanol extraction. HPLC was performed by using a C-18 column (mobile phase: gradient of acetonitrile and 0.1% trifluoroacetic acid) to separate and quantify oxyethidium (product of DHE and O$_2^-$) and ethidium (a product of DHE auto-oxidation). O$_2^-$ production was determined by conversion of DHE into oxyethidine.

**Electrophoretic mobility shift assay (EMSA) for AP-2α and NF-κB DNA-binding activities**

Subcellular fractions were prepared by using NE-PER Nuclear and Cytoplasmic Extract kit (Cat78833) from PIERCE. EMSA were performed as described in a previous study. AP-2α kit (AY1002) is from Panomics Company. NF-κB kit (E3050) is from Promeag Company.

**ChIP assay for transcriptional factor and gene promoter binding**

ChIP assays were performed by using a ChIP-IT kit (Upstate, 17-295), according to the manufacturer’s protocol. 1×10^6 cells were seeded on a 10 cm dish. Proteins were cross-linked to DNA by adding formaldehyde directly to culture medium at a final concentration of 1% and incubating for 10 min at 37°C. The cells were harvested in SDS lysis buffer and added protease inhibitors. Cell lysates were sonicated to shear DNA to lengths between 200 and 1000 bp. Sheared chromatin was precleared with protein G beads prior to incubation overnight at 4°C with 4 µg of anti-AP-2α antibody, or control IgG antibody. Purified, immunoprecipitated chromatin fragments from IP samples were subjected to PCR. For IκBα promoter analysis, the primer sequences were as follows: Forward: 5' CTTTTTCTGGTCTGACTGGCT 3' and reverse: 5' TGGAACATGGC CGGACGAGC 3'. PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide.
Reverse transcription PCR
Total cellular RNA was isolated using a Qiagen reagent and reverse transcribed to cDNA with specific antisense primers using the ThermoScript RT-PCR System protocol (Invitrogen). Samples (2 μl) of reverse transcribed product were PCR-amplified in a total volume of 25 μl with 10 pmoles each of forward and reverse primer. For IκBα mRNA analysis, the primer sequences were as follows: Forward: 5’GCTGATCCTGAGCTCCGAGAC3’ and reverse: 5’CATCAGG CAGCTTC CCTC3’. For GAPDH mRNA analysis, the primer sequences were as follows: Forward: 5’GCTGATCCTGAGCTCCGAGAC3’ and reverse: 5’CATGTGGGCCAGGTCCACCAC3’.

Immunohistochemistry (IHC)
The thoracic aorta was fixed in 4% paraformaldehyde overnight, and then processed, embedded in paraffin, and sectioned at 4 µm. The deparaffinized, rehydrated section from thoracic aorta and cryosections from aortic root (5 µm) were microwaved in citrate buffer for antigen retrieval. Sections were incubated in endogenous peroxidase (DAKO) and protein block buffer, and then with primary antibodies indicated overnight at 4°C. Slides were rinsed with washing buffer and incubated with labeled polymer-horseradish peroxidase- antimouse/antirabbit antibodies followed by DAB+ chromogen detection (DAKO). After final washes, sections were counterstained with hematoxylin. All positive staining was confirmed by ensuring that no staining occurred under the same conditions with the use of non-immune rabbit or mouse control IgG.

Atherosclerotic lesion analysis
After being fed the Western diet for 8 weeks, the mice were fasted for 14 h and then were anesthetized and euthanized. The heart and aortic tissue were removed from the ascending aorta to the ileal bifurcation and placed in 4% paraformaldehyde for 16 h. After fixation, the adventitia was thoroughly cleaned under a dissecting microscope. For analyzing the lesion area in the aortic root, the heart was dissected from the aorta, embedded in Polyfreeze tissue freezing medium (Polysciences, Inc) and sectioned (5 µm thickness). Four consecutive sections were collected from each mouse and stained with
Oil Red O for neutral lipids, and counterstained with hematoxylin to visualize the nuclei. Plaques were captured under the Olympus microscope connected to a QImaging Retiga CCD camera. The aortic lesion size of each animal was obtained by the averaging of lesion areas in four sections from the same mouse. For analyzing the lesion area in the aortic arch, the intimal surface was exposed by a longitudinal cut from the ascending arch to 5 mm distal of the left subclavian artery to allow the lumen of the aortic arch to be laid flat. The aorta was rinsed for 5 min in 75% ethanol, stained with 0.5% Sudan IV in 35% ethanol and 50% acetone for 15 min, destained in 75% ethanol for 5 min, then rinsed with distilled water. Digital images of the aorta were captured under a stereomicroscopy, and the lesion area was quantified from the aortic arch to 5 mm distal of the left subclavian artery using Alpha Ease FC software (version 4.0 Alpha Innotech). The plaque vulnerable index was calculated according to the ratio of area I (Oil Red$^+$ + CD68$^+$) to area II ($\alpha$-SMA$^+$ + Collagen$^+$) as described previously$^8$.

**Measurement F$_2$-isoprostanes in urine and blood**

The concentration of iPF$_2$-$\alpha$-VI in urine or blood was determined by LC-MS/MS as previously described$^9$. In brief, 0.1 ml of 10 ng/ml deuterated internal standard (8iPF2$\alpha$-d4; Cayman chemical, Ann Arbor, MI, USA) was added to 1 ml urine or blood. The sample was then subjected to solid phase extraction (Oasis HLB, Waters, Milford, MA, USA). The eluate was taken to dryness under a stream of nitrogen at room temperature, and afterwards redissolved in 100 μl 10% acetonitrile of which, 40 μl was injected on a reverse-phase XTerra MS C18 column (Waters, Milford, MA, USA; 3.5 μm, 2.1X100 mm). Urinary F2-isoprostanes were quantified using a Quattro Micro (Waters) mass spectrometer. To calculate the iPF$_2$-$\alpha$-VI concentration, the analyte to internal standard peak area ratio was compared with a standard curve from 2 to 16 ng/ml iPF$_2$-$\alpha$-VI (Cayman chemical, Ann Arbor, MI, USA). The intra-run coefficient of variation (CV) was 4.8% and the inter-run CV was 10.1%.

**Statistical analysis**

All quantitative data are reported as mean ± SEM. and were analyzed using a two-way ANOVA. Bonferroni corrections were applied to multiple comparisons and $P<0.05$ was considered significant.
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Supplementary Figure S1. Quantitative analysis of the aspirin-induced effect of atherosclerotic plaque growth and vulnerability in *Apoe*<sup>−/−</sup> mice. 

(A) The protocol of collar placement and aspirin administration in *Apoe*<sup>−/−</sup> mice. At the end of experiments, all mice were sacrificed under anaesthesia. (B) Quantitative data for sizes of atherosclerotic lesion in left common carotid arteries. (C) Quantitative data for macrophages numbers. (D) Quantitative data for lipid content. (E) Quantitative data for collagens. (F) Quantitative data for vascular smooth muscle cells numbers. (G) Vulnerability index was determined by using the ration of CD68<sup>+</sup> (%) plus Oil Red (%) to α-SMA (%) plus collagen (%). (H) Quantitative data for phosphorylated AP-2α. N is 10-15 in each group. *P<0.05 vs. Control (Aspirin at 0). NS indicates no significant difference.
Supplementary Figure S2. Infection of lentivirus containing control or AP-2α shRNA silences AP-2α protein expression in aorta and carotid in Apoe<sup>−/−</sup> mice. (A and B) The protocol of lentivirus infection and aspirin administration in Apoe<sup>−/−</sup> mice fed with high fat diet was illustrated in A. 4 weeks later after injection, aortic roots were isolated from mice to detect AP-2α protein expression by western blot in B. (C-E) The protocol of collar placement, lentivirus infection, and aspirin administration in Apoe<sup>−/−</sup> mice was illustrated in C. 4 weeks later after infection, carotids were isolated from mice to detect AP-2α protein expression by IHC in D and by western blot in E.
Supplementary Figure S3. Aspirin increases the binding of AP-2α to the promoter of IκBα in VSMCs. (A) VSMCs were incubated with aspirin (200 μg/ml) for 24 hours. Binding of AP-2α on the promoter of IκBα analyzed by ChIP assay. The picture is a representative of three individual experiments. (B) Proposed mechanisms of the aspirin-induced suppressive effects on the growth and instability of atherosclerotic plaque.
Supplementary Figure S4. Knockdown of AP-2α abolishes the effects of aspirin on oxidative stress in Apoe<sup>−/−</sup> mice with vulnerable atherosclerotic plaque. The protocol and experimental designs were described in Supplementary Methods and Figure S2C. (A) Urine levels of F<sub>2</sub>-isoprostanes. (B) Blood levels of F<sub>2</sub>-isoprostanes. Data were expressed by mean ± SEM. N is 10-15 in each group. *P<0.05 vs. control shRNA alone. NS indicates no significance.
Supplementary Table S1. Serum lipids, blood glucose, and monocyte count in AP-2α-knockdown mice

| Groups                        | TC (mM)      | TG (mM)    | GLU (mM) | Monocytes (10⁹/L) |
|-------------------------------|--------------|------------|----------|-------------------|
| Control shRNA                 | 10.73 ± 1.35 | 1.74 ± 0.22| 6.80 ± 0.63| 1.94 ± 0.76       |
| Control shRNA + Aspirin       | 9.97 ± 0.61  | 1.67 ± 0.09| 5.76 ± 1.28| 1.28 ± 0.57       |
| AP-2α shRNA                   | 10.25 ± 1.55 | 1.96 ± 0.28| 5.69 ± 0.61| 1.83 ± 0.66       |
| AP-2α shRNA + Aspirin         | 10.64 ± 1.49 | 1.88 ± 0.08| 5.16 ± 0.87| 1.75 ± 0.91       |

TC, total cholesterol; TG, triglycerides; GLU; glucose. N is 12 in each group.

Data were expressed by mean ± s.e.m. *P<0.05 vs. Control shRNA.
**Supplementary Table S2. Demographic data for control individuals and patients taking aspirin**

| ID | Gender | Ages (years old) | Aspirin (dose and time) | WBC ($10^9$/L) | Platelet ($10^9$/L) |
|----|--------|------------------|-------------------------|----------------|---------------------|
| 1  | Male   | 44               | None                    | 5.93           | 118                 |
| 2  | Male   | 41               | None                    | 6.18           | 267                 |
| 3  | Male   | 48               | None                    | 5.36           | 186                 |
| 4  | Female | 62               | None                    | 4.17           | 195                 |
| 5  | Male   | 57               | None                    | 7.08           | 214                 |
| 6  | Female | 65               | None                    | 8.24           | 227                 |
| 7  | Male   | 56               | None                    | 6.92           | 176                 |
| 8  | Female | 70               | None                    | 7.84           | 254                 |
| 9  | Male   | 45               | 100 mg/day for 3 months | 6.57           | 193                 |
| 10 | Male   | 47               | 100 mg/day for 6 months | 5.09           | 207                 |
| 11 | Female | 64               | 100 mg/day for 4 months | 9.13           | 238                 |
| 12 | Male   | 67               | 100 mg/day for 2 months | 8.26           | 190                 |
| 13 | Female | 58               | 100 mg/day for 2 months | 6.17           | 217                 |
| 14 | Male   | 72               | 100 mg/day for 3 months | 7.36           | 137                 |
| 15 | Male   | 49               | 100 mg/day for 3 months | 8.29           | 202                 |
| 16 | Female | 77               | 100 mg/day for 3 months | 5.37           | 183                 |
| ID | Gender | Ages (years old) | Body Weight (kg) | Aspirin (dose and time) |
|----|--------|------------------|------------------|-------------------------|
| 1  | Male   | 40               | 75               | 100 mg/day for 4 weeks  |
| 2  | Male   | 35               | 70               | 100 mg/day for 4 weeks  |
| 3  | Male   | 25               | 60               | 100 mg/day for 4 weeks  |
| 4  | Female | 25               | 55               | 100 mg/day for 4 weeks  |
| 5  | Female | 30               | 60               | 100 mg/day for 4 weeks  |
| 6  | Female | 35               | 65               | 100 mg/day for 4 weeks  |