

### Low-Dose Aspirin Treatment Attenuates Male Rat Salt-Sensitive Hypertension via Platelet Cyclooxygenase 1 and Complement Cascade Pathway

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**Background**—The role of platelets in the development of vascular inflammation and endothelial dysfunction in the pathogenesis of hypertension is well established at this time. Aspirin is known to relieve pain, decrease fever, reduce inflammation, impair platelet aggregation, and prevent clotting, yet its effect in the context of salt-sensitive hypertension remains unclear. The present study investigated the importance of aspirin in inhibiting the abnormal activation of platelets and promoting the normal function of the vascular endothelium in a rat model of salt-sensitive hypertension.

*Method and Results*—Dahl salt-sensitive rats and salt-resistant rats were fed a normal-salt diet (4% NaCl), a high-salt diet (8% NaCl), or a high-salt diet with aspirin gavage (10 mg/kg per day) for 8 weeks. Blood pressure, platelet activation, vascular function, inflammatory response, and potential mechanism were measured. Low-dose aspirin (10 mg/kg per day) decreased the high-salt diet—induced elevation of blood pressure, platelet activation, leukocyte infiltration, and leukocyte—platelet aggregation (CD45+CD61+), as well as vascular endothelial and renal damage. These effects were related to the ability of aspirin to prevent the adhesion of leukocytes to endothelial cells via inhibition of the platelet cyclooxygenase 1 but not the cyclooxygenase 2 pathway. Aspirin also reversed the high-salt diet—high-salt diet—induced abnormal activation of complement and coagulation cascades in platelets.

*Conclusions*—These results highlight a new property of aspirin in ameliorating vascular endothelial dysfunction induced by platelet activation, which may be beneficial in the treatment of salt-sensitive hypertension. (*J Am Heart Assoc.* 2020;9:e013470. DOI: 10.1161/JAHA.119.013470.)

Key Words: aspirin • cyclooxygenase • platelet • salt-sensitive hypertension • vascular endothelium

The endothelium is a pivotal player in blood pressure (BP) homeostasis by producing and interacting with a variety of circulating factors, including platelets and platelet-triggered immune cells.<sup>1-3</sup> Abnormal platelet activation and platelet-leukocyte aggregation have been found in patients with multiple thrombotic and inflammatory conditions. These contribute to endothelial dysfunction and vascular remodeling, which are considered to be the key links between inflammation and cardiovascular disease.<sup>4-7</sup> Platelet-leukocyte interaction in the circulation is increased in salt-sensitive

hypertension.<sup>5,8,9</sup> In our previous study, we found that a highsalt (HS) diet contributes to abnormal platelet activation and platelet–leukocyte aggregation in salt-sensitive hypertensive rats.<sup>10</sup>

Antiplatelet drugs, including the cyclooxygenase inhibitor aspirin, are well-established therapies for the treatment of various cardiovascular diseases (CVDs) and their complications.<sup>11,12</sup> The efficacy of aspirin in reducing ischemic cardiovascular events and overall mortality in patients with CVD has been consistently shown in numerous clinical trials.<sup>13–15</sup> Some

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Accompanying Tables S1 through S4 and Figures S1 through S6 are available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.013470

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#### **Clinical Perspective**

#### What Is New?

- Low-dose aspirin ameliorates salt-sensitive hypertension and target organ damage by inhibiting platelet-activated vascular endothelial dysfunction in Dahl salt-sensitive rats.
- Low-dose aspirin inhibits cyclooxygenase 1 activity and complement and coagulation cascades in Dahl saltsensitive rats rat platelets without increasing bleeding time, which enhances the efficacy and safety of low-dose aspirin in the treatment of salt-sensitive hypertension.

#### What Are the Clinical Implications?

- Low-dose aspirin inhibiting platelet cyclooxygenase 1 activity and complement and coagulation cascades may be an effective therapy to alleviate salt-sensitive hypertension caused by preventing vascular endothelial dysfunction, which might be promising for clinical application.
- The determination of platelet activity and complement and coagulation cascades may be useful in the evaluation of novel therapeutic strategies for salt-sensitive hypertension.

but not all uncontrolled, open-label, short-term trials have shown a BP-lowering effect of low-dose aspirin, and the effect may be altered depending on the time of dosing.<sup>16–19</sup> Thus, aspirin, given at bedtime, has been reported to decrease ambulatory BP in humans with hypertension.<sup>17</sup> Animal studies also suggest that low-dose (10 mg/kg per day) aspirin can limit increases in systemic systolic BP and/or diastolic BP in spontaneously hypertensive rats and Sprague-Dawley rats fed an HS diet.<sup>18,19</sup> By contrast, high-dose (100 mg/kg per day) aspirin increased the BP of stroke-prone spontaneously hypertensive rats.<sup>20</sup> However, there have been no reports on the effects of aspirin in salt-sensitive hypertension, which is responsible for  $\approx$ 50% of cases of essential hypertension in humans.<sup>21</sup> Therefore we hypothesized that low-dose aspirin may prevent HS-induced salt-sensitive hypertension via blocking abnormal platelet activation and aggregation.

To investigate the role and mechanism of aspirin-mediated modulation of salt-sensitive hypertension, the effects of a low dose (10 mg/kg per day) of aspirin,<sup>22</sup> gavaged into Dahl salt-sensitive (DSS) rats, on BP, leukocyte–platelet aggregation, vascular infiltration, and vascular endothelial function were studied. We also considered whether the effect of aspirin, if any, on BP and platelet activation is related to the cyclooxy-genase platelet pathway.

#### Methods

The authors declare that all supporting data are available within the article (and its online supplementary files).

#### Animals

Studies were performed in male 2-month-old DSS (body weight, 190 $\pm$ 10 g; n=38) and salt-resistant SS13<sup>BN</sup> rats (body weight, 189.1 $\pm$ 9.6 g; n=27) purchased from Beijing Vital River Laboratory Animal Center. All experimental procedures were performed in accordance with the guidelines of our Institutional Animal Care and Use and Committee (ILAS-YZW2016004).

#### Materials

Aspirin (A2039) and carboxymethyl cellulose (419273) were purchased from Sigma. For surface staining of platelet proteins, anti-CD61 (104306), anti-CD62P (148310) (Biolegend), anti-CD45 (202220), anti-CD45R (553090), anti-CD11b (562140), anti-CD3 (563949), and anti-CD25 (565608) (BD Biosciences) were used. Other reagents included anti-interleukin (IL) 17A (4306419) (eBioscience); anti–IL-6 (21865-1-AP), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ; 60291-1-lg), and IL-1 (sc-130325) (Santa Cruz Biotechnology); anti-von Willebrand factor (vWF) antibody (ab6994), and endothelin-1 (ET-1) (12191-1-AP) (Abcam); endothelial NO synthase (eNOS) (#32027) (Cell Signaling); inducible NO synthase (ab15323) (Abcam); and goat anti-rabbit IgG-HRP (sc-2004), lumiracoxib (S2903), and triflusal (S3200) (Selleckchem). Protein concentration was quantified using a bicinchoninic acid protein concentration assay kit (Biyuntian, P0010). An animal peripheral blood leukocyte isolation kit (2010c1119) (TBD science) was used.

#### **Telemetry Probe Implantation**

The rats were anesthetized with 2% isoflurane and the abdominal aortae were exposed through a midline incision. A BP sensor (model PTA-M) was inserted into the aorta to femoral bifurcation, extending up to the renal artery along the femoral artery. A transmitter was stitched to the inside of the abdominal muscle wall. All incisions were sutured and carprofen (5 mg/kg) was given for analgesia. After surgery, the rats were housed individually.

#### **Radio Telemetry Recording**

A Stellar Telemetry System (TSE Systems) was used to acquire and log BP data. The system consists of a small implantable recording device with an antenna and USB base station for communicating with the implanted units. A radio transmitter, once implanted and turned on by a magnetic switch, provided a continuous measure of the arterial pressure waveform. The Acqknowledge software program was used to configure the Stellar experiments and analyze BP.

# BP Measurement in DSS and Salt-Resistant SS13<sup>BN</sup> Rats

The rats were randomly divided into 3 groups: (1) rats fed a normal-salt (NS; 0.4% NaCl) diet; (2) rats fed an HS (8% NaCl) diet; and (3) rats fed an HS diet with aspirin gavage (HS+ASA; 10 mg/kg per day, dosing time was 6 PM) for 8 weeks. BP was measured with the tail-cuff method (BP-2000 system) and a radio telemetry recording method in conscious rats (3 times per week).<sup>23</sup> The weekly BP data are the average of 3 measurements per week at 8:30 AM. At the end of the treatments (on the 56th day), systolic BP was also measured directly from a catheter inserted into the carotid artery that was threaded into the aorta of 2% isoflurane-anesthetized rats, as previously described.<sup>24</sup> After the rats were euthanized with 2% sodium pentobarbital, the blood, kidneys, and arteries were obtained.

#### Hematologic Analysis

Complete blood cell count and hematocrit level were measured by Adicon Clinical Laboratories, Inc, with a ProCyte Dx hematology analyzer (IDEXX Laboratories).

#### **Flow Cytometry**

Single aortic cells obtained by enzymatic digestion and peripheral blood samples treated with EDTA (1.5%) were collected. The blood samples, lysed with BD FACS Lysing Solution (1X final solution; BD Bioscience) and washed, were stained for 30 minutes in the dark on ice with fluorescently labeled antibodies (CD45-Percep-cy5.5, CD3e-PE, CD4-APC, CD8a-FITC, CD61-PE-Cy7, and CD62P-PE-Cy7) for identification of the different leukocyte cell populations. After processing the samples, the cells were fixed immediately with 1% paraformaldehyde to prevent transitory activation of platelets. Labeled and fixed samples were immediately or within 24 hours analyzed by flow cytometry on FACS Canto II (BD Bioscience). Before every run, BD cytometer setup and tracking beads (BD Bioscience) were used for internal calibration. Appropriate controls were prepared for each sample to allow compensation and detection of nonspecific binding. The cellular fluorescence was quantified as mean fluorescence intensity or percentage of the double-positive cells at all time points. All results were analyzed using BD FACS Diva software (BD Bioscience).

#### Tail Bleeding/Clot Stability Assay

After the rats were anesthetized with 2% pentobarbital (0.3 mL/mg, intraperitoneally), the tails were prewarmed for 5 minutes in 10 mL of saline at  $37^{\circ}$ C in a water bath. Then, the tails were lifted out of the saline and a 3-mm portion of

DOI: 10.1161/JAHA.119.013470

the tail tip was excised. The tail was then immediately placed back into the saline solution. Bleeding time was the time between the start of the bleeding and the cessation of the bleeding. Clot stability time was measured as the time between the cessation of the bleeding and the start of the second bleeding.

#### Platelet Adhesion on Collagen-Coated Plates

Ninety-six-well plates were coated for 2 hours with 100  $\mu$ g/mL collagen at 4°C. Then, the plates were blocked with fetal bovine serum (5 mg/mL). The washed platelets (2×10<sup>7</sup>/mL, 200  $\mu$ L), labeled with calcein, were allowed to spread on the collagen-coated surfaces at 37°C for 60 minutes. Then, the plates were washed 3 times with PBS to remove nonadherent platelets. The adhered platelets were fixed with 2% paraformaldehyde for 15 minutes and then washed with PBS. The adherent platelets were viewed with an inverted fluorescence microscope (Nikon Ti-S).

#### Vascular Relaxation

Vascular relaxation studies were performed, as previously described.<sup>25</sup> Briefly, mesenteric arterial (internal diameter between 150 µm and 250 µm) were harvested from DSS rats and placed in chilled buffer. The arterial rings were suspended in 5-mL organ baths containing oxygenated Krebs-Henseleit buffer (118 mmol/L of NaCl, 25 mmol/L of NaHCO<sub>3</sub>, 4.6 mmol/L of KCl, 1.2 mmol/L of MgSO<sub>4</sub>, 1.2 mmol/L of KH<sub>2</sub>PO<sub>4</sub>, 1.25 mmol/L of CaCl2, 10 mmol/L of glucose, and 0.025 mmol/L of EDTA; PH: 7.4 at 37°C). After equilibration for 60 minutes, contractile responses were recorded. Endothelium-dependent and -independent relaxation activity of the arterial rings were confirmed by the vasodilation response to varying concentrations of acetylcholine  $(10^{-9} 10^{-4}$  mol/L) or sodium nitroprusside  $(10^{-9}-10^{-3}$  mol/L), respectively, in vessels precontracted with norepinephrine  $(1 \times 10^{-6} \text{ mol/L})$ , recorded with a PowerLab system.

### Transmission Electron Microscopy Imaging of Gold Nanorods in Aortic Rings

Aortic rings were incubated with gold nanorods for 8 hours in M199 media at  $37^{\circ}$ C and 5% CO<sub>2</sub>, washed 3 times with Krebs–Henseleit buffer, and 3 more times with a heparin sodium solution (100 USP unit/mL) to get rid of nanoparticles that were electrostatically adsorbed to the tissue. The washed aortic rings were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), post-fixed in 2% osmium tetroxide in sodium cacodylate, stained en bloc with 2% uranyl acetate, dehydrated with a graded series of ethanol, and embedded in Epon-Araldite resin. Thin sections (65 nm), cut with a diamond

knife on a Leica EM UC6 ultramicrotome (Leica Microsystems), were collected on copper grids and stained with uranyl acetate and lead citrate. The specimens were visualized in a JEM 1230 transmission electron microscope (JEOL USA Inc) at 110 kV and imaged with an UltraScan 4000 CCD camera and First Light Digital Camera Controller (Gatan Inc).

#### Immunoblotting

Rat thoracic aorta homogenates were prepared for immunoblotting.<sup>9</sup> The samples were immunoblotted with well-characterized anti-rat eNOS, ET-1, and vWF antibodies. Uniformity of protein loading and membrane transfer were determined by immunoblotting for GAPDH.

# Hematoxylin and Eosin Staining and Immunofluorescence

Thoracic aortae obtained from DSS rats (NS, HS, and HS+ASA) were fixed in 10% formalin at a pH of 7.4. Dehydration, clarification, and inclusion were performed soon afterward. After blocks were obtained, sections were obtained using a microtome (Leica RM2235; Leica Microsystems) with a thickness of 5  $\mu$ m. Thoracic aortae were stained with hematoxylin and eosin. The antibodies eNOS (ab76198), vascular cell adhesion molecule-1 (ab134047), and collagen I (ab96723) were also used for the primary incubation, while fluorescent antibody rabbit antimouse immunoglobulin G and rabbit antimouse immunoglobulin G (A11059 and A11061; both Thermo Fisher Scientific) were included for the second incubation. From each aorta's description, all sections were obtained using a section-scanning system (Leica SCN400; Leica Microsystems).

# Quantitative Real-Time Polymerase Chain Reaction

Total RNA contents from kidneys and platelets were extracted using TRIzol reagents (Invitrogen) and reverse-transcribed for quantitative real-time polymerase chain reaction analysis with SYRB green using an ABI7500 quantitative real-time polymerase chain reaction system. The Master Mix and all gene probes were purchased from Invitrogen. The primers used are described in Table S1. The expressions of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , cyclooxygenase 1 (COX-1), and cyclooxygenase 2 (COX-2) were normalized to those of  $\beta$ -actin and analyzed with the 2- $\Delta\Delta$ Ct method.

#### **Cell Preparation and Adhesion Test**

Rat pulmonary microvascular endothelial cells (PMVECs) were cultured in monolayers in 96-well plates with DMEM high glucose (4500 mg/L) medium, supplemented with 10% fetal

bovine serum, 100 µg/mL penicillin, and 10 µg/mL streptomycin. Peripheral blood leukocytes (PBLs) and platelets were isolated from DSS and SS13<sup>BN</sup> rats, respectively. The PBLs  $(1.4 \times 10^9)$  were isolated by an animal peripheral blood leukocyte isolation kit, stained with 2'7'-bis-(2-carboxyethyl)-5-carboxyfuorescein acetoxymethyl ester for 15 minutes at 37°C. Platelets  $(2 \times 10^8)$  were isolated by density gradient centrifugation, as previously described, <sup>10</sup> and then treated with vehicle, aspirin (100 µmol/L), triflusal (COX-1 inhibitor, 0.1 mmol/L), or lumiracoxib (COX-2 inhibitor, 0.1 mmol/L) for 15 minutes. The treated PBLs and platelets were incubated with the cultured PMVECs, grown in monolayers in 96-well plates, incubated at the lower end of the normal serum sodium concentration (133 mmol/L)<sup>26</sup> or high (173 mmol/L) NaCl for 1 hour at 37°C. Thereafter, the plates were washed 3 times with PBS to remove the nonadherent PBLs. Fluorescent signals were captured using an inverted fluorescence microscope.

#### **TRAQ-Based Quantitative Proteomics**

#### Sample collection and preparation

Protein samples were obtained from platelets as previously described and stored at  $-80^{\circ}$ C in a refrigerator for future use.

#### iTRAQ-based quantitative proteomics analysis

The analytic processes were conducted by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. and included protein digestion, isobaric tags for relative and absolute quantitation (iTRAQ) labeling, strong cation exchange fractionation, liquid chromatography–mass spectrometry (MS)/MS analysis, protein identification, and protein quantitation. Briefly,<sup>27</sup> trypsin digestion and iTRAQ labeling were performed. Then, the mixed peptides were fractionated by strong cation exchange chromatography on an ultimate high-performance liquid chromatography system. MS analysis of the iTRAQ-labeled samples was performed on a Q Exactive LC-MS/MS Mass Spectrometer (Thermo Fisher Scientific) and 3 independent MS/MS runs were performed for each sample. The Proteome Discoverer software program (Thermo Fisher Scientific) was used for data acquisition and quantification.

#### **Bioinformatic analysis**

The data were analyzed using the free online platform of the Majorbio Cloud Platform (www.majorbio.com). All differentially expression proteins (DEPs) identified previously were submitted to the Gene Ontology Consortium, and PANTHER database analysis tools were applied for functional annotation and enrichment analysis.<sup>28</sup> Pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Predicted protein–protein interactions were generated and visualized using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) software.<sup>29</sup>

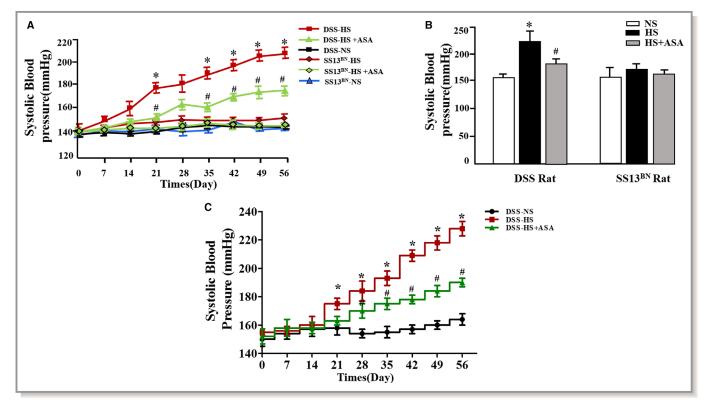
#### **Data Analyses**

All data are expressed as mean $\pm$ SEM. All statistical analyses were performed with Prism software (GraphPad). Statistical comparisons were made using 1-way ANOVA followed by Tukey test for comparisons of >2 groups. A *P*<0.05 was considered statistically significant.

#### Results

# Aspirin Attenuates the Increase in BP Caused by an HS Diet in DSS Rats

Before the initiation of the HS (8% NaCl) diet, there was no significant difference in systemic BP (tail-cuff plethysmography) between DSS and SS13<sup>BN</sup> rats. The HS diet in the DSS rats caused a progressive increase in systolic BP from an initial value of  $148\pm4.9$  mm Hg to  $212\pm6.3$  mm Hg at 8 weeks postinitiation of the HS diet (*P*=0.008 versus NS) (Figure 1A). Meanwhile, there was no change in BP in the NS group (baseline= $140\pm4.9$  mm Hg, 8 weeks= $145\pm4.3$  mm Hg). Aspirin (HS+ASA) attenuated the increase in BP  $(30\pm5.6$ versus  $64\pm6.8$ ) caused by the HS diet (HS+SAS: baseline BP=142±5.3 mm Hg, BP at 8 weeks=178±3.9 mm Hg). At the end of the study, the aortic systolic BP as measured via carotid artery cannulation was also lower in the DSS+HS+ASA group than the DSS+HS group (183±2.9 mm Hg versus 217±3.4 mm Hg, P=0.034) (Figure 1B). Radio telemetry units were also implanted into male DSS rats (NS: n=5, HS: n=5, and HS+ASA: n=5) to measure intra-arterial pressures more precisely. As shown in Figure 1C and Figure S1, aspirin treatment significantly downregulated HS-increased high BP, which was consistent with the trend observed by tail-cuff measurements. Systolic BP was significantly increased  $(232\pm11.9 \text{ mm Hg})$  in the HS group at the eighth week as compared with in the NS group ( $161\pm9.3$  mm Hg), but BPs in the HS+ASA group were markedly decreased (189 $\pm$ 7.9 mm Hg) as compared with in the HS group (P=0.021). In contrast, neither HS diet nor aspirin treatment had any effect on systolic BP, as measured by tail-cuff plethysmography or directly from the aorta via the carotid artery, in SS13<sup>BN</sup> rats. Variations among treatments



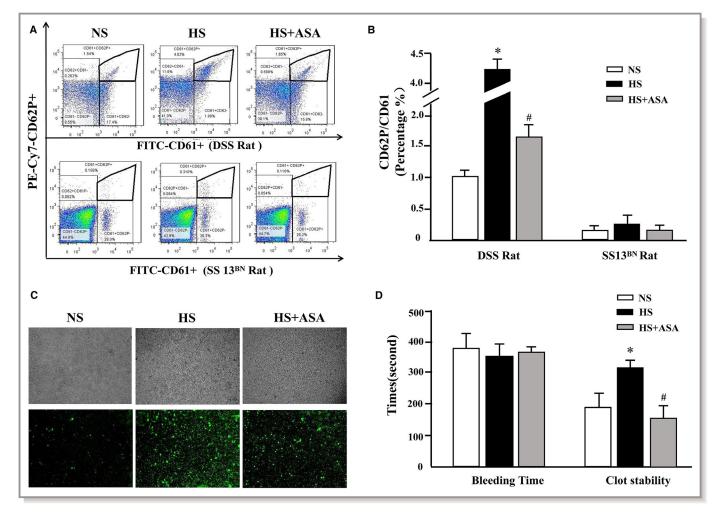
**Figure 1.** Aspirin attenuates the increase in blood pressure caused by a high-salt (HS) diet in Dahl salt-sensitive (DSS) rats. **A**, Systolic blood pressure (SBP) was measured in conscious rats, 3 times a week by tail-cuff plethysmography. Male DSS and salt-resistant rats (SS13<sup>BN</sup>) rats were fed a normal-salt (0.4% NaCl; NS, DSS, n=13; SS13<sup>BN</sup>, n=8) or high-salt (8% NaCl; HS, DSS, n=13; SS13<sup>BN</sup>=8) diet. In other groups of rats fed an HS diet, vehicle or aspirin were given by gavage (10 mg/kg per day; DSS+HS+ASA, n=12; SS13<sup>BN</sup>+HS+ASA, n=11). \**P*=0.008 vs NS. <sup>#</sup>*P*=0.038 vs HS, 1-way ANOVA, Tukey test. **B**, At the end of the study (day 56), SBP was measured from the aorta (via the left carotid artery) in pentobarbital-anesthetized rats. \**P*=0.007 vs NS, <sup>#</sup>*P*=0.034 vs HS, 1-way ANOVA, Tukey test. n=6 per group. C. SBP was measured by Stellar Telemetry System and the data were analyzed by Acqknowledge software (NS: n=5; HS: n=5; HS+ASA: n=5). \**P*=0.006 vs NS, <sup>#</sup>*P*=0.021 vs HS, 1-way ANOVA, Tukey test. Values are expressed as mean±SEM.

(NS, HS, and HS+ASA) did not impact the age-related increase in body weight (Tables S2 and S3) or food intake (Table S4) in DSS rats and SS13<sup>BN</sup> rats in different ways.

### Aspirin Blunts the Platelet Activation Caused by an HS Diet in DSS Rats

The expression of releasable platelet P-selectin was greater in DSS rats fed the HS diet than DSS rats fed the NS diet (P=0.03). The increase in releasable platelet P-selectin level caused by the HS diet in DSS rats was reduced by aspirin (P=0.036). The releasable platelet P-selectin levels were much lower in SS13<sup>BN</sup> than DSS rats with the NS or HS diet; ASA had no effect on the release of platelet P-selectin in SS13<sup>BN</sup> rats (Figure 2A and 2B).

The bleeding times were similar in the DSS rats fed the NS  $(368.8\pm20.7 \text{ sec})$  or HS diet without  $(378\pm29.1 \text{ seconds})$  or with  $(359\pm18.9 \text{ seconds})$  aspirin treatment (Figure 2C). By contrast, clot stability time was longer in the DSS rats fed the HS diet  $(326\pm19.7 \text{ seconds})$  than DSS rats fed the NS diet  $(199\pm19.6 \text{ seconds})$ . The increase in clot stability time in DSS rats fed the HS diet  $(203\pm25 \text{ seconds})$  was normalized by aspirin (HS+ASA,  $187\pm21 \text{ seconds})$  (Figure 2C). The adhesion between immobilized collagen and platelets in DSS rats was markedly enhanced by the HS diet that was blocked by aspirin treatment (Figure 2D). Neither the HS diet nor aspirin had an effect on red blood cell count, white blood cell count, lymphocyte count, platelet count, hematocrit, hemoglobin, mean platelet volume, and platelet distribution width (Table).



**Figure 2.** Aspirin blunts the platelet activation caused by a high-salt (HS) diet in Dahl salt-sensitive (DSS) rats. **A** and **B**, Surface expression of P-selectin determined by flow cytometry in DSS and salt-resistant SS13<sup>BN</sup> rats fed a normal-salt (NS, 0.4% NaCl), an HS (8% NaCl), or an HS diet with aspirin gavage (HS+ASA, 10 mg/kg per day). Results are expressed as mean fluoresce intensity, (n=7 per group). Values are expressed as mean $\pm$ SEM. \**P*=0.003 vs NS, <sup>#</sup>*P*=0.024 vs HS, 1-way ANOVA, Tukey test. **C**. Bleeding time indicates duration of first bleeding and clot stability indicates the time between the first and second bleeding (n=10 per group). Values are expressed as mean $\pm$ SEM, \**P*=0.008 vs NS, <sup>#</sup>*P*=0.035, vs HS, 1-way ANOVA, Tukey test. **D**. Platelets isolated from DSS rats with different treatments (NS, HS, HS+ASA) were prestained with the fluorescent dye 2'7'-bis-(2-carboxyethyl)-5-carboxyfuorescein acetoxymethyl ester, and then co-cultured with collagen; the fluorescent signals were captured using a confocal microscope (Leica). Green color shows the adhered platelet (n=5 per group, ×100).

#### Table. Hematologic Analysis

	NS	HS	HS+ASA	P Value (NS vs HS)	P Value (HS vs HS+ASA)
RBCs, 10 <sup>12</sup> /L	8.22±0.43	7.76±0.39	7.68±0.22	0.45	0.13
WBCs, 10 <sup>9</sup> /L	4.17±1.09	5.63±1.12	5.48±0.65	0.54	0.15
Lymphocytes, 10 <sup>9</sup> /L	2.70±0.57	3.40±0.74	3.16±0.44	0.20	0.70
Platelets, 10 <sup>9</sup> /L	709.00±66.01	782.75±44.32	689.60±47.69	0.15	0.09
Hematocrit, %	45.30±0.02	43.75±0.01	44.60±0.01	0.21	0.34
Hemoglobin, g/L	147.30±7.30	144.00±5.80	144.00±2.90	0.57	0.61
MPV, fL	6.40±0.05	6.40±0.01	6.32±0.05	0.73	0.70
PDW, fL	14.93±0.03	15.07±0.06	15.10±0.03	0.33	0.70

Values are expressed as mean±SEM. No abnormalities or significant differences in hematologic parameters were observed in the normal-salt (NS), high-salt (HS), and HS diet with aspirin gavage (HS+ASA) groups in Dahl salt-sensitive rats. MPV indicates mean platelet volume; PDW, platelet distribution width (n=10 per group); RBCs, red blood cells; WBCs, white blood cells.

#### Aspirin Impairs Platelet–Leukocyte Aggregation Caused by an HS Diet in DSS Rats

There is increasing recognition that abnormal platelet activation leads to inappropriate leukocyte recruitment in different organs/tissues in CVD.<sup>25</sup> The number of leukocytes (CD45+) in the peripheral blood were increased by the HS diet in both DSS and SS13<sup>BN</sup> rats but the increase was greater in DSS than SS13<sup>BN</sup> rats; both were normalized to their NS diet values by aspirin (Figure 3A and 3B). The aggregation between leukocytes (CD45+) and platelets (CD41+) was increased by the HS diet in DSS but not SS13<sup>BN</sup> rats (Figure 3C and 3D). The HS diet-stimulated leukocyte and platelet aggregation in DSS rats fed the HS diet was decreased by aspirin (Figure 3C and 3D). The lineage of the heterotypic aggregates (leukocytes-platelets CD45+CD61+) was studied further. The neutrophil-platelet (CD11b+CD61+), T lymphocyte-platelet (CD3e+CD61+), and T helper-platelet (CD4+CD61+) aggregations were increased by the HS diet and decreased by aspirin (Figure 3E and 3F). By contrast, the HS diet decreased regulatory T cell-platelet (CD25+CD61+) aggregation but was also normalized by aspirin (Figure 3E and 3F). The aggregation of platelets and B lymphocytes (CD45R+CD61+), as well as with T cytotoxic cells (CD8a+CD61+), was not significantly affected by NS diet, HS diet, and HS+ASA diet (Figure S2A and S1B). Scanning electron microscopy demonstrated that in DSS rats, the HS diet increased the number of platelets adherent to leukocytes, which were normalized by aspirin (Figure 3G).

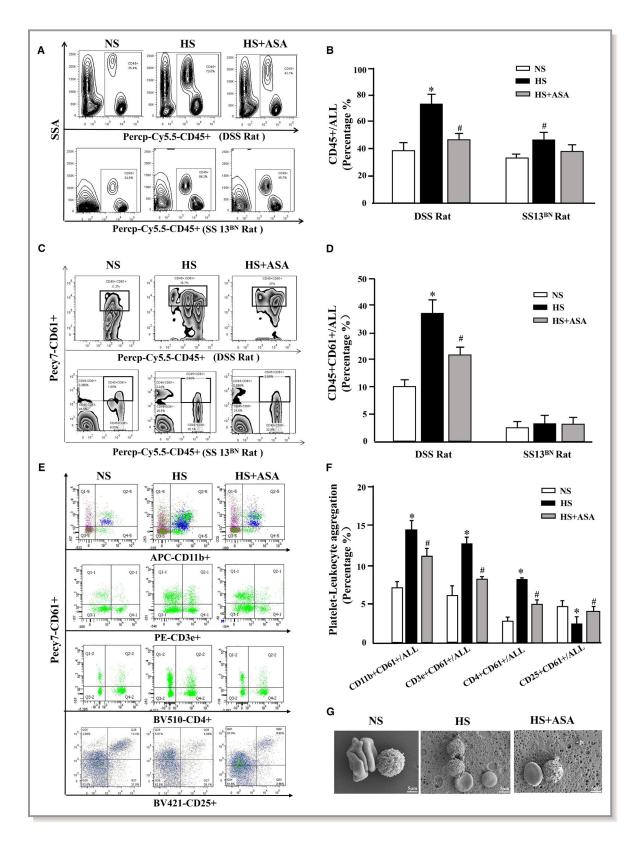
#### Aspirin Decreases the Inflammatory Reaction Caused by an HS Diet in DSS Rats

Leukocyte infiltration into target cardiovascular organs is crucial in the pathogenesis of hypertension.<sup>30</sup> Therefore, the number of leukocytes (CD45+) in the kidney and aorta were quantified in DSS rats fed an NS and HS diet with or without

aspirin treatment. Flow cytometric analysis (Figure 4A and 4B) and immunohistochemistry (Figure 4C and 4D) revealed that DSS rats fed the HS diet had increased leukocyte infiltration in their aortas (Figure 4A through 4C) and kidneys (Figure 4D) compared with control rats (NS diet). Low-dose aspirin given by gavage in DSS rats markedly attenuated the renal and aortic infiltration of leucocytes (CD45+), especially neutrophils (CD11b+) and T lymphocytes (CD3+), in the aortas, caused by the HS diet (Figure 4A and 4B). There was also a skewing toward inflammatory T-cell subsets gated to CD4+T cells. In the DSS rats, intracellular fluorescence-activated cell sorting (FACS) analysis revealed that the HS diet, relative to the NS diet, increased T cells positive for Th17 cell (IL-17) and negative for regulatory T cell (CD25+). Low-dose aspirin decreased the increased infiltration of IL-17 cells but restored to normal the decreased expression of CD25+ cells (Figure 4A and 4B). By contrast, no significant differences were observed in B lymphocytes (CD45R+) and T cytotoxic lymphocytes (CD8a+) among the groups (NS diet, HS diet, and HS+ASA diet) (Figure S3A and S3B). Transmission electron microscopy images of DSS aortas from the NS and ASA groups displayed intact endothelial layers (Figure 4C). Remarkably, the aortas isolated from the HS group without aspirin had deformed endothelial layers that were infiltrated by leukocytes and decreased by aspirin (HS+ASA) (Figure 4C). The renal mRNA (Figure 4E) and protein (Figure 4F and 4G) expressions of the inflammatory factors IL-1, IL-6, and TNF- $\alpha$  were also increased by the HS diet but were decreased by aspirin.

#### Aspirin Attenuates the Vascular Dysfunction Caused by an HS Diet in DSS Rats

Vascular dysfunction is a hallmark of hypertension and an important precursor to atherosclerosis and cardiovascular events.<sup>31,32</sup> We examined the mesenteric arteries to examine the effects of aspirin on the vascular function in 3 groups of



DSS rats and recorded the concentration-relaxation curves using the PowerLab system.<sup>33</sup> We determined that the HS diet impaired the endothelium-dependent vasodilatation but not the endothelium-independent vasodilatation in DSS rats

(Figure 5A and 5B). In contrast, aspirin largely restored the HS-impaired vasodilatory effect of the mesenteric arteries in response to acetylcholine (Figure 5A). Vasodilation with sodium nitroprusside was similar in all of the groups

**Figure 3.** Aspirin impairs the platelet–leukocyte aggregation induced by a high-salt (HS) diet in Dahl salt-sensitive (DSS) rats. **A** and **B**, Peripheral blood lymphocytes enriched from fresh blood in DSS rats fed a normal-salt (NS; 0.4%, n=8) diet, an HS (8%, n=10) diet, or HS with aspirin gavage (HS+ASA; 10 mg/kg per day, n=10) were analyzed by flow cytometry using the leucocyte surface marker CD45+. Values are expressed as mean $\pm$ SEM, 1-way ANOVA, Tukey test, DSS rat: \**P*=0.006 vs NS, \**P*=0.037 vs HS, salt-resistant rats (SS13<sup>BN</sup>): \**P*=0.041 vs HS, HS+ASA. **C** and **D**, CD45+CD61+/ALL indicates the ratio of leukocytes aggregated to platelets. Values are expressed as mean $\pm$ SEM, 1-way ANOVA, Tukey test, \**P*=0.004 vs NS. \**P*=0.031 vs HS. **E** and **F**, Percentages of CD11b+CD61+ and CD3+CD61+ gate were circled within the CD45+CD61+gate; CD4+CD61+ gate was obtained from the CD3+CD61+ gate; CD25+CD61+ was obtained from the CD4+CD61+ gate. Values are expressed as mean $\pm$ SEM, 1-way ANOVA, Tukey test, CD11b+CD61+ and CD3+CD61+ was obtained from the CD4+CD61+ gate. Values are expressed as mean $\pm$ SEM, 1-way ANOVA, Tukey test, CD11b+CD61+/ALL: \**P*=0.006 vs NS. \**P*=0.031 vs HS, CD4+CD61+ gate; CD25+CD61+/ALL: \**P*=0.004 vs NS. \**P*=0.031 vs HS, CD4+CD61+/ALL: \**P*=0.004 vs NS. \**P*=0.043 vs HS. **G**, Scanning electron microscopy of aggregated platelets and leukocytes (×4000, scan bar 5 µm).

(Figure 5B). We then used the thoracic aorta<sup>34</sup> to examine the effects of aspirin on vascular integrity by electron microscopy and hematoxylin and eosin staining. The control aorta presented a normal histology consisting of a luminal monolayer of endothelial cells, a media containing smooth muscle cells, and an adventitia rich in collagen fibers with a typical periodicity of 67 nm (Figure 5C). The HS diet caused a loss of undulation and severe damage of all layers of the vessel wall, but the internal elastic lamina remained intact (Figure 5C). The vascular structure was partially normalized by aspirin. Immunofluorescence was also used to verify endothelial damage, and we observed increased vascular cell adhesion molecule-1, collagen I, and decreased eNOS levels throughout the aorta cross-section after the HS diet, especially in the intimal region (Figure 5C). The expressions of the vascular injury markers inducible NO synthase, ET-1, and vWF  $^{\rm 35,36}$ were also increased in the thoracic aorta of the rats with the HS diet. In contrast, the HS diet decreased the aortic expression of eNOS (Figure 5D and 5E). These effects of an HS diet were ameliorated by aspirin. Hypertrophy of the heart and kidney were observed after HS diet intake, with effects decreased by aspirin (Figure S4).

#### Aspirin Attenuates the Leukocyte-Endothelial Cell Adhesion Induced by Platelet Activation via COX-1 But Not COX-2 Pathway in DSS Rats Fed an HS Diet

The HS diet increased COX-1 but not COX-2 expression in platelets of DSS rats but had no effect in SS13<sup>BN</sup> rats (Figure 6A). The HS diet also increased serum thromboxane A2 (TXA2) in DSS but not SS13<sup>BN</sup> rats (Figure 6B). The adhesion between PBL and PMVECs was markedly enhanced by platelets from DSS but not SS13<sup>BN</sup> rats incubated in medium with high salt (173 mmol/L NaCl) (Figure 6C). The increase in PBL/PMVEC adhesion caused by high salt in DSS rats was prevented in platelets pretreated with aspirin (COX-1, COX-2 inhibitor) and triflusal (COX-1 inhibitor) but not lumiracoxib (COX-2 inhibitor) (Figure 6D). The activation of platelets (P-selectin, CD62P+) by the HS diet in DSS rats was also blunted by aspirin and triflusal (COX-1 inhibitor) (Figure 6D). These results support the notion

that COX-1 but not COX-2 is involved in the HS diet–related PBL/PMVEC adhesion and platelet activation.

#### Analysis of Proteins in Platelets From DSS Rats Treated With Aspirin Using Parallel Adaptor Capture Proteomics

To understand the mechanism by which aspirin exerts its beneficial effects, as related to the detrimental effects of an HS diet in DSS rats, gene ontology functional classification analysis and KEGG pathway analysis were performed and the results are presented in Figure 7. The classifications of platelets proteins by gene ontology cover 3 processes (biological process, cellular component, and molecular function), according to their domains in molecular and cellular biology (Figure 7A). We also performed pathway mapping analysis using KEGG, which ranks the related pathways in the order according to the number of mapped platelet proteins and found that 3 of the top 10 significantly mapped pathways were related to signal transduction, signaling molecules and interaction, and the immune system (Figure 7B). Sample correlation analysis and principal component analysis suggested large differences between the NS and HS groups, which were restored to the NS data, by aspirin to a large extent (Figure 7C and 7D). The Venn diagram showed that 75 overlapping proteins were sensitive to high salt and aspirin treatment (Figure 8A). Gene ontology and KEGG analyses were performed and of the top 10 mapped pathways (Figure 8B and 8C), the complement and coagulation cascades had the highest enrichment ratio with 11 proteins having altered amounts (marked by red star, Figure 8D). The Western blots of platelet proteins extracted from DSS rats showed that coagulation factor XIIIa, carboxypeptidase B2, and complements C1, C3, C6, and C7 were increased by the HS diet compared with the NS group, effects that were reversed by aspirin treatment (Figure 8E).

#### Discussion

The major findings of the current study are as follows: (1) aspirin ameliorated the increased BP and platelet activation induced by the HS diet in DSS rats, effects that were not



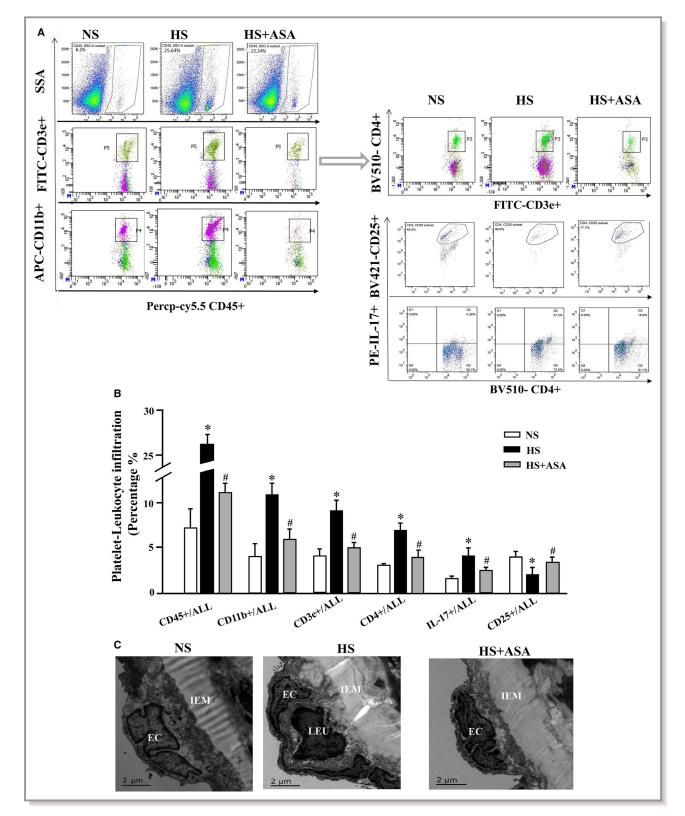
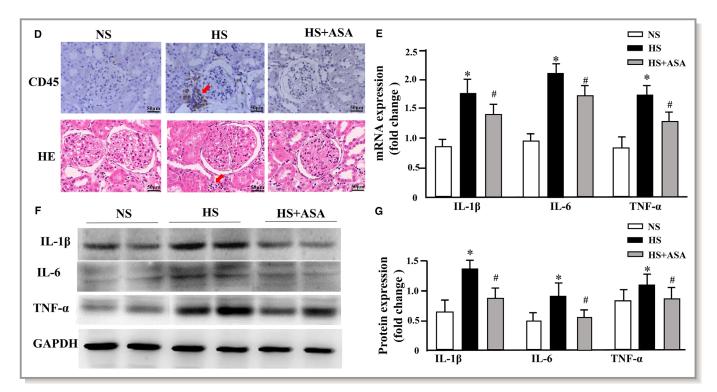


Figure 4. Continued.

observed in salt-resistant SS13<sup>BN</sup> rats; (2) aspirin ameliorated the increased leukocyte–platelet aggregation and expression of inflammatory cytokines (ie, IL-6, IL-1, and TNF- $\alpha$ ) in the

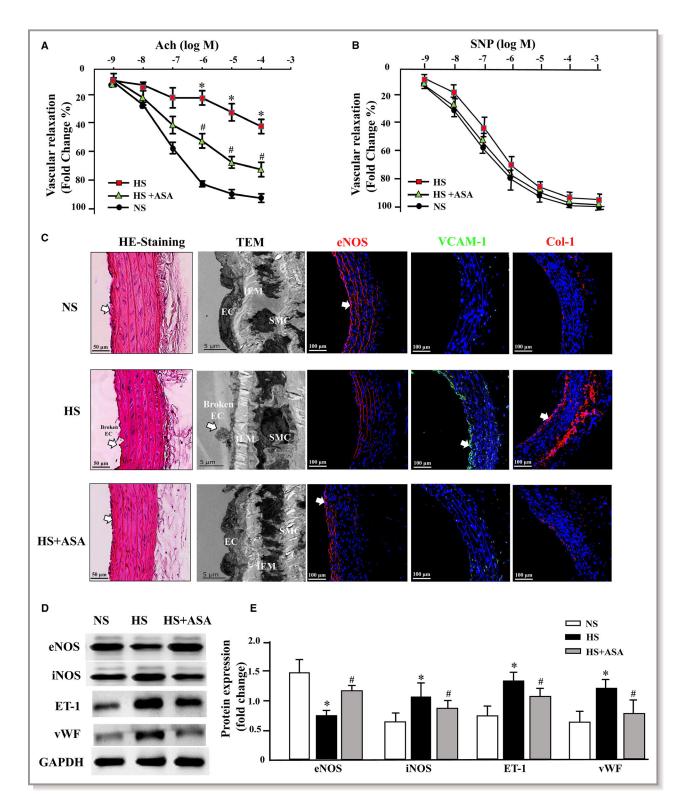
kidneys and T-cell infiltration in the aorta and kidneys as induced by the HS diet in DSS rats; (3) aspirin ameliorated the vascular dysfunction induced by the HS diet in DSS rats; (4) a



**Figure 4.** Aspirin decreases the inflammatory reaction caused by high-salt (HS) diet in Dahl salt-sensitive (DSS) rats. **A** and **B**. The aorta was digested into single-cell suspensions, and white blood cells were detected by fluorescent antibodies; the fluorescence signal was captured by a flow cytometer. CD3+CD4+ gate was selected from CD45+CD3+ gate; CD4+CD25+ and CD4+IL-17+ gate was selected from the CD3+CD4+ gate. Values are expressed as mean $\pm$ SEM, 1-way ANOVA, Tukey test, CD45+/ALL: \**P*=0.002 vs the normal-salt (NS) diet group, "*P*=0.017 vs HS, CD11b+/ALL: \**P*=0.005 vs NS, "*P*=0.037 vs HS, CD3e+/ALL: \**P*=0.004 vs NS, "*P*=0.030 vs HS, CD4+/ALL: \**P*=0.005 vs NS, "*P*=0.034 vs HS, IL-17+/ALL: \**P*=0.004 vs NS, "*P*=0.033 vs HS, CD25+/ALL: \**P*=0.008 vs NS, "*P*=0.045 vs HS.\* **C**, DSS rat (n=3) aortas were imaged by TEM, as described in the Materials and Methods section. Low magnification (×1000) imaging was used to identify endothelial cell (EC), internal elastic membrane (IEM), and leukocyte (LEU) (bar 2 µm). **D**, Leukocyte (CD45+) infiltration in the kidney was identified by immunohistochemistry; hematoxylin and eosin staining showed the inflammatory morphology near the glomerulus (bar 50 µm). **E** and **G**, The expression of inflammation factors was quantified by real-time polymerase chain reaction and immunoblotting in the kidneys of DSS rats. Results are corrected for expression of GAPDH. Values are expressed as mean $\pm$ SEM, 1-way ANOVA, Tukey test, mRNA expression: interleukin (IL) 1 $\beta$ , \**P*=0.008 vs NS, "*P*=0.039 vs HS; IL-6: \**P*=0.039 vs HS; tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ): \**P*=0.007 vs NS, "*P*=0.032 vs HS, protein expression: IL-1 $\beta$ , \**P*=0.007 vs NS, "*P*=0.039 vs HS; IL-6: \**P*=0.008 vs NS, "*P*=0.036 vs HS, TNF- $\alpha$ : \**P*=0.007 vs NS, "*P*=0.038 vs HS. HS+ASA indicates HS diet with aspirin gavage.

positive correlation between the COX-1 pathway and platelet activation by the HS concentration in vitro was observed; and (5) platelet proteomics indicated the activation of the complement cascade induced by the HS diet in DSS rats. Overall, these results suggest that the increase in BP induced by the HS diet in DSS rats is related to endothelial dysfunction caused by an abnormal activation of inflammatory, complement, and coagulation cascades in platelets.

Aspirin has been recommended for the primary prevention of CVD by the US Preventive Services Task Force.<sup>37</sup> The benefit of combining antiplatelet therapy with aspirin for the secondary prevention of CVD is well established and supported by strong data from clinical trials.<sup>38–40</sup> However, evidence supporting the use of aspirin for the primary treatment of hypertension is not as robust.<sup>16</sup> In the large Hypertension Optimal Treatment study, the efficacy of aspirin in reducing major cardiovascular events (in particular, myocardial infarction but not stroke) was evident, but aspirin also increased the risk of major and minor nonfatal bleeding with no effect on mortality observed.<sup>41,42</sup> Further, aspirin did not lower BP in that study, and a reanalysis showed that aspirin was associated with a small nonsignificant increase in both systolic BP and diastolic BP.43 A meta-analysis of other studies showed that aspirin did not affect the efficacy of other antihypertensive drugs.<sup>16</sup> However, as aforementioned in the study by Hermida et al,<sup>17</sup> aspirin (100 mg/d) given at bedtime did decrease the ambulatory BP in patients with hypertension. In animal experiments, Schirner and Taube<sup>44</sup> reported that oral aspirin could reduce or increase BP in spontaneously hypertensive rats, depending on whether the initial BP was above or below 160 mm Hg. In another study involving spontaneously



hypertensive rats, a single intravenous injection of aspirin decreased the BP of male but not female spontaneously hypertensive rats.<sup>45</sup> Elsewhere, Taube C showed that low-dose (10 mg/kg per day) but not high-dose (100 mg/kg per day) aspirin can significantly decrease high BP in rats.<sup>46</sup> In the current study using DSS rats, we found that low-dose

aspirin (10 mg/kg per day) administered by daily gavage inhibits the HS diet-induced increase in BP and ameliorates the impaired endothelium-dependent acetylcholine vasodilation.

Platelets participate in the pathogenesis of endothelial dysfunction and hypertension.<sup>1,2,5,16</sup> Our in vivo functional

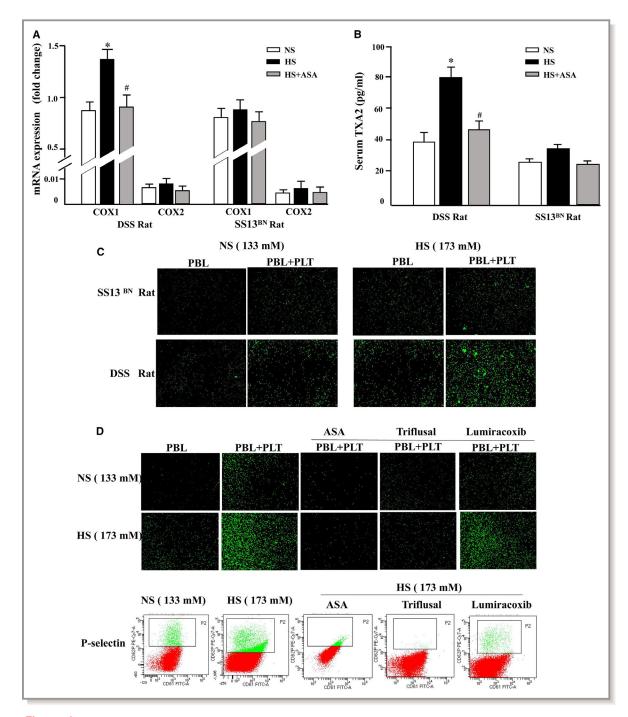
**Figure 5.** Aspirin attenuates the vascular (aorta) dysfunction caused by a high-salt (HS) diet in Dahl salt-sensitive (DSS) rats. **A** and **B**, Mesenteric arterial was obtained to examine vasodilatation induced by sodium nitroprusside (SNP) and acetylcholine (Ach) and the results were quantified by a PowerLab system (n=5). Values are expressed as mean $\pm$ SEM, 1-way ANOVA, Tukey test, Ach(log-6): \**P*=0.004 vs the normal-salt (NS) diet group, "*P*=0.039 vs HS. Ach(log-5): \**P*=0.003 vs NS, "*P*=0.031 vs HS, Ach(log-4): \**P*=0.003 vs NS, "*P*=0.035 vs HS. **C**, Thoracic aorta was taken for measurement. Hematoxylin and eosin (HE) staining in the thoracic aorta of DSS rats locked destroyed or integrated vascular endothelium in HS and NS (HS diet with aspirin gavage [HS+ASA]), respectively (bar 50 µm). Transmission electron microscopy (TEM) cross-section images of the thoracic aorta of DSS rats fed an NS, an HS, or an HS+ASA diet (bar 5 µm). Immunofluorescence indicated the endothelial cell marker (endothelial NO synthase [eNOS], red; vascular cell adhesion molecule-1 (VCAM-1), green; Col-1, collagen I, red). Cell nuclei are stained blue (DAPI). Scale bars=100 µm. **D** and **E**, The expression of Vascular injury markers was quantified by immunoblotting in thoracic aortas of DSS rats. Results were corrected for expression of GAPDH protein. Values are expressed as mean $\pm$ SEM, 1-way ANOVA, Tukey test, eNOS: \**P*=0.005 vs NS, "*P*=0.037 vs HS. Inducible NO synthase (iNOS): \**P*=0.008 vs NS, "*P*=0.041 vs HS, endothelin-1 (ET-1): \**P*=0.007 vs NS, "*P*=0.045 vs HS, von Willebrand factor (vWF): \**P*=0.006 vs NS, "*P*=0.030 vs HS (n=6 per group). EC indicates endothelial cells; IEL, internal elastic lamina; SMC, smooth muscle cells.

study revealed that low-dose aspirin (10 mg/kg per day) administered by daily gavage prevented an HS diet-induced increase in BP and ameliorated impaired endotheliumdependent vasodilation in DSS rats. Endothelial dysfunction is important in the development of hypertension, which is often used as a target of pharmacological treatment.<sup>28,29,47-</sup> <sup>51</sup> Endothelial cells produced vasoactive substance NO, and the vasodilator ET-1 is the most important in BP regulation.<sup>52,53</sup> In our study, we found that aspirin reversed HSpromoted decreased eNOS and elevated ET-1 protein expression, while immunofluorescence also found the reverse was true of eNOS and vascular cell adhesion molecule-1. A recent analysis of the Framingham population indicated that aortic stiffening precedes the development of overt hypertension.<sup>54</sup> Further, we found that low-dose aspirin also decreases the levels of collagen I in the thoracic aorta, which is predicted to contribute to vascular remodeling and potentially dysfunction.55,56 Our in vitro studies showed a positive linear relationship between platelet activation and sodium concentration in the incubation medium. Long-term HS intake in DSS rats activated platelets but is prevented by the intake of lowdose aspirin, which inhibits platelet activation without affecting the tail bleeding time and promoting the treatment of endothelial dysfunction and hypertension.

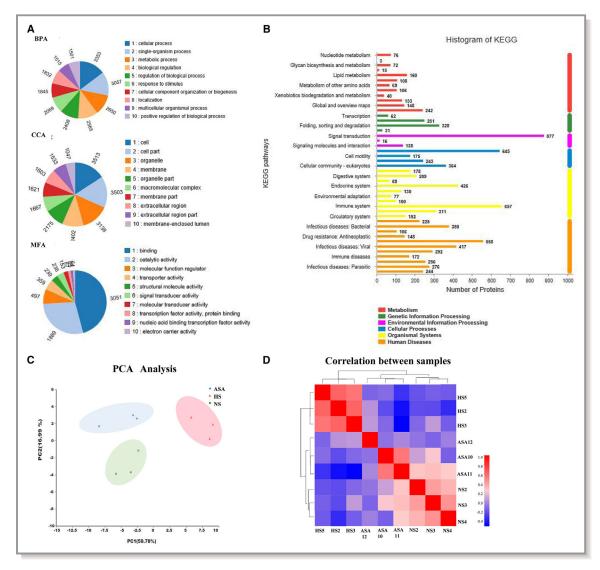
Platelet activation causes the release of platelet granules and lysosomes that contain a multitude of biologically active molecules with many consequences, in addition to coagulation.<sup>57–59</sup> These factors induce leukocyte recruitment, the formation of heterotypic aggregates (platelets–leukocytes), inflammation, oxidative stress, and remodeling of the vascular wall, among others. Platelets can interact with blood vessels via several pathways, as follows: (1) during hemostasis, when platelets adhere to and are activated on subendothelial matrix proteins exposed by vascular damage, leukocytes are recruited to the growing thrombus; (2) platelets adherent to endothelial cells are activated and act as bridges for bloodborne leukocytes to the vessel wall; and (3) adhesion between platelets and leukocytes in the blood leads to the formation of heterotypic aggregates before contact with endothelial cells. The third process is that which most likely happened in our current study because we detected an increase in leukocyte– platelet aggregates in the peripheral blood of DSS rats fed an HS diet, which was associated with an increase in the blood vessel infiltration of leukocytes, including monocytes and T lymphocytes. All lesions were prevented by aspirin treatment. There is evidence to suggest that immune cells' infiltration is associated with increased vasoconstriction in response to norepinephrine and the impairment of endothelial-dependent (ACh-induced) vasorelaxation.<sup>34,60–62</sup> Activated T cells infiltrate the vascular adventitia that induces collagen deposition, leading to aortic dysfunction and ultimately hypertension.<sup>30,32,63–65</sup> Therefore, aspirin may effectively block endothelial dysfunction and salt-sensitive hypertension by inhibiting platelet-stimulated immune cell infiltration.

Salt-sensitive hypertension is considered to be a chronic inflammatory disease.<sup>66</sup> Imbalances between proinflammatory effector responses and anti-inflammatory responses of regulatory T cells determine, to a large extent, the severity of inflammation.<sup>67</sup> An HS diet may also cause hypertension via autoimmunity by inducing T helper 17 cells.<sup>68</sup> We now report that DSS rats fed an HS diet have decreased regulatory T cells but increased T helper cells. Aspirin treatment trended to normalize the ratio of regulatory T and T helper 17 cells. Thus, aspirin-mediated normalization of T-lymphocyte function may, in part, be responsible for the beneficial effect of aspirin in salt-sensitive hypertension.

The mechanism by which aspirin affects platelet function involves the inhibition of the cyclooxygenase and TXA2 pathways.<sup>69</sup> There are experimental and epidemiological pieces of evidence linking abnormalities in the cyclooxygenase/ prostaglandin system to salt-sensitive hypertension.<sup>70</sup> The cyclooxygenase family is composed of 3 isozymes: "constitutive" COX-1, "inducible" COX-2, and maybe COX-3.<sup>71–73</sup> Inhibition of COX-2 in hematopoietic cells, in addition to renal tubule cells, results in salt-sensitive hypertension<sup>74</sup> and may interfere with the BP-lowering effects of antihypertensive medications.<sup>75</sup> By contrast, COX-1 (but not COX-2) inhibition improves the impaired kidney function induced by ischemia-



**Figure 6.** Aspirin inhibits the peripheral blood leukocyte (PBL)–pulmonary microvascular endothelial cell adhesion via the platelet cyclooxygenase 1 (COX-1) but not the cyclooxygenase 2 (COX-2) pathway. **A**, mRNA expression of platelet COX-1 and COX-2 were measured by real-time polymerase chain reaction. Values are expressed as mean $\pm$ SEM, 1-way ANOVA, Tukey test, Dahl salt-sensitive (DSS) rat-COX-1: \**P*=0.005 vs the normal-salt (NS) group, \**P*=0.030 vs the high-salt (HS) group. Salt-resistant rats (SS13<sup>BN</sup>) rat-COX-1: \**P*=0.044 vs HS. **B**, Serum thromboxane A2 (TXA2) was detected by ELISA kit. Values are expressed as mean $\pm$ SEM, 1-way ANOVA, Tukey test, DSS rat-TXA2: \**P*=0.004 vs NS, \**P*=0.033 vs HS. SS13<sup>BN</sup> rat-TXA2: \**P*=0.046 vs HS (**C** and **D**). PBL was dyed with 2,7-bis-(2-carboxyethyl)-5 carboxy furancillin acetoxymethyl ester, triflusal was used as the COX-1 inhibitor and lumiracoxib was used as the COX-2 inhibitor, normal salt (133 mmol/L NaCl), high salt (173 mmol/L NaCl), and aspirin (100 µmol/L). The fluorescence signal was captured using a confocal microscope (Leica) and the green fluorescence concentration was proportional to the amount of adhered PBL. Flow cytometry was performed to detect the expression of platelet P-selectin (CD62P+). HS+ASA indicates HS diet with aspirin gavage; PLT, platelet.

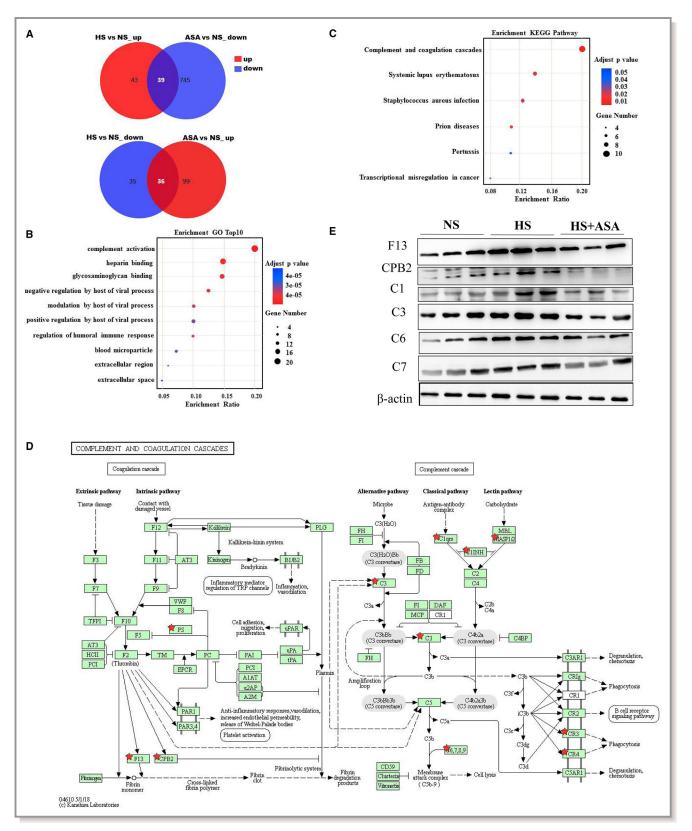


**Figure 7.** Gene ontology (GO) classification and GO enrichment-based clustering analysis of the differentially expressed platelet proteins. **A**, GO functional classification of the upregulated or downregulated acetylated proteins; GO enrichment-based clustering analysis of the differentially expressed platelet proteins. **B**, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. **C**, Principal component analysis (PCA). **D**, Correlation of genes altered by salt intake and aspirin. ASA indicates high-salt diet with aspirin gavage BPA, biological process analysis; CCA, cellular component analysis; HS, high-salt diet; MFA, molecular function analysis; NS, normal-salt diet.

reperfusion.<sup>76</sup> COX-1 may also be the cause of endothelial dysfunction in heart failure.<sup>77</sup> In the current study in DSS rats, we found that platelet COX-1 but not COX-2 mRNA and protein (Figure S5) are increased by an HS diet, but the increased expression can be inhibited by aspirin. In fact, aspirin also has a plethora of favorable vascular effects in addition to preventing COX-1–dependent TXA2 synthesis. Aspirin was found to acetylate lysine of eNOS, which evokes the activation of eNOS independently of COX-1 inhibition and TXA2 production.<sup>78–80</sup> In 2 clinical trials, aspirin increased in conjunction with HO-1 and decreased in conjunction with ADMA expression, which contributed to NO production.<sup>81,82</sup> Furthermore, our study found that aspirin can slightly attenuate HS intake–induced

prostaglandin I2 suppression and also downregulated prostaglandin E2 levels in DSS rats, which shows that aspirin may return anti-inflammatory efficacy by depressing prostaglandin E2, while partly restores the prostaglandin I2/TXA2 balance to avoid the adverse effects of salt-sensitive hypertension (Figure S6). Considering multiple functions of aspirin, we plan to further explore novel mechanisms of aspirin on HS-induced abnormal platelet activation.

Because platelets have no nuclei, proteomics is a perfect tool to study their biological functions. The complement and coagulation pathways were found as the most relevant pathway in the present study. The complement system is involved in the development of inflammatory responses and is a major effector



**Figure 8.** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed platelet proteins in Dahl saltsensitive (DSS) rats. **A**, The Venn diagram demonstrates the overlap of upregulated and downregulated platelet proteins in DSS rats fed a normal-salt (NS), a high-salt (HS), or an HS diet with aspirin gavage (ASA). **B** and **C**, Gene ontology (GO) and KEGG enrichment analysis of the upreulated and downregulated platelet proteins. **D**, The pathway (complement and coagulation cascades) with the highest enrichment ratios, using GO and KEGG enrichment analysis. **E**, Western blotting of the altered proteins in the complement and coagulation cascades. Coagulation factor XIII (F13); Carboxypeptidase B2 (CPB2); complement 1 (C1); complement 3 (C3); complement 6 (C6); complement 1 (C7). mechanism of innate and adaptive immunity.<sup>83</sup> It is activated by 3 pathways (classical, lectin, and alternative pathway). The stimulation of specific factors, induced by the formation of convertase-generated membrane attack complex, participates in various biological processes, such as inflammatory and cytolytic responses.<sup>84</sup> Inflammation and coagulation pathways are activated by complement components such as C5a.85 Inappropriate activation of coagulation and complement is evident in numerous diseases, including atherosclerosis and venous thromboembolic disease.<sup>86–88</sup> Zhou et al<sup>84,89</sup> emphasized the beneficial relationship between C3 deficiency and hypertension, as well as renal injury in the model of unilateral ureteral obstruction. Treatment with a C5a receptor antagonist in deoxycorticosterone acetate-salt hypertension mice also results in the improvement of cardiac remodeling and vascular inflammation.<sup>90,91</sup> Our finding in DSS rats fed an HS diet of the overexpression of several components in complement and coagulation pathways, such as coagulation factor XIIIa, carboxypeptidase B2, and complements C1, C3, C6, and C7, indicates that these pathways may participate in the pathogenesis of salt-sensitive hypertension.

#### Conclusions

In male DSS rats, the HS diet-induced aggravated elevation of BP is associated with platelet activation, leukocyte infiltration and leukocyte-platelet aggregation (CD45+CD61+), and activation of the complement and coagulation pathways. These contribute to vascular endothelial and renal damage. Aspirin ameliorates the HS diet-induced aggravation of high BP by preventing the adhesion of leukocytes to endothelial cells via the inhibition of the platelet COX-1 but not the COX-2 pathway. Aspirin treatment also reverses the abnormal complement and coagulation cascades in male DSS rats fed an HS diet. These results highlight a new property of aspirin in protecting vascular endothelial dysfunction, which may be beneficial in the treatment of salt-sensitive hypertension.

#### Sources of Funding

These studies were supported in part by grants from the Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (CIFMS, 2016-I2M-1-016, 2019-I2M-1-001), the National Natural Science Foundation (China) (81600334, 81670387, 81800402), Life Science Society Consortium "Youth Talent Promotion Project," and the National Institutes of Health, USA, R01DK039308, P01HL074940, and R01HL092196.

#### **Disclosures**

None.

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# **Supplemental Material**

**Table S1. Primers information.** 

	Forward primer	Reverse primer		
COX-1	CCAAGACCTGCCCCTATGTC	ATAGGAATGGCGGCTCCC		
COX-2	TGAGTACCGCAAACGCTTCT	ACACAGGAATCTTCACAAATGGA		
IL-1β	GTGGTGGTTCTCATCGTGGT	AGCACGTAGTTGGGGGCTTAG		
IL-6	AGCGATGATGCACTGTCAGA	TAGCACACTAGGTTTGCCGA		
TNF- α	AGAACTCAGCGAGGACACCAA	GCTTGGTGGTTTGCTACGAC		

Forward primer and reverse primer Primers information

### Table S2. Body weight in DSS rats.

	NS	HS	HS+ASA	P value (NS.VS.HS)	P value (HS.VS.HS+ASA)
The first week	219.61±2.43	217.23±1.59	215.71±2.32	0.54	0.63
The second week	246.42±3.07	243.63±2.81	247.48±4.65	0.36	0.32
The third week	260.48±4.50	259.60±5.62	267.29±2.13	0.27	0.19
The fourth week	271.14±9.30	270.75±4.32	281.65±4.69	0.35	0.09
The fifth week	294.49±4.10	291.75±7.83	298.57±2.47	0.21	0.34
The sixth week	310.33±7.30	308.60±5.82	309.53±2.90	0.57	0.61
The seventh week	318.73±5.40	319.23±7.39	320.50±5.53	0.12	0.69
The eighth week	324.96±3.01	325.07±6.47	329.89±0.03	0.33	0.70

Body weights from 1 to 8 weeks of DSS rats on NS diet, HS diet, and HS+ASA diet.

### Table S3. Body weight in SS13<sup>BN</sup> rats.

	NS	HS	HS+ASA	P value (NS.VS.HS)	P value (HS.VS.HS+ASA)
The first week	210.61±2.06	211.23±1.39	208.71±3.21	0.71	0.33
The second week	250.40±4.17	246.80±1.91	249.43±2.65	0.52	0.41
The third week	267.32±3.30	269.60±4.52	276.38±3.13	0.47	0.39
The fourth week	277.60±5.73	280.10±7.82	285.05±2.69	0.29	0.16
The fifth week	295.57±4.19	299.75±7.83	300.57±2.47	0.31	0.29
The sixth week	318.33±4.30	320.60±6.40	319.71±2.38	0.47	0.35
The seventh week	323.20±5.90	334.58±5.27	330.50±2.53	0.14	0.19
The eighth week	350.76±4.98	354.27±6.23	345.31±5.53	0.42	0.38

Body weights from 1 to 8 weeks of SS13<sup>BN</sup> rats on NS diet, HS diet, and HS+ASA diet.

	1W	2W	3W	4W	5W	6W	7W	8W
DSS+NS	20.82±4.12	25.85±2.10	27.88±2.23	28.87±3.20	29.23±1.45	30.23±1.70	30.77±3.14	34.66±2.15
DSS+HS	19.83±5.27	23.86±4.08	26.86±6.16	29.45±4.34	31.88±4.78	32.78±4.78	32.78±4.11	35.09±4.98
DSS+HS+ASA	21.72±5.14	24.67±6.11	28.87±4.28	29.07±3.10	30.67±3.56	32.21±3.34	32.99±2.09	35.56±5.17
SS13 <sup>BN</sup> +NS	19.43±3.21	24.43±2.09	27.39±1.20	27.28±5.36	28.25±4.78	29.83±1.55	31.09±4.55	33.77±2.23
SS13 <sup>BN</sup> +HS	21.82±2.54	23.12±4.16	27.43±7.14	29.54±3.90	30.12±3.91	31.56±1.23	32.12±3.86	34.12±1.16
SS13 <sup>BN</sup> +HS+ASA	19.41±4.91	22.44±3.56	26.09±3.75	28.54±2.86	29.44±4.45	30.98±3.06	32.56±5.44	34.45±4.96

Table S4. Food intake.

Food intake (g) of Dahl Salt-sensitive Rat and 13BN Salt Resistant Rat treated with normal salt, high salt or high salt with aspirin. Foods intake was recorded three times/week averaged for each week. (n=8/group).

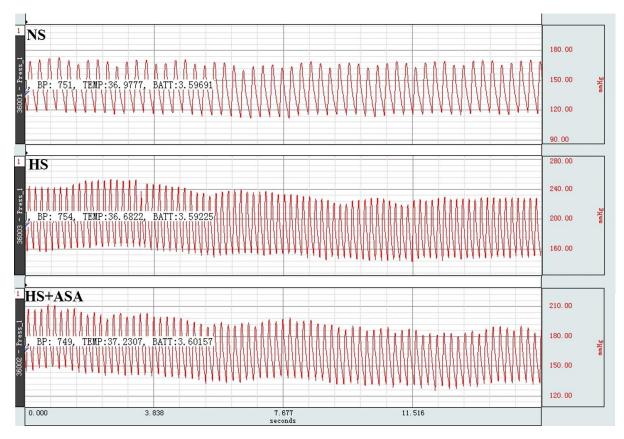
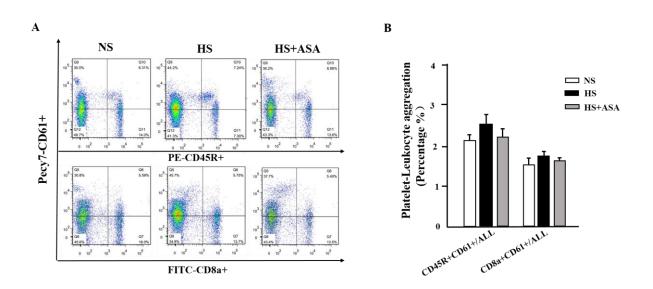


Figure S1. Aspirin Attenuates the Increase in BP Caused by HS Diet in DSS Rats.

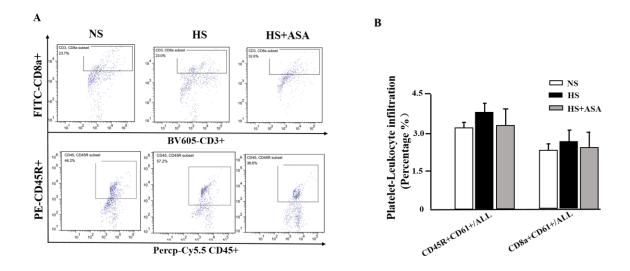
Stellar Telemetry System were used to acquire and logs blood pressure data. Acqknowledge software were used to analyzed blood pressure (systolic blood pressure).

Figure S2. There is no effect of HS diet or HS diet and aspirin, relative to NS diet, in the aggregation of B-lymphocytes and platelets or T-cytotoxic cells and platelets.



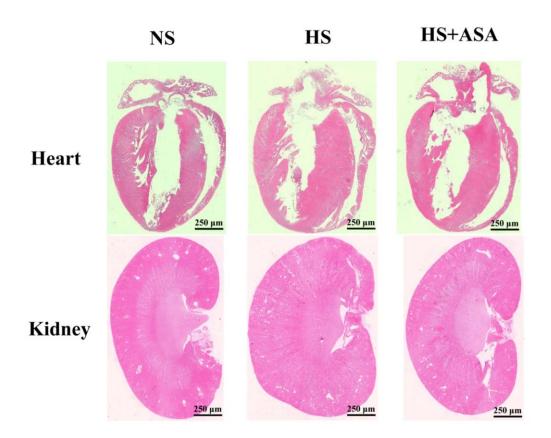
**A-B.** Peripheral blood lymphocytes enriched from fresh blood in DSS rats fed NS (normal salt, 0.4%, n=8), HS (high salt, 8%, n=10), or HS+ASA (high salt with aspirin gavage, 10 mg/kg/d, n=10) were analyzed by flow cytometry; B lymphocytes and T cytotoxic cells were labeled with surface marker CD45R+ and CD8a+, respectively. Values are mean ± SEM, one-way ANOVA, Tukey test; no significant differences among the groups.

Figure S3. There is no effect of HS diet or HS diet and aspirin, relative to NS diet, in the infiltration of B lymphocytes and T cytotoxic cells.



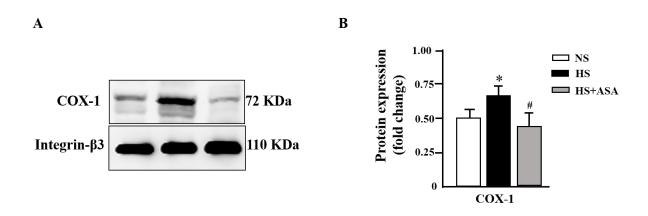
**A-B**. The aorta was digested into single-cell suspensions, and white blood cells were detected by fluorescent antibodies; the fluorescence signal was captured by a flow cytometer. CD3+CD8+ gate was selected from CD45+CD3+ gate; CD45+CD45R+ gate was selected from the CD45+SSC+ gate. Values are the mean  $\pm$  SEM, one-way ANOVA, Tukey test; no significant differences among the groups.

Figure S4. Aspirin blunts the hypertrophy of target organs, such as heart and kidney, in DSS rats fed HS diet, relative to DSS rats, fed NS diet.



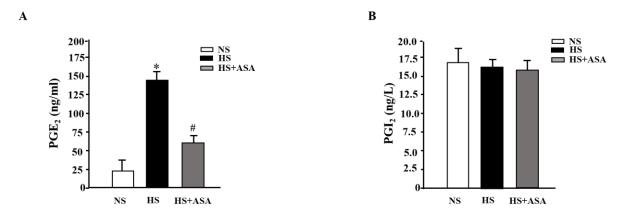
The heart and kidney were stained by H&E, bar 250  $\mu m.$ 

Figure S5. Aspirin inhibits the increased expression in COX-1 caused by HS diet in DSS rats.



**A-B** The expression of platelet COX-1was quantified by immunoblotting Results were corrected for expression of integrin-3. Values are the mean  $\pm$  SEM, one-way ANOVA, Tukey test, \*P= 0.009 vs NS and HS+ASA. <sup>#</sup>P =0.044 vs HS and NS (n= 6/group).

Figure S6. A. Serum PGE<sub>2</sub> (ng/ml) was detected by ELISA kit, Values are the mean  $\pm$  SEM, one-way ANOVA, Tukey test \*P = 0.003 vs NS, #P =0.017 vs HS (n= 6/group).



B. Serum  $PGI_2$  (ng/l) was detected by ELISA kit, There was no significant difference among this three groups. Values are the mean  $\pm$  SEM, one-way ANOVA, Tukey test, P =0.559. All values are expressed as Mean  $\pm$  SEM.