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Inhibition of semicarbazide-sensitive amine oxidase reduces atherosclerosis in apolipoprotein E-deficient mice

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1 **Inhibition of Semicarbazide-sensitive Amine Oxidase Reduces Atherosclerosis in**
2 **Apolipoprotein E-deficient Mice**

3

4 **Running title:** Inhibition of SSAO Reduces Atherosclerosis

5

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16

17 **Key words:** vascular adhesion protein-1, semicarbazide-sensitive amine oxidase,
18 atherosclerosis, coronary artery disease

19

1 **Abbreviations**

- 2 AGEs = advanced glycated end-products; ALEs = advanced lipoxidation end-products;
- 3 VAP-1= vascular adhesion protein-1; SSAO = semicarbazide-sensitive amine oxidase;
- 4 CAD = coronary artery disease; ApoE = apolipoprotein E; SMC = smooth muscle
- 5 cells; ROS = reactive oxygen species; LDL = low-density lipoprotein; H₂O₂ =
- 6 hydrogen peroxide; advanced glycation end-products; LOX = lysyl oxidase; S1P =
- 7 sphingosine-1-phosphate; OGTT = oral 75-g glucose tolerance test; LDL-C =
- 8 low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol;
- 9 ALT = alanine transaminase; AST = aspartate aminotransferase; MCP-1 = monocyte
- 10 chemoattractant protein-1; TLR-4 = Toll-like receptor-4; RAGE = receptor for
- 11 advanced glycation end-products; MMP-9 = matrix metalloproteinase-9; VCAM-1 =
- 12 vascular cell adhesion molecule-1; ICAM-1 = intercellular adhesion molecule-1;
- 13 PCNA = proliferating cell nuclear antigen; TNF- α = tumor necrosis factor alpha;
- 14 LOX-1 = lectin-like oxidized low-density lipoprotein receptor-1; HUVEC = human
- 15 umbilical vein endothelial cells; ECM = endothelial Cell Medium; HUVEC
- 16 hSSAO/VAP-1 cells = HUVEC overexpressing VAP-1/SSAO; A7r5 hSSAO/VAP-1
- 17 cells = A7r5 cells overexpressing VAP-1/SSAO; LPS = lipopolysaccharide;
- 18 H₂DCF-DA = dichlorofluorescein diacetate; PDGF = platelet-derived growth factor;
- 19 BMI = body mass index

1 **Background**

2 Vascular adhesion protein-1 (VAP-1) participates in inflammation and has
3 semicarbazide-sensitive amine oxidase (SSAO) activity. However, the effect of
4 VAP-1/SSAO inhibition on atherosclerosis remains controversial.

5

6 **Translational Significance**

- 7 • Plasma VAP-1/SSAO concentrations are positively associated the presence
8 and the extent of coronary artery disease in humans.
- 9 • PXS-4728A, a specific VAP-1/SSAO inhibitor, can reduce atherosclerosis in
10 cholesterol-fed ApoE-deficient mice, through suppression of many key steps
11 for atherosclerosis, including reactive oxygen species generation, endothelial
12 dysfunction, adhesion and transmigration of monocytes, recruitment and
13 activation of macrophages, as well as migration and proliferation of SMC.
- 14 • VAP-1/SSAO inhibition is a potential treatment for atherosclerotic
15 cardiovascular disease.

16

17 **Abstract**

18 Inflammation, oxidative stress and formation of advanced glycated/lipoxidation
19 end-products (AGEs/ALEs) are important for atherosclerosis. Vascular adhesion

1 protein-1 (VAP-1) participates in inflammation and has semicarbazide-sensitive amine
2 oxidase (SSAO) activity, which catalyzes oxidative deamination to produce hydrogen
3 peroxide and aldehydes, leading to generation of AGEs/ALEs. However, the effect of
4 VAP-1/SSAO inhibition on atherosclerosis remains controversial, and no studies used
5 coronary angiography to evaluate if plasma VAP-1/SSAO is a biomarker for coronary
6 artery disease (CAD). Here, we examined if plasma VAP-1/SSAO is a biomarker for
7 CAD diagnosed by coronary angiography in humans and investigated the effect of
8 VAP-1/SSAO inhibition by a specific inhibitor PXS-4728A on atherosclerosis in cell
9 and animal models. In the study, VAP-1/SSAO expression was increased in plaques in
10 humans and apolipoprotein E (ApoE)-deficient mice, and colocalized with vascular
11 endothelial cells and smooth muscle cells (SMC). Patients with CAD had higher
12 plasma VAP-1/SSAO than those without CAD. Plasma VAP-1/SSAO was positively
13 associated with the extent of CAD. In ApoE-deficient mice, VAP-1/SSAO inhibition
14 reduced atheroma and decreased oxidative stress. VAP-1/SSAO inhibition attenuated
15 the expression of adhesion molecules, chemoattractant proteins, and pro-inflammatory
16 cytokines in aorta, and suppressed monocyte adhesion and transmigration across
17 human umbilical vein endothelial cells. Consequently, the expression of markers for
18 macrophage recruitment and activation in plaques was decreased by VAP-1/SSAO
19 inhibition. Besides, VAP-1/SSAO inhibition suppressed proliferation and migration of

1 A7r5 SMC. Our data suggest plasma VAP-1/SSAO is a novel biomarker for the
2 presence and extent of CAD in humans. VAP-1/SSAO inhibition by PXS-4728A is a
3 potential treatment for atherosclerosis.
4

Accepted Manuscript

1 **Introduction**

2 Atherosclerosis is the leading cause of death in developed countries.^{1,2} The
3 World Health Organization (WHO) estimated that 17.5 million people died from
4 cardiovascular diseases, representing 31% of all global deaths.³ Inflammation has
5 been shown to be another important mechanism for the progression of
6 atherosclerosis.^{4,5} Circulating leukocytes, especially monocytes, transmigrate from the
7 vasculature to the inflammation site and are activated. Various chemokines, cytokines,
8 enzymes, and reactive oxygen species (ROS) are produced and secreted, which may
9 modify low-density lipoprotein (LDL), propagate inflammation, and result in
10 atherosclerosis. Therefore, therapeutic agents able to suppress inflammation and
11 reduce ROS in the arterial wall are of great potential as new anti-atherosclerotic
12 drugs.

13 Vascular adhesion protein-1 (VAP-1) is an endothelial adhesion molecule
14 involved in leukocyte rolling, adhesion and transmigration into inflammatory sites.⁶
15 VAP-1 is expressed in endothelial cells, smooth muscle cells (SMC), and
16 adipocytes.^{7,8} Different from other adhesion molecules, VAP-1 has an enzymatic
17 function, called semicarbazide-sensitive amine oxidase (SSAO) [EC 1.4.3.21], which
18 catalyzes the oxidative deamination of primary amines into aldehydes (e.g.,
19 formaldehyde, methylglyoxal), hydrogen peroxide (H₂O₂), and ammonia.^{9,10} Under

1 enhanced oxidative stress, aldehydes can cross-link proteins and result in the
2 generation of advanced glycation/lipoxidation end-products (AGEs/ALEs), both of
3 which can induce endothelial injury and promote monocyte activation.¹¹⁻¹⁴ H₂O₂ is a
4 source of ROS, which can modify LDL in the arterial wall. Besides, both hydrogen
5 peroxide and ammonia are cytotoxic to vascular cells at high concentrations. In other
6 words, all these end-products of VAP-1/SSAO can result in the development of
7 atherosclerosis. Indeed, chronic administration of methylamine, a substrate of SSAO,
8 resulted in increased atheroma area in mice.¹⁵

9 On the other hand, there is a soluble form of VAP-1 that can be measured in
10 plasma. Soluble VAP-1 is derived from the transmembranous form of VAP-1 by
11 proteolytic cleavage.¹⁶⁻¹⁹ Soluble VAP-1 is upregulated and released in certain
12 inflammatory disorders, such as diabetes^{20,21} and hepatitis¹⁶ among others. In humans,
13 serum VAP-1 is associated with atherosclerosis in carotid arteries^{22,23} and the
14 incidence of major cardiovascular events²⁴. Moreover, serum VAP-1 can predict
15 cardiovascular mortality in both type 2 diabetic subjects²⁵ and general population²⁴.
16 Taken together, findings from these reports suggest that VAP-1/SSAO is involved in
17 atherogenesis and can be a novel target for the treatment of atherosclerosis.

18 So far, there are limited studies that investigate the effects of VAP-1/SSAO
19 inhibition on atherosclerosis.²⁶⁻²⁸ Two of them used semicarbazide to inhibit

1 VAP-1/SSAO activity in LDL receptor knock-out mice, with conflicting results. In
2 one report, semicarbazide aggravated atherosclerotic lesions.²⁷ However,
3 semicarbazide could stabilize the established atherosclerotic lesions in another
4 report.²⁶ Off-target effects of semicarbazide may be one of the reasons for the
5 inconsistent findings, since semicarbazide is not a specific inhibitor for VAP-1/SSAO
6 and also inhibits enzymes which are involved in atherogenesis, such as lysyl oxidase
7 (LOX) and sphingosine-1-phosphate (S1P) lyase.²⁹⁻³⁴ Besides, another study using a
8 small molecule VAP-1 inhibitor (LJP1586) for 4 weeks did not find a significant
9 regression of atherosclerosis.²⁸ However, a 4-week treatment length may not be long
10 enough so that it may underestimate the treatment efficacy.

11 PXS-4728A is a highly specific inhibitor of VAP-1/SSAO, and does not have any
12 significant off-target effects when tested against other amine oxidases or over 100
13 different macromolecular targets.³⁵ In this study, we investigated whether
14 VAP-1/SSAO inhibition reduced atherosclerosis using this specific inhibitor in
15 apolipoprotein E (ApoE)-deficient mice for 15 weeks. Then, we explored the potential
16 mechanisms in cell models. Although there were some studies which investigated the
17 role of plasma VAP-1/SSAO as a biomarker for atherosclerosis,^{21,22,24,36-38} none of
18 them used coronary angiography to confirm the presence and the extent of coronary
19 artery disease (CAD). Therefore, we conducted a human study, which examined the

1 association of plasma VAP-1/SSAO to the presence and the extent of CAD diagnosed
2 by coronary angiography.

3

4 **Materials and methods**

5 **▪ Human study**

6 *Patients*

7 From 2008 to 2009, we conducted a cross-sectional study at National Taiwan
8 University Hospital.³⁹ Subjects who were suspected to have CAD based on positive
9 results of noninvasive stress tests and who received selective coronary angiography
10 were invited to participate in this study. All subjects were evaluated by cardiologists.
11 They were asked to answer questionnaires and receive physical examinations and
12 blood tests, with the aid of trained nurses. The choice of noninvasive stress tests was
13 determined by the attending physician, such as treadmill electrocardiography and
14 thallium perfusion scanning. None of the study subjects took medications which may
15 affect the activity of serum SSAO or VAP-1.⁴⁰ Written informed consent was obtained
16 from each participant. The study protocol was approved by the institutional review
17 board and the study was performed in accordance with the Declaration of Helsinki of
18 Human Research.

19 *Measurements and assays*

1 Plasma samples were collected from each participant after overnight fasting and
2 then stored at -80°C before the measurement of VAP-1/SSAO concentration. A
3 standard oral 75-g glucose tolerance test (OGTT) was performed to measure 2-h
4 postprandial plasma glucose. Plasma glucose and lipid profiles were measured with an
5 automatic analyzer (Toshiba TBA 120FR, Toshiba Medical Systems Co., Ltd., Tokyo,
6 Japan). Plasma VAP-1/SSAO concentration was measured by a quantitative sandwich
7 enzyme immunoassay (R&D Systems, Minneapolis, USA). The assay utilized a
8 specific monoclonal antibody against VAP-1 as a capturer on a microplate. Detection
9 of bound VAP-1 was performed using a different polyclonal antibody specific for
10 VAP-1 conjugated to horseradish peroxidase. A substrate solution was added and the
11 intensity of the color was measured, which was in proportion to the amount of plasma
12 VAP-1 bound in the initial step. Plasma VAP-1/SSAO concentration was quantified on
13 the basis of a reference sample of highly purified recombinant human plasma
14 VAP-1/SSAO. The intra-assay and inter-assay coefficients of variation were 1.90%
15 and 3.58%, respectively.

16 ***Definition***

17 CAD was determined by coronary angiography and defined as the presence of at
18 least one stenosis of $\geq 50\%$ diameter in any of the major epicardial coronary arteries.⁴¹
19 The extent of CAD was assessed based on the number of atherosclerotic segments and

1 the number of major epicardial coronary arteries with $\geq 50\%$ stenosis. Diabetes was
2 diagnosed if fasting plasma glucose ≥ 126 mg/dL, 2-h postprandial plasma glucose
3 ≥ 200 mg/dL, hemoglobin A1c $\geq 6.5\%$ or the patient used pharmacological treatment
4 for diabetes.⁴² Hypertension was defined if systolic blood pressure ≥ 140 mmHg,
5 diastolic blood pressure ≥ 90 mmHg, or the patient used antihypertensive drugs.⁴³

6
7 **▪ In vivo and in vitro study**

8 ***Animal groups and treatment***

9 Male 8-week ApoE-deficient mice were purchased from Animal Resources
10 Centre (Canning Vale, WA, Australia) and kept under a C57/BL6 background. All
11 procedures were performed in accordance with the local institutional guidelines for
12 animal care of the National Taiwan University and complied with the “Guide for the
13 Care and Use of Laboratory Animals” NIH publication No. 86-23, revised 2011. The
14 protocol was approved by the National Taiwan University College of Medicine and
15 College of Public Health Institutional Animal Care and Use Committee (IACUC NO:
16 20130274). Animals were randomly distributed into five groups: Group I (Control),
17 fed with standard chow diet (n=12); Group II (Cholesterol Diet), fed with
18 cholesterol-enriched diet (Purina Mills, Inc., USA) containing 21% fat (12%
19 saturated fat) and 0.15% cholesterol for 15 weeks (n=12); Group III (Cholesterol

1 Diet/PXS-4728A, the prevention group), fed with cholesterol-enriched diet and
2 PXS-4728A (10 mg/kg/day in 50 μ l of 5 mM Na₂HPO₄ solution, Pharmaxis,
3 Australia) orally for 15 weeks (n=12); Group IV (Cholesterol Diet/PXS-4728A 7W,
4 the treatment group), fed with cholesterol-enriched diet for 15 weeks and PXS-4728A
5 (10 mg/kg/day) orally from 9-15 weeks (n=12); Group V (Cholesterol
6 Diet/atorvastatin, the positive therapeutic control group), fed with
7 cholesterol-enriched diet and atorvastatin (2.5 mg/kg/day, Pfizer, USA) orally for 15
8 weeks (n=12). Fasting blood samples were collected one day before the experiment
9 commencement (0 time) and at the end of the experiment (after 15 weeks) for
10 measurement of plasma triglycerides, total cholesterol, low-density lipoprotein
11 cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), glucose,
12 creatinine, alanine transaminase (ALT), and aspartate aminotransferase (AST)
13 (Randox Laboratories, Ltd., UK). After 15 weeks, mice were anaesthetized with
14 pentobarbital (150mg/kg i.p.). The aortic sinus and thoracic aorta were gently
15 removed and the adipose tissue peeled off the aortic wall. Subsequently, the aortic
16 sinus and thoracic aorta were fixed with 4% paraformaldehyde solution before
17 cryosection.

18 ***SSAO activity analysis***

19 SSAO activity was determined as a result of the turnover of hydrogen peroxide,

1 which was measured by Amplex Red Monoamine Oxidase Assay Kit (A12214,
2 Molecular Probes, Eugene, OR, USA). Briefly, 100 μ g of protein was incubated at
3 room temperature with clorgyline 1 μ M (monoamine oxidase-A inhibitor), pargyline 3
4 μ M (monoamine oxidase-B inhibitor), benzylamine 2 mM (substrate of SSAO), 100
5 μ M Amplex Red reagent, and 1 U/ml HRP, with or without semicarbazide 1mM.
6 Absorbance at 570 nm was measured every 5 minutes for 30 minutes. A standard
7 curve was plotted using different solutions with known hydrogen peroxide
8 concentrations. Production rate of hydrogen peroxide is calculated and expressed as
9 [H₂O₂]/min/ μ g protein. SSAO activity was determined by the difference between the
10 production rates of hydrogen peroxide with and without semicarbazide. The linearity
11 (R²) of this assay is 0.9989~1.0000 and the intra- or inter-assay CV is 0.5~3.8% in
12 our lab.

13 *Oil Red O staining*

14 Atherosclerotic plaque development was quantified by histomorphometry of Oil
15 Red O-stained aortic sinus and thoracic aorta. Aortic sinus and thoracic aorta stained
16 with Oil Red O were performed according to a standard protocol. Briefly, after being
17 rinsed with water, the aorta was exposed to isopropanol (60%) for 2 min, followed by
18 1 h of staining with a solution of Oil Red O dissolved in 60% isopropanol. The excess
19 stain was removed with isopropanol and water, and the aorta was photographed using

1 a microscope equipped with a digital camera (Leica). The Oil Red-stained areas
2 representing atherosclerotic plaques were measured using Image Pro Plus. The results
3 were expressed as the ratio of total plaque area over total vessel area. The aortic sinus
4 and thoracic aorta were fixed in 4% paraformaldehyde, embedded in OCT, and
5 cross-sectioned for morphometric analysis and immunohistochemistry. The aortic
6 sinus and thoracic aorta were cut serially into 8 μm frozen sections, and every tenth
7 section was stained with Oil Red O for lesion area calculation. The analyses were
8 performed microscopically; the images were analyzed with Image Pro Plus.

9 ***Evaluation of atherosclerotic lesions and immunohistochemical staining***

10 For the pathomorphological examination, 8 μm -thick serial sections were
11 collected throughout the length of each segment. All sections were
12 immunohistochemically stained with CD31 (1:200, endothelial marker, Abcam,
13 Cambridge, UK), α -actin (1:200, SMC marker, GeneTex, Irvine, California), Iba-1
14 (1:500, macrophage marker, Wako, Richmond, VA), monocyte chemoattractant
15 protein-1 (MCP-1) (1:100, Pepotech, Rocky Hill, NJ), Toll-like receptor-4 (TLR-4)
16 (1:100, Proteintech, Chicago, IL, USA), CD36 (1:100, Abcam, Cambridge, UK),
17 receptor for advanced glycation end-products (RAGE) (1:100, Abbiotec, San Diego,
18 CA), matrix metalloproteinase-9 (MMP-9), VAP-1, vascular cell adhesion molecule-1
19 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, proliferating cell

1 nuclear antigen (PCNA) (1:100, all from Santa Cruz, Delaware Avenue, CA), tumor
2 necrosis factor alpha (TNF- α), lectin-like oxidized low-density lipoprotein receptor-1
3 (LOX-1) (1:100, all from GeneTex, Irvine, California), nitrotyrosine (1:100, upstate
4 Biotechnology, Charlottesville, VA) and then reacted with rhodamine- and
5 FITC-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, USA), and
6 observed by fluorescence microscopy.

7 ***Western blot detection***

8 The cells were lysed with RIPA lysis buffer (Cell Signaling, Beverly, MA, USA).
9 A total of 20 μ g of protein was separated by 10-12% SDS-PAGE and transferred onto
10 PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5% BSA at
11 room temperature for 1 h, the membranes were incubated with primary antibodies (all
12 1:1000 in 1.5% BSA in TBST) at 4°C overnight. The membranes were then incubated
13 with HRP-conjugated secondary antibodies (1:6000 in 1.5% BSA in TBST) at room
14 temperature for 1 h. Immunoreactivity was detected with ECL (GE Healthcare
15 Bioscience, Piscataway, NJ, USA). The intensities of the bands were quantified using
16 Gel-Pro software (Media Cybernetics, Rockville, MD, USA). β -actin or GAPDH was
17 used as an internal control (1:6000 in 1.5% BSA in TBST; GeneTex, Irvine,
18 California). The intensities of the target proteins were normalized by the intensities of
19 the internal control bands.

1 ***Tissue adhesion assay***

2 The cultured U937 cells were stained with the BCECF-AM. The stained U937
3 cells were added to the artery for 30 min at 37 °C. They were then washed and
4 observed by fluorescence microscopy.

5 ***Cell cultures***

6 A7r5, purchased as cryopreserved tertiary cultures (Cascade Biologics Inc., Portland,
7 OR, USA), were grown in DMEM containing 10% FBS and 1% antibiotic/antimycotic
8 solution at 37°C in a 5% CO₂ atmosphere. The growth medium was changed every other
9 day until confluence, and then the cells were passaged every 3-5 days. Cells between
10 passages 3 and 8 were used for the subsequent experiments. Before conducting
11 experiments, A7r5 were pre-cultured in serum-starved medium for 24 h. Human umbilical
12 vein endothelial cells (HUVEC) were obtained from the Bioresource Collection and
13 Research Center (BCRC). The cells were grown in Endothelial Cell Medium (ECM)
14 (Lonza, Walkersville, MD, USA) containing penicillin-streptomycin (1%) and endothelial
15 cell growth supplements at 37°C in a humidified atmosphere of 95% air and 5% CO₂ and
16 were used between passages 2 and 5. HUVEC overexpressing VAP-1/SSAO (HUVEC
17 hSSAO/VAP-1 cells) and A7r5 cells overexpressing VAP-1/SSAO (A7r5 hSSAO/VAP-1
18 cells) were developed by Dr. Mercedes Unzeta and Dr. Montse Solé (Universitat
19 Autònoma de Barcelona (UAB), Barcelona, Spain). The details were described in previous

1 publications.^{44,45} HUVEC hSSAO/VAP-1 and A7r5 hSSAO/VAP-1 cells were cultured
2 similarly ways as described for HUVEC and A7r5 cells. U937 cells were obtained from
3 ATCC (American Type Culture Collection). Cells were grown in RPMI-1640 medium
4 (GIBCO) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100
5 Ag/ml streptomycin at 37°C in an incubator containing 5% CO₂.

6 ***ROS detection***

7 Cells were grown in 6 cm culture dishes. After the cells were pre-treated with
8 300 nM PXS-4728A for 1 h, and 1 µg/mL of lipopolysaccharide (LPS) or 10 ng/mL
9 TNF-α for another 1 h, the cells were stained with dichlorofluorescein diacetate
10 (H₂DCF-DA) and then were trypsinized for FACScan cytometer analysis.

11 ***Monocyte adhesion assay***

12 HUVEC hSSAO/VAP-1 cells were grown in 24-well culture plates. After reaching
13 confluence, cells were pre-treated with 300 nM PXS-4728A or
14 N-acetyl-L-cysteine (NAC) (5, 10 mM) for 1 h, and 10 ng/mL TNF-α treatment for
15 another 24 h. After treatment, the U937 cells were added to treated cells for 60 min at
16 37 °C. They were then washed and observed under an inverted phase-contrast
17 microscope.

18 ***Monocyte transmigration assay***

19 HUVEC hSSAO/VAP-1 cells were grown in transwells. After reaching

1 confluence, cells were pre-treated with 300 nM PXS-4728A for 1 h, and 10 ng/mL
2 TNF- α treatment for another 24 h. After treatment, the U937 cells were added into
3 transwells for another 6 h. The transmigrated cells were determined after 6 h by
4 Coomassie blue staining and count.

5 ***Cell cycle analysis***

6 A7r5 hSSAO/VAP-1 cells were grown in 6-well culture plates and then
7 serum-starved as described above. After pretreatment with 300 nM PXS-4728A or NAC
8 (1, 2 mM) for 1 h and 20 ng/mL platelet-derived growth factor (PDGF)-BB for another 24
9 h, the cells were trypsinized and stained with propidium iodide (PI). The cell-cycle
10 progression was detected on a FACScan cytometer (BD Biosciences, San Jose, CA) and
11 was analyzed by ModFit software.

12 ***Wound-healing assay***

13 A7r5 hSSAO/VAP-1 cells were grown in 6 cm culture dishes. After the cells
14 were pre-treated with 300 nM PXS-4728A or NAC (1, 2 mM) for 1 h, wounds were
15 inflicted by dragging a sterile pipette tip across the monolayer, creating a 250 μ m
16 cell-free path after which 20 ng/mL PDGF-BB (Peprotech, Rocky Hill, NJ, USA) was
17 added. After 24 h, the migration rate of the cells and the wound closure area/original
18 wound area ratio were calculated using a real-time cultured cell monitoring system
19 (ASTECC, Japan) and Metamorph and Image-Pro Plus 4.5 software.

1 *Statistical analyses*

2 Continuous variables with normal distribution were presented as means \pm SD or
3 means \pm SEM. Variables with skewed distribution were presented as medians
4 (interquartile ranges) and were analyzed after logarithmic transformation. The
5 statistical significance of the differences in different subgroups was tested with
6 Student's *t* test or χ^2 test. Unadjusted Pearson's correlation coefficients and partial
7 correlation coefficients adjusting for age and gender were used to determine the
8 relationship of plasma VAP-1/SSAO with cardiovascular risk factors, the number of
9 vessels with stenosis and the number of segments with stenosis. The association
10 between CAD and plasma VAP-1/SSAO was evaluated by logistic regression;
11 whereas the association between the number of vessels with stenosis, the number of
12 segments with stenosis, and plasma VAP-1/SSAO was evaluated by linear regression
13 analyses. Clinically important variables associated with CAD were included in
14 multivariate analyses, including age, gender, hypertension, diabetes, smoking and
15 LDL-C. A two-tailed *p*-value below 0.05 was considered significant. Stata/SE 14.0 for
16 Windows (StataCorp LP, College Station, TX) was used for statistical analyses.

17

18 **Results**

19 *Expression of VAP-1/SSAO in atherosclerotic lesions in human and ApoE-deficient*

1 *mice*

2 To examine VAP-1/SSAO expression during atherosclerosis in humans and mice,
3 immunohistochemical staining was performed with antibodies against VAP-1/SSAO.
4 In the human normal coronary artery, little VAP-1/SSAO expression was seen (Figure
5 1A). Compared to normal artery, VAP-1/SSAO expression was significantly stronger
6 in the atherosclerotic plaques. To examine the cellular localization of VAP-1/SSAO
7 during the formation of atherosclerosis in humans and ApoE-deficient mice,
8 immunohistochemical staining with antibodies against VAP-1/SSAO, endothelial
9 cells, or SMC was carried out in human coronary artery and the mice aorta sections
10 (Figure 1A and 1B). VAP-1/SSAO staining overlaid with markers staining for
11 endothelial cells and SMC (Figure 1A and 1B), thus confirming that endothelial cells
12 and SMC in atherosclerotic plaques express VAP-1/SSAO. In Supplemental Figure 1,
13 VAP-1/SSAO expression in thoracic aorta increased by time after
14 cholesterol-enriched diet was given. However, there was no significant difference in
15 plasma VAP-1/SSAO concentrations at different time after cholesterol-enriched diet
16 was given (means \pm standard deviations, at day 0, 1.007 ± 0.7418 ng/ml; at the 9th
17 week, 1.972 ± 1.726 ng/ml; at the 12th week, 3.276 ± 2.330 ng/ml; at the 15th week,
18 1.639 ± 0.613 ng/ml, $p > 0.05$).

19

1 ***Plasma VAP-1/SSAO concentrations are positively associated with the presence and***
2 ***the extent of CAD in humans***

3 Baseline characteristics of the subjects enrolled in the human study are shown in
4 Table 1. A total of 127 (70%) subjects were diagnosed with CAD. Compared to those
5 without CAD, subjects with CAD were more likely to be male, had a higher
6 prevalence to use statins, and had lower plasma HDL-C. There were no significant
7 differences between the two groups regarding age, smoking, body mass index (BMI),
8 blood pressure, history of hypertension or diabetes, plasma concentrations of fasting
9 and 2-h postprandial glucose, hemoglobin A1c, total cholesterol, triglyceride, and
10 LDL-C. Notably, plasma VAP-1/SSAO concentrations were significantly higher in
11 subjects with CAD than in the control group (median 579 [interquartile range 490-710]
12 ng/ml vs. 544 [460-644] ng/ml, $p=0.003$, Table 1 and Figure 2A). There was a
13 positive association between plasma VAP-1/SSAO concentrations, the number of
14 coronary arteries with stenosis (p for trend <0.001 , Figure 2B), and the number of
15 coronary arterial segments with stenosis (p for trend $=0.001$, Figure 2C).

16 Table 2 showed the relationships between plasma VAP-1/SSAO concentrations
17 and clinical characteristics. In univariate analysis, plasma VAP-1/SSAO positively
18 correlated with age ($r=0.1485$, $p=0.047$), fasting plasma glucose ($r=0.2349$,
19 $p=0.0015$), 2-h postprandial plasma glucose ($r=0.3435$, $p<0.0001$), the number of

1 coronary arteries with stenosis ($r=0.2569$, $p=0.0005$), and the number of coronary
2 arterial segments with stenosis ($r=0.2505$, $p=0.0007$). The relationships remained
3 significant after controlling for age and gender. There was a significant correlation
4 between plasma VAP-1/SSAO and plasma triglyceride concentrations ($r=0.1731$,
5 $p=0.021$), adjusted for age and gender. Besides, plasma VAP-1/SSAO concentrations
6 were similar between subjects receiving statins and subjects who did not receive
7 statins (median 570 [interquartile range 475-674] ng/ml vs. 578 [489-652] ng/ml,
8 $p=0.99$).

9 To determine whether plasma VAP-1/SSAO was an independent predictor of the
10 presence and the extent of CAD, we performed logistic and linear regression analyses
11 (Table 3). Plasma VAP-1/SSAO concentrations were associated with the presence of
12 CAD (odds ratio=2.09, 95% CI 1.29-3.38, $p=0.003$, adjusted for age and gender).
13 There was a positive association between plasma VAP-1/SSAO concentrations and the
14 extent of CAD, as measured by the number of coronary arteries with stenosis ($\beta=0.32$,
15 $p<0.001$) and the number of coronary arterial segments with stenosis ($\beta=0.62$,
16 $p<0.001$). The relationship of plasma VAP-1/SSAO concentrations to the presence and
17 the extent of CAD remained significant after further adjustment for history of
18 hypertension, diabetes, plasma LDL-C and smoking.

19

1 ***PXS-4728A inhibits VAP-1/SSAO activity and the consequent H₂O₂ generation and***
2 ***oxidative stress in cholesterol-fed ApoE-deficient mice***

3 To detect the effects of PXS-4728A treatment on VAP-1/SSAO activity, we
4 measured SSAO-specific H₂O₂ production rate in cholesterol-fed ApoE-deficient
5 mice. PXS-4728A significantly inhibited VAP-1/SSAO activity and SSAO-specific
6 H₂O₂ production in thoracic aortic tissues, lung, and epididymal fat (Figure 3A-C).
7 The oxidative stress expression was determined by nitrotyrosine staining. The result
8 showed that the oxidative stress expression was decreased significantly by
9 PXS-4728A treatment (Figure 3D).

10

11 ***SSAO inhibition by PXS-4728A reduces atherosclerosis in cholesterol-fed***
12 ***ApoE-deficient mice***

13 The effects of PXS-4728A on the atherosclerotic plaque area were quantified by
14 histomorphometric analysis of the aortic sinus and thoracic aortic sections in
15 ApoE-deficient mice 15 weeks after cholesterol-enriched diet. The atherosclerotic
16 plaque area was determined by Oil Red O staining analysis (Figure 3E-F). The ratios
17 of atherosclerotic plaque area to total area of the aortic sinus and thoracic aorta were
18 significantly higher in the cholesterol-fed group than in the control group. Compared
19 with the cholesterol-fed group, PXS-4728A significantly reduced the ratio of

1 atherosclerotic plaque area to total area, both in the prevention group and in the
2 treatment group ($51.69\% \pm 2.27\%$ in the cholesterol-fed group; $36.05\% \pm 3.93\%$ in
3 the prevention group [PXS-4728A given along with cholesterol-enriched diet for 15
4 weeks], $p < 0.05$ vs. cholesterol-fed group; $36.65\% \pm 4.29\%$ in the treatment group
5 [cholesterol-enriched diet was given for 8 weeks followed by cholesterol-enriched
6 diet plus PXS-4728A for another 7 weeks] , $p < 0.05$ vs. cholesterol-fed group). Similar
7 data were observed in the ratios of atherosclerotic plaque area to total area of thoracic
8 aorta. Importantly, the effect of VAP-1/SSAO inhibition in the prevention group was
9 similar to the effect of atorvastatin (2.5 mg/kg/day for 15 weeks), the treatment of
10 choice for primary and secondary prevention for cardiovascular diseases in humans.

11

12 ***Effects of VAP-1/SSAO inhibition by PXS-4728A on serum biochemical parameters***
13 ***of cholesterol-fed ApoE-deficient mice***

14 As shown in Table 4, plasma total cholesterol, LDL-C, triglyceride and glucose
15 concentrations were significantly increased in the cholesterol-fed group, compared
16 with the control group ($p < 0.05$). The elevation of plasma total cholesterol, LDL-C,
17 and glucose concentrations were significantly reduced by PXS-4728A (the prevention
18 group) or atorvastatin (all $p < 0.05$). In the PXS-4728A 7W group (the treatment group),
19 plasma glucose concentrations decreased significantly, but plasma total cholesterol

1 and LDL-C were not significantly different from that in the cholesterol-fed group. In

2 all groups, there was no significant difference in plasma creatinine, AST and ALT.

3

4 ***SSAO inhibition by PXS-4728A reduces the expression of adhesion molecules and***
5 ***inflammatory cytokines in atherosclerotic plaques***

6 To test the effects of PXS-4728A on inflammation, immunohistochemical

7 staining and western blot with antibodies against adhesion molecules and

8 inflammatory cytokines were carried out on thoracic aorta sections. The expression of

9 adhesion molecules, including VAP-1, vascular cell adhesion molecule-1 (VCAM-1),

10 intercellular adhesion molecule-1 (ICAM-1) and E-selectin, were higher in thoracic

11 aorta of ApoE-deficient mice in the cholesterol-fed group than that in the control

12 group (Figure 4A and Supplemental Figure 2). VAP-1/SSAO inhibition by

13 PXS-4728A (both the prevention and the treatment group) significantly decreased the

14 expression of these adhesion molecules, compared with the cholesterol-fed group

15 (Figure 4A and Supplemental Figure 2).

16 Since leukocyte recruitment plays an important role in the initiation and

17 progression of atherosclerosis,^{4,5} we used labeled-U937 cells to directly test whether

18 VAP-1/SSAO inhibition can inhibit early monocyte recruitment into atherosclerotic

19 lesions. In Figure 4B-C, the number of labeled-U937 cells on artery was higher in the

1 cholesterol-fed group than that in the control group (Figure 4B-C). PXS-4728A (both
2 in the prevention and the treatment groups) significantly decreased the number of
3 monocytes adhered to the arteries, compared with the number in the cholesterol-fed
4 group. In Figure 4D and Supplemental Figure 2, the expression of pro-inflammatory
5 molecules TNF- α and MCP-1 were higher in the cholesterol-fed group and were
6 reduced by PXS-4728A (both in the prevention and the treatment groups). All
7 together, the results suggest that, VAP-1/SSAO inhibition by PXS-4728A reduce
8 inflammation and leukocyte recruitment to atherosclerotic lesions in cholesterol-fed
9 ApoE-deficient mice.

10

11 ***SSAO inhibition by PXS-4728A reduces the expression of markers for macrophage***
12 ***recruitment and activation in atherosclerotic plaques***

13 TLR-4, CD36 and LOX-1 activation by oxidized LDL (oxLDL) contribute to the
14 pathogenesis of atherosclerosis.⁴⁶ During the development of atherosclerosis,
15 macrophages are recruited to plaques, where they are activated by AGEs and
16 oxLDL.⁴⁷ Activated macrophages form foam cells, which consequently propagate
17 inflammation and progresses to atherosclerosis.^{48,49} To test the effect of VAP-1/SSAO
18 inhibition by PXS-4728A on AGEs-LDL-activated macrophages in the atherosclerotic
19 plaques, immunohistochemical staining and western blot with antibodies against Iba-1

1 (a macrophage marker), TLR-4, CD36, RAGE and LOX-1 were performed in
2 ApoE-deficient mice thoracic aorta sections. As shown in Figure 4E and
3 Supplemental Figure 2, the cholesterol-enriched diet resulted in a significant increase
4 in the recruitment of macrophages and the expression of TLR-4, CD36, RAGE and
5 LOX-1 in the atherosclerotic plaques. These effects were markedly decreased by
6 PXS-4728A, except CD36 and LOX-1 expression in the cholesterol-fed/PXS7W
7 group. All together, these data showed that VAP-1/SSAO inhibition by PXS-4728A
8 reduces the expression of markers for macrophage recruitment and activation in
9 atherosclerotic plaques.

10

11 ***SSAO inhibition by PXS-4728A reduces the adhesion and the transmigration of***
12 ***monocytes in TNF- α -treated HUVEC overexpressing hSSAO/VAP-1 by inhibiting***
13 ***ROS production***

14 In order to elucidate the role of VAP-1/SSAO in endothelial cells on
15 atherosclerosis, HUVEC hSSAO/VAP-1 cells were used. In Figure 5A-B, HUVEC
16 hSSAO/VAP-1 cells expressed more VAP-1/SSAO protein than HUVEC, as
17 determined by western blot and immunofluorescence analysis. The VAP-1/SSAO
18 activity of HUVEC hSSAO/VAP-1 cells was inhibited completely by PXS-4728A
19 treatment (Figure 5C).

1 Since TNF- α -induced endothelial dysfunction and ROS production play a pivotal
2 role in the pathogenesis of atherosclerosis,⁵⁰ the effect of VAP-1/SSAO inhibition was
3 also tested in the presence of TNF- α stimulation. As shown in Figure 5D, the
4 accumulation of intracellular ROS was increased by TNF- α treatment, and the
5 increased effect was significantly inhibited by PXS-4728A treatment, suggesting that
6 VAP-1/SSAO inhibition can reduce TNF- α -induced ROS production. During
7 inflammation, monocytes are adhered in the vascular wall and then transmigrate into
8 subendothelial space.⁵¹ Therefore, we evaluated the effect of VAP-1/SSAO inhibition
9 on the adhesion and transmigration of monocytes. As shown in Figure 5E and F, the
10 number of adhered and transmigrated monocytes were increased by TNF- α treatment.
11 PXS-4728A pre-treatment significantly reduced the number of monocytes adhered to
12 and transmigrated across TNF- α -treated HUVEC hSSAO/VAP-1 cells. Besides,
13 pretreatment with NAC, a ROS scavenger, also reduced the number of adhered and
14 transmigrated monocytes. Taken together, these findings suggest that VAP-1/SSAO
15 inhibition by PXS-4728A reduced the adhesion and transmigration of monocytes in
16 TNF- α -treated HUVEC hSSAO/VAP-1 cells by inhibiting the production of ROS.

17

18 ***SSAO inhibition by PXS-4728A reduces the expression of MMP-9, SMC, and***

19 ***PCNA in atherosclerotic plaques***

1 The proliferation and migration of vascular SMC play an important role in the
2 progression of atherosclerosis. SMC migrate from the tunica media into the intima via
3 degradation of the extracellular matrix mediated by MMP-9 and other proteinases.^{52,53}
4 In Figure 4F and Supplemental Figure 2, analysis of MMP-9 and the SMC marker,
5 α -actin, revealed stronger MMP-9 expression and more α -actin-positive cells in the
6 thickened intima of the cholesterol-fed group as compared to the intima of the control
7 group. These effects were significantly reduced by PXS-4728A treatment. To detect
8 cell proliferation in the atherosclerotic plaque, PCNA staining was used. As shown in
9 Figure 4F and Supplemental Figure 2, the expression and the number of
10 PCNA-positive cells in the thickened plaques were higher in the cholesterol-fed group
11 than that in the control group and in PXS-4728A groups. Taken together, these
12 findings suggest that VAP-1/SSAO inhibition by PXS-4728A reduces the expression
13 of markers for SMC migration and proliferation in the development of atherosclerosis.

14
15 ***SSAO inhibition by PXS-4728A reduces the cell proliferation and migration in***
16 ***A7r5 cells overexpressing hSSAO/VAP-1 by inhibiting ROS production***

17 In order to elucidate the role of VAP-1/SSAO in SMC on atherosclerosis, A7r5
18 hSSAO/VAP-1 cells were used. In Figure 6A-C, A7r5 hSSAO/VAP-1 cells expressed
19 more VAP-1/SSAO protein than A7r5 cells, and had VAP-1/SSAO activity. Treatment

1 with PXS-4728A inhibited the VAP-1/SSAO activity of A7r5 hSSAO/VAP-1 cells
2 completely (Figure 6C).

3 ROS have been reported to accelerate the proliferation and migration of vascular
4 SMC.⁵⁴ As shown in Figure 6D, LPS treatment led to intracellular ROS accumulation
5 in A7r5 hSSAO/VAP-1 cells, which was significantly reduced when cells were treated
6 with PXS-4728A. The proliferation and migration of vascular SMC are important in
7 the progression of atherosclerosis.⁵⁵ First, we measured the effects of PXS-4728A on
8 SMC proliferation by flow cytometry. As shown in Figure 6E, following PDGF-BB
9 stimulation, the proportion of A7r5 hSSAO/VAP-1 cells in S phase increased from
10 $7.72 \pm 0.43\%$ to $12.79 \pm 0.65\%$ ($p < 0.05$), which was prevented by PXS-4728A
11 pre-treatment ($12.79 \pm 0.65\%$ vs. $8.45 \pm 0.25\%$, $p < 0.05$) and accompanied by a
12 significant increase in the proportion of cells in G0/G1 phase (from $73.33 \pm 2.19\%$ to
13 $78.05 \pm 1.33\%$, $p < 0.05$). These data indicate that PXS-4728A suppresses the
14 proliferation of PDGF-BB-treated A7r5 hSSAO/VAP-1 cells via G0/G1 arrest.
15 Besides, pre-treatment with ROS scavenger NAC also reduced PDGF-BB-stimulated
16 proliferation of A7r5 hSSAO/VAP-1 cells. For SMC migration, wound-healing assays
17 were done. As shown in Figure 6F, the migration of A7r5 hSSAO/VAP-1 cells
18 increased significantly after stimulation with PDGF-BB. PXS-4728A or NAC
19 treatment reduced migration rate of PDGF-BB-treated A7r5 hSSAO/VAP-1 cells

1 significantly. Taken together, these findings indicate that VAP-1/SSAO inhibition by
2 PXS-4728A reduces the proliferation and migration of PDGF-BB-treated A7r5
3 hSSAO/VAP-1 cells by inhibiting ROS production.

4

5 **Discussion**

6 In the present study, we have demonstrated that plasma VAP-1/SSAO
7 concentrations were higher in subjects with CAD and were positively associated with
8 the number of coronary arteries or coronary arterial segments with stenosis in humans.
9 The findings are different from the results in a study by Salmi *et al.*,³⁷ who did not
10 found any significant association between serum VAP-1/SSAO and CAD. Since the
11 diagnosis of CAD in the study was established by self-reporting and verified with
12 drug reimbursement records, but not by coronary angiography as in the present study,
13 the possible ascertainment bias may reduce the statistical power. Aside from CAD,
14 there are several articles which report a significant relationship between serum
15 VAP-1/SSAO and atherosclerosis in carotid arteries in humans. Serum VAP-1/SSAO
16 activity has been reported to be associated with carotid intima-medial thickness and
17 plaques in women²² and in diabetic subjects.³⁶ In general population, we have
18 demonstrated that acute change of serum VAP-1/SSAO to hyperglycemia was
19 correlated to blood AGEs concentrations and carotid intima-medial thickness.²³

1 Furthermore, we have reported that serum VAP-1/SSAO can predict cardiovascular
2 mortality and all-cause mortality in subjects with diabetes;²⁵ whereas in subjects older
3 than 50 years, a Finnish study has shown that serum VAP-1/SSAO can predict the
4 incidence of major cardiovascular events and cardiovascular mortality.²⁴ All these
5 results suggest that blood VAP-1/SSAO is a biomarker for atherosclerosis and can be
6 used to predict cardiovascular events and mortality.

7 A recent study supports our findings that endothelial cells in atherosclerotic
8 plaques displayed increased VAP-1 expression compared to normal artery, and
9 VAP-1/SSAO inhibition reduced the macrophage recruitment in atherosclerotic
10 plaques.²⁸ In LDL receptor-deficient mice expressing only apolipoprotein B100
11 (LDLR^{-/-} ApoB^{100/100}), VAP-1 was expressed on the luminal surface of endothelial
12 cells in atherosclerotic plaques. However, endothelial cells lining non-atherosclerotic
13 vessel wall were VAP-1 negative. When a small molecular VAP-1 inhibitor (LJP1586)
14 was given after introducing high-fat diet for 8 weeks, it reduced the density of
15 macrophages in plaques. In contrast to our study, there was no significant difference
16 in the size of plaques after LJP1586 treatment for 4 weeks. This discrepancy may be
17 explained by a shorter treatment length (4 weeks), which may not be long enough to
18 observe the significant regression of atherosclerosis. Besides, mice were shifted to a
19 normal chow diet during LJP1586 treatment, which may slow down the progression

1 of atherosclerosis. Therefore, the effect of LJP1586 on atherosclerosis was minimized,
2 so that no significant difference in the size of plaques was found between the
3 treatment and control groups.

4 RAGE is a member of immunoglobulin superfamily that is able to bind diverse
5 molecules such as AGEs, ALEs and high mobility group box 1 (HMGB1), and
6 function as a signaling transduction receptor.⁵⁶ The interaction of RAGE and its
7 ligands can induce the release of inflammatory cytokines, increase expressions of
8 adhesion molecules, and promote SMC proliferation, thereby contributing to
9 atherogenesis.⁵⁷ RAGE inactivation has been shown to inhibit atherosclerosis through
10 reducing oxLDL-induced pro-inflammatory responses and oxidative stress in LDL
11 receptor-deficient mice.⁵⁸ In the present study, VAP-1/SSAO inhibition decreased
12 H₂O₂ production and the expression of RAGE in the vessel walls, suggesting two
13 possible mechanisms whereby VAP-1/SSAO inhibition reduces atherosclerosis.

14 In the present study, plasma VAP-1/SSAO concentrations correlated positively
15 with plasma fasting glucose and 2-h postprandial glucose in humans (Table 2), and
16 VAP-1/SSAO inhibition by PXS-4728A significantly lowered fasting plasma glucose
17 in ApoE-deficient mice (Table 4). In the literature, serum VAP-1/SSAO activity has
18 been reported to correlate with plasma glucose concentrations in humans.^{20,21,37}
19 Besides, a population study found an association of serum VAP-1/SSAO activity with

1 type 1 diabetes in both sexes and with type 2 diabetes in men.²² There are two
2 possible mechanisms linking VAP-1/SSAO and glucose homeostasis. First, vascular
3 VAP-1/SSAO participates in inflammation of adipose tissue. Adipose macrophages
4 can secrete various cytokines, such as $\text{TNF}\alpha$, $\text{IL-1}\beta$ and $\text{IFN}\gamma$, which results in insulin
5 resistance in adipose tissue and promotes lipolysis.⁵⁹ Increased circulating fatty acids
6 may lead to ectopic lipid accumulation and insulin resistance in liver and skeletal
7 muscle through the generation of diacylglyceride and protein kinase C. In
8 VAP-1/SSAO knockout mice, leukocyte infiltration in adipose tissue was reduced.⁶⁰
9 Besides, these mice had an increased fat mass, suggesting that their fat storage
10 capacity was increased and may be more resistant to insulin resistance resulting from
11 ectopic lipid accumulation. In contrast, adipose VAP-1/SSAO may play a beneficial
12 role by enhancing glucose uptake induced by amines, which has been shown in the
13 experiments using fat cells from VAP-1/SSAO knockout mice⁴⁴ and in KKAY mice
14 using another SSAO inhibitor (E)-2-(4-fluorophenethyl)-3-fluoroallylamine (FPFA)⁶¹.
15 In KKAY mice, a single injection of FPFA resulted in elevation of plasma glucose.
16 However, chronic treatment of FPFA on glucose homeostasis was not evaluated in the
17 study. Taken together, since inhibition of adipose inflammation may take a longer time
18 to develop, we hypothesize that VAP-1/SSAO inhibition may increase plasma glucose
19 concentrations through the inhibition of adipose glucose uptake in a short period of

1 time, but may improve glucose homeostasis through the reduction of adipose
2 inflammation in a long period of time, as shown in the present study. Further studies
3 are needed to prove the hypothesis in the future.

4 Hypercholesterolemia is an important risk factor for atherosclerosis. In humans,
5 serum VAP-1/SSAO activity has been reported to correlate with plasma LDL-C²² and
6 total cholesterol.⁶² In the present study, VAP-1/SSAO inhibition by PXS-4728A for 15
7 weeks significantly reduced plasma LDL-C levels in cholesterol-fed apolipoprotein
8 E-deficient mice. However, plasma LDL-C levels was not significantly suppressed
9 with PXS-4728A for 7 weeks, suggesting that treatment duration is an important
10 factor for plasma LDL-C reduction. Indeed, inhibition of VAP-1/SSAO by another
11 specific inhibitor LJP1586 for 4 weeks failed to show any change in plasma LDL-C in
12 LDLR^{-/-} ApoB^{100/100} mice.²⁸ The mechanism for the effect remains unknown. Several
13 pro-inflammatory cytokines and stimulants, such as TNF α , IL-1 and
14 lipopolysaccharide, have been reported to increase serum cholesterol levels by
15 enhancing hepatic cholesterol synthesis and 3-hydroxy-3-methylglutaryl-CoA
16 (HMG-CoA) reductase activity, and reducing bile acid synthesis in rodent models.⁶³
17 However, in humans, subjects with inflammatory diseases, such as rheumatoid
18 arthritis and psoriasis, have lower levels of plasma LDL-C, which can be elevated by
19 anti-inflammatory agents.^{64,65} In these patients, there are changes in quality of LDL-C,

1 such as generation of small dense LDLs and increased susceptibility toward oxidation
2 etc.⁶³ Therefore, they have an increased risk of atherosclerosis despite a lower plasma
3 LDL-C levels. Further studies are needed to clarify the mechanisms how
4 VAP-1/SSAO inhibition reduces plasma LDL-C, considering the treatment duration
5 and differences in research models. Clinically, statins are the treatment of choice for
6 primary and secondary prevention of cardiovascular events, mainly through the
7 reduction in plasma LDL-C.^{66,67} However, concerns about the association between
8 statins and new-onset diabetes have recently been raised. In the present study,
9 VAP-1/SSAO inhibition has shown to successfully reduce plaque size, and the
10 efficacy of VAP-1/SSAO inhibition for primary prevention of atherosclerosis is
11 comparable to that of atorvastatin. Since PXS-4728A for 7 weeks reduced plaque size
12 without a significant decrease in plasma LDL-C levels, this suggests that
13 VAP-1/SSAO inhibition can alleviate the progression of atherosclerosis through not
14 only lipid-dependent mechanisms but also lipid-independent mechanisms, such as
15 decreasing ROS production, lowering plasma glucose, suppressing endothelial
16 activation, inhibiting recruitment and activation of macrophages, and attenuating
17 migration and proliferation of SMC. Taken together, our findings suggest that
18 VAP-1/SSAO inhibition is a novel way with different mechanisms to statins for the
19 treatment of atherosclerosis.

1 The first step to initiate atherosclerosis is the activation of endothelial cells,
2 which facilitates leukocyte trafficking to inflammatory sites, thereby kicks off the
3 downstream pathological immune response. Therefore, the role of VAP-1/SSAO on
4 endothelial cells serves as a crucial cornerstone for the anti-atherosclerotic effects of
5 PXS-4728A. VAP-1/SSAO on endothelial cells not only serves as an adhesion
6 molecule⁶ but also acts as an ectoenzyme contributing to leukocyte rolling, firm
7 adhesion and transmigration⁶⁸⁻⁷¹. A working model has been proposed to explain how
8 VAP-1/SSAO modulates the leukocyte extravasation cascade, based on study results
9 using anti-VAP-1 antibodies and VAP-1/SSAO inhibitors and HUVEC transfected
10 with an enzyme-inactive mutant of VAP-1.¹⁰ First, VAP-1 on endothelial cells serves
11 as an adhesin to interact with leukocytes through antibody-defined epitopes. Then, the
12 leukocyte surface molecule is used as a substrate for oxidative deamination reaction
13 of SSAO. This leads to the formation of a transient covalent interaction (Schiff base)
14 which brings endothelial cells and leukocytes together. Finally, after the enzyme
15 reaction proceeds, the leukocyte surface molecule is catalyzed to aldehyde and H₂O₂,
16 which can serve as a signaling molecule to regulate leukocyte emigration and modify
17 inflammatory cascades in the local microenvironment. In the present study,
18 VAP-1/SSAO inhibition reduced monocyte adhesion to aorta (Figure 4B and 4C), as
19 well as adhesion to and transmigration through HUVEC hSSAO/VAP-1 monolayers

1 (Figure 5E and Figure 5F). Our results indicate that abrogation of VAP-1/SSAO
2 activity inhibits a sequential process associated with endothelial activation. First,
3 VAP-1/SSAO inhibition reduced the expressions of E-selectin, P-selectin, and
4 ICAM-1 on aorta (Figure 4A and Supplemental Figure 2), which supports the
5 concepts that the catalytic activity of VAP-1/SSAO can up-regulate several other
6 endothelial adhesion molecules.^{72,73} Notably, we found that inhibition of
7 VAP-1/SSAO activity also reduced its own protein expression on endothelial cells
8 (Figure 4A and Supplemental Figure 2). This finding implies that there might be a
9 positive feedback mechanism by which VAP-1/SSAO activity could regulate its own
10 protein expression, but further studies are warranted to prove this hypothesis.
11 Moreover, we found that VAP-1/SSAO inhibition suppressed intracellular ROS
12 production in HUVEC hSSAO/VAP-1 cells (Figure 5D). In the literature, intracellular
13 ROS have been shown to regulate the expression of adhesion molecules.^{74,75} In
14 addition, we have proven that the number of adhered and transmigrated monocytes
15 was decreased by antioxidant NAC (Figure 5E and 5F). Taken together, our results
16 suggest that VAP-1/SSAO inhibition can reduce monocyte adhesion and
17 transmigration, which may result from direct enzymatic effect, suppressed
18 intracellular ROS production and reduced expression of adhesion molecules.

19 During inflammation, chemotactic proteins such as MCP-1 are released to recruit

1 leukocytes, especially monocytes, into the inflammatory site.⁴ Once monocytes have
2 migrated into the intima, they can be stimulated by various cytokines and differentiate
3 into macrophages. In the present study, cholesterol-enriched diet resulted in the
4 upregulation of the expression of MCP-1 and various pro-inflammatory cytokines,
5 and more macrophages were recruited in the atherosclerotic plaques. By contrast,
6 VAP-1/SSAO inhibition reduced the expression of MCP-1 and these cytokines, and
7 suppressed macrophage recruitment. These findings are in agreement with previous
8 reports.^{73,76} In a rat model, VAP-1/SSAO inhibition has been shown to suppress the
9 expression of MCP-1 and TNF- α , resulting in reduced choroidal neovascularization.⁷⁶
10 Similarly, decreased expression of MCP-1 and TNF- α by VAP-1/SSAO inhibition has
11 also been demonstrated in a mice model of intracerebral hemorrhagic stroke.⁷³

12 Proliferation and migration of vascular SMC are crucial to atherogenesis.⁵⁵ In the
13 present study, SMC displayed strong expression of VAP-1/SSAO, which is also found
14 in a previous report.⁷⁷ During differentiation of SMC, the mRNA expression, protein
15 expression, and VAP-1/SSAO activity increase significantly.⁷⁸ Mercier *et al.* have
16 demonstrated that VAP-1/SSAO deficient mice had larger arterial diameters,
17 suggesting a role of VAP-1/SSAO in arterial wall remodeling.⁷⁹ MMP-9, with its
18 proteolytic activity, is a key molecule in SMC migration and vessel remodeling.^{52,53} In
19 the literature, intracellular ROS can also regulate the migratory and proliferative

1 activities of SMC, aside from various inflammatory cytokines and growth factors.⁸⁰⁻⁸²
2 ROS participate in pro-migratory signaling pathways, such as lamellipodia formation,
3 actin cytoskeleton remodeling, focal adhesion turnover, and contraction of cell body.⁸¹
4 ROS also promote SMC proliferation and modulate the activity and gene expression
5 of MMP.⁸² In the present study, VAP-1/SSAO inhibition decreased MMP-9 expression
6 in aorta (Figure 4F and Supplemental Figure 2), reduced ROS production in A7r5
7 hSSAO/VAP-1 SMC (Figure 6D), and attenuated the migratory and proliferative
8 ability of PDGF-BB-treated A7r5 hSSAO/VAP-1 SMC (Figure 6E and 6F). Moreover,
9 we have demonstrated that inhibition of oxidative stress by NAC suppressed the
10 proliferation and migration of SMC (Figure 6E and 6F). Taken together, our findings
11 indicate that VAP-1/SSAO inhibition can regulate proliferation and migration of SMC,
12 directly or indirectly via its effect on MMP-9 expression and intracellular redox state.

13 In the present study, we found that VAP-1/SSAO inhibition by PXS-4728A
14 reduced atherosclerosis. However, the findings are different from the two reported
15 studies, both used semicarbazide to inhibit VAP-1/SSAO in LDL receptor knock-out
16 mice on western-type diet.^{26,27} When semicarbazide was given after introducing
17 western-type diet for 6 or 9 weeks, it increased collagen content and cap thickness of
18 the plaques, and induced phenotypic switch of SMC, but did not result in regression
19 of the atherosclerotic lesions.²⁶ By contrast, when semicarbazide was given along with

1 western-type diet, it resulted in an increase in the size of atherosclerotic lesions.²⁷ One
2 of the potential reasons for the discrepant findings is the off-target effects of
3 semicarbazide. Semicarbazide is not a specific inhibitor for VAP-1/SSAO and can act
4 on other enzymes involved in atherogenesis.^{29,30} For instance, semicarbazide can
5 inhibit LOX, another amine oxidase involved in extracellular matrix maturation and
6 proliferation, as well as SMC migration. The disturbance of LOX expression could
7 contribute to endothelial dysfunction and plaque progression.³¹ In addition,
8 semicarbazide can also inhibit S1P lyase,²⁹ a key regulator of S1P signaling. S1P has
9 diverse effects on a variety of cell types in atherogenesis, including attachment and
10 migration of monocytes, SMC proliferation, and production of pro-inflammatory
11 cytokines.^{32,33} Thus, disturbance of S1P signaling may have pro- or anti-atherogenic
12 effects, depend on the cell and animal model studied.³⁴ By contrast, we used a potent
13 and highly specific VAP-1/SSAO inhibitor PXS-4728A, which has more than
14 500-fold selectivity for VAP-1/SSAO over all the related human amine oxidases
15 (including LOX) or over 100 different macromolecular targets.³⁵ Therefore, off-target
16 effects are minimized in this study.

17 The present study is limited in that the human study measured plasma
18 VAP-1/SSAO protein concentrations alone, rather than plasma VAP-1/SSAO protein
19 concentrations and VAP-1/SSAO activity. However, serum VAP-1/SSAO protein

1 concentrations have been shown to correlate well with serum VAP-1/SSAO
2 activity,^{22,37} and treatment with antibody against VAP-1 can reduce more than 95% of
3 serum VAP-1/SSAO activity.³⁷ Therefore, it is reasonable to speculate that subjects
4 with CAD in this study not only have elevated plasma VAP-1/SSAO protein
5 concentrations but also have higher plasma VAP-1/SSAO activity. Besides, there was
6 a marked male predominance in subjects with CAD, which may result from chance
7 since we recruited study subjects consecutively and did not select them by gender.
8 Since the relationship between plasma VAP-1 and the presence or the extent of CAD
9 was similar after adjustment for gender, this limitation has limited effect on our
10 findings.

11 In conclusion, plasma VAP-1/SSAO concentrations are associated with CAD in
12 humans, and can be used as a novel biomarker for assessing the presence and the
13 extent of CAD. PXS-4728A, a specific VAP-1/SSAO inhibitor, can reduce
14 atherosclerosis in cholesterol-fed ApoE-deficient mice, through suppression of many
15 key steps for atherosclerosis, including ROS generation, endothelial dysfunction,
16 adhesion and transmigration of monocytes, recruitment and activation of macrophages,
17 as well as migration and proliferation of SMC. Our data suggest that VAP-1/SSAO
18 inhibition is a potential treatment for atherosclerotic cardiovascular disease.

19

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9

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1 **Figure legends**

2 **Figure 1. Expression of vascular adhesion protein-1 (VAP-1) is increased in**

3 **atherosclerotic plaques and is colocalized with endothelial cells (EC) and smooth muscle**

4 **cells (SMC).** Immunohistochemical staining with the antibodies against EC, SMC, and

5 VAP-1 by double immunofluorescent staining in (A) coronary artery sections in humans (n =

6 3-4) and in (B) aorta sections in apolipoprotein E (ApoE)-deficient mice (n = 3-4). VAP-1

7 staining (white arrows) is stronger in atherosclerotic plaques than that in normal parts in

8 human aorta. VAP-1 staining is colocalized with EC and SMC. The scale bar = 100 μ m in (A)

9 and 50 μ m in (B). Nuclei are stained with DAPI. Dashed white line represents internal elastic

10 lamina.

11

12 **Figure 2. Plasma vascular adhesion protein-1 (VAP-1) is positively associated with the**

13 **presence and extent of coronary arterial disease (CAD) in humans.** (A) Subjects with

14 CAD (n = 127) had higher plasma VAP-1 concentrations compared with those without CAD

15 (n = 53). (B) Plasma VAP-1 concentrations were positively associated with the number of

16 coronary arteries with clinical significant stenosis (n = 53, 38, 39, and 50, respectively). (C)

17 Plasma VAP-1 concentrations were positively associated with the number of coronary arterial

18 segments with clinical significant stenosis (n = 53, 26, 18, 25, 21, 18, and 19, respectively).

19 Means and standard errors (error bars) of log-transformed (Ln) plasma VAP-1 are shown.

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Figure 3. PXS-4728A inhibits semicarbazide-sensitive amine oxidase (SSAO) activity and reduces cholesterol diet-induced atherosclerosis in apolipoprotein E (ApoE)-deficient mice. Effects of PXS-4728A on SSAO activity in (A) thoracic aorta, (B) lung, and (C) epididymal fat. SSAO activity is expressed as the SSAO-specific production rate of H_2O_2 (n = 4-8). (D) Representative images and quantification of the intensity of nitrotyrosine staining within the thoracic aorta. The values are the mean \pm SEM (n = 3-5 per group). The scale bar = 50 μ m. (E) Oil Red O staining of aortic sinus and quantification of the Oil Red O positive area (n = 5-8). The scale bar = 100 μ m. (F) En face Oil Red O staining of thoracic aorta, and quantification of the Oil Red O positive area represented as percentage of total aorta area (n = 6-9). The values are the mean \pm SEM. *p <0.05. (CTRL, control group; cholesterol-fed, cholesterol-enriched diet for 15 weeks; cholesterol-fed/PXS, PXS-4728A (10 mg/kg/day) was given along with cholesterol-enriched diet for 15 weeks, "the prevention group"; cholesterol-fed/PXS 7W, cholesterol-enriched diet was given for 8 weeks followed by cholesterol-enriched diet plus PXS-4728A (10 mg/kg/day) for another 7 weeks, "the treatment group"; cholesterol-fed/atorvastatin, atorvastatin (2.5 mg/kg/day) was given along with cholesterol-enriched diet for 15 weeks, " the positive therapeutic control group".)

1 **Figure 4. Semicarbazide-sensitive amine oxidase (SSAO) inhibition by PXS-4728A**
2 **suppresses expression of adhesion molecules, inflammatory cytokines, markers for**
3 **macrophage recruitment and activation, as well as markers for smooth muscle cells**
4 **(SMC) migration and proliferation in apolipoprotein E (ApoE)-deficient mice. (A)**
5 Expression of adhesion molecules including vascular adhesion protein-1(VAP-1), vascular
6 cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and
7 E-selectin in the atherosclerotic plaques by immunohistochemical staining in different groups.
8 The negative control containing only a rhodamine-conjugated secondary antibody without
9 primary antibody incubation (w/o 1° primary antibody) showed the species specificity of the
10 antibody. (B) En face microscopy images illustrate BCECFAM-labeled U937 bound to
11 endothelium of thoracic aorta. (C) Quantification of the number of monocytes adhered to
12 aorta in different groups, represented as the percentage of control. The values are the mean \pm
13 SEM (n = 3-5 per group). *p<0.05 (D) Expression of inflammatory cytokines including
14 monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor- α (TNF- α) in the
15 atherosclerotic plaques by immunohistochemical staining in different groups. (E) Expression
16 of markers for macrophage recruitment and activation including Iba-1 (macrophage),
17 Toll-like receptor-4 (TLR-4), CD36, receptor for advanced glycation end-product (RAGE),
18 and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in the atherosclerotic
19 plaques by immunohistochemical staining in different groups. (F) Expression of matrix

1 metalloproteinase-9 (MMP-9), smooth muscle cell marker α -actin (SMC), and proliferative
2 cell nuclear antigen (PCNA) in the atherosclerotic plaques by immunohistochemical staining
3 in different groups. The internal elastic lamina is indicated by the arrows. The negative
4 control containing only a rhodamine- or FITC-conjugated secondary antibody without
5 primary antibody incubation (w/o 1° primary antibody) showed the species specificity of the
6 antibody. The scale bar = 100 μ m. (CTRL, control group; cholesterol-fed,
7 cholesterol-enriched diet for 15 weeks; cholesterol-fed/PXS, PXS-4728A was given along
8 with cholesterol-enriched diet for 15 weeks; cholesterol-fed/PXS 7W, cholesterol-enriched
9 diet was given for 8 weeks followed by cholesterol-enriched diet plus PXS-4728A for another
10 7 weeks.)

11
12 **Figure 5. Semicarbazide-sensitive amine oxidase (SSAO) inhibition by PXS-4728A**
13 **reduces reactive oxygen species (ROS) production and monocyte transmigration in**
14 **tumor necrosis factor- α (TNF- α)-induced human umbilical vein endothelial cells**
15 **(HUVEC) overexpressing SSAO/VAP-1 (HUVEC hSSAO/VAP-1 cells).** (A-C) vascular
16 adhesion protein-1 (VAP-1) expression and SSAO activity were determined by western blot,
17 immunofluorescence and SSAO activity assay in HUVEC and HUVEC hSSAO/VAP-1 cells.
18 (D) Cells were pre-treated with PXS-4728A (300 nM) for 1h and then treated with TNF- α (10
19 ng/ml) for another 1h. After treatment, the cells were then loaded with ROS indicator

1 dichlorofluorescein diacetate (H₂DCF-DA), and the fluorescence intensity was assessed by
2 flow cytometry and quantified. (E) SMC were pretreated with PXS-4728A (300 nM) or
3 N-acetyl-L-cysteine (NAC) (5, 10 mM) for 1 h and then treated with 10 ng/ml TNF- α for
4 another 24 h. The monocyte adhesion assay was performed to evaluate dysfunction of
5 TNF- α -treated HUVEC hSSAO/VAP-1 cells. (F) Cells were pre-treated with PXS-4728A
6 (300 nM) or NAC (5, 10 mM) for 1h and then treated with TNF- α (10 ng/ml) for another 24 h.
7 After treatment, monocytes were added in the upper well. Monocytes transmigrated for 6 h
8 onto the lower surface of the filter were stained with coomassive blue and counted. The bar
9 graphs represent the numbers of cells transmigrated. The values are the mean \pm SEM (n = 3-6
10 per group). *p <0.05. The scale bar = 100 μ m. CTRL, control; PXS, PXS-4728A.

11

12 **Figure 6. Semicarbazide-sensitive amine oxidase (SSAO) inhibition by PXS-4728A**
13 **reduced reactive oxygen species (ROS) production, proliferation and migration in**
14 **lipopolysaccharide (LPS)- or platelet-derived growth factor (PDGF)-BB-induced A7r5**
15 **cells overexpressing SSAO/VAP-1 (A7r5 hSSAO/VAP-1 cells).** (A-C) vascular adhesion
16 protein-1 (VAP-1) expression and SSAO activity were determined by western blot,
17 immunofluorescence and SSAO activity assay in A7r5 smooth muscle cells and A7r5
18 hSSAO/VAP-1 cells. (D) Cells were pre-treated with PXS-4728A (300 nM) for 1h and then
19 treated with LPS (1 μ g/ml) for another 1h. After treatment, the cells were loaded with ROS

1 indicator dichlorofluorescein diacetate (H₂DCF-DA), and fluorescence intensity was assessed
2 by flow cytometry and quantified. (E) Cells were left untreated or were treated with 300 nM
3 PXS-4728A or N-acetyl-L-cysteine (NAC) (1, 2 mM) for 1h and then with PDGF-BB for
4 additional 24 h. The proportion of cells in the S phase was determined by FACScan analysis.
5 (F) Cell migration was examined via wound-healing assay. The migration rate of the cells
6 was determined. The values are the mean \pm SEM (n = 3-5 per group). *p < 0.05. The scale bar
7 = 100 μ m. CTRL, control; PXS, PXS-4728A.

8

9

1 Table 1. Clinical characteristics of total population stratified by the presence of coronary
 2 arterial disease (CAD).

Variable	CAD (-)	CAD (+)	p
N	53	127	
Numbers of coronary arteries with stenosis (0/1/2/3, N)	53/0/0/0	0/38/39/50	<0.001
Numbers of coronary arterial segments with stenosis	0	3.5 ± 2.0	<0.0001
Age (years)	57.9 ± 12.2	61.5 ± 11.6	0.066
Gender (male/female)	31/22	109/18	<0.001
Smoking (no/yes/quit)	29/11/13	59/27/41	0.5
BMI (kg/m ²)	25.6 ± 3.9	26.5 ± 4.1	0.15
Systolic blood pressure (mmHg)	135 ± 19	140 ± 23	0.3
Diastolic blood pressure (mmHg)	74 ± 9	76 ± 12	0.2
Use of anti-hypertensive drugs (N, %)	34 (64)	80 (63)	0.9
Hypertension (N, %)	46 (87)	115 (91)	0.5
Fasting plasma glucose (mg/dL)	96 ± 19	108 ± 61	0.2
2-h postprandial plasma glucose (mg/dL)	139 ± 59	157 ± 86	0.2
Hemoglobin A1c (%)	6.3	6.2	0.7
Diabetes (N, %)	22 (42)	51 (40)	0.9
Total cholesterol (mg/dL)	178 ± 30	170 ± 35	0.11
Triglyceride (mg/dL)	110 (79-151)	116 (86-179)	0.072
LDL-C (mg/dL)	99 ± 25	94 ± 32	0.3
HDL-C (mg/dL)	44 ± 11	40 ± 12	0.03
Use of statin medications (N, %)	7 (13)	58 (46)	<0.001
Plasma VAP-1 (ng/ml)	544 (460-644)	579 (490-710)	0.003

3 Means ± SDs or medians (interquartile ranges) are shown.

4 Abbreviations: BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C,
 5 high-density lipoprotein cholesterol; VAP-1, vascular adhesion protein-1

6

1 Table 2. The relationship between plasma vascular adhesion protein-1 (VAP-1) and clinical
 2 characteristics. Pearson's correlation coefficients (r) and partial correlation coefficients
 3 (partial r) are shown. Plasma VAP-1 and triglyceride levels were log transformed for the
 4 analysis.

Variable	r	p	partial r^*	p^*
Age (years)	0.1485	0.047		
BMI (kg/m ²)	0.0246	0.7	0.0794	0.30
Systolic blood pressure (mmHg)	0.1063	0.16	0.0871	0.25
Diastolic blood pressure (mmHg)	-0.0307	0.68	0.0015	0.98
Fasting plasma glucose (mg/dL)	0.2349	0.0015	0.2524	0.0007
2-h postprandial plasma glucose (mg/dL)	0.3435	<0.0001	0.3687	<0.0001
Hemoglobin A1c (%)	0.0897	0.3	0.0866	0.3
Total cholesterol (mg/dL)	-0.0479	0.5	-0.0593	0.43
Triglyceride (mg/dL)	0.1426	0.056	0.1731	0.021
LDL-C (mg/dL)	-0.1224	0.10	-0.1324	0.081
HDL-C (mg/dL)	-0.0597	0.4	-0.1150	0.13
The number of coronary arteries with stenosis	0.2569	0.0005	0.2869	0.0001
The number of coronary arterial segments with stenosis	0.2505	0.0007	0.2717	0.0003

5 * Adjust for age and gender

6 Abbreviations: BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C,
 7 high-density lipoprotein cholesterol

8

9

1 Table 3. The relationship between plasma vascular adhesion protein-1 (VAP-1) and the presence or the extent of coronary arterial disease (CAD)
 2 in adjusted models. Odds ratios (OR) or regression coefficients (β) for every 1 standard deviation increase in plasma VAP-1 are shown.

	The presence of CAD		The number of coronary arteries with stenosis		The number of coronary arterial segments with stenosis	
	OR (95% CI)	p	β	p	B	p
Crude	1.81 (1.19-2.76)	0.006	0.30	<0.001	0.61	<0.001
Model 1	2.09 (1.29-3.38)	0.003	0.32	<0.001	0.62	<0.001
Model 2	2.27 (1.36-3.77)	0.002	0.34	<0.001	0.66	<0.001

3 Model 1, adjusted for age and gender.

4 Model 2, adjusted for age, gender, hypertension, diabetes, plasma low-density lipoprotein cholesterol and smoking.

5

6

1 Table 4. The clinical and biochemical characteristics of apolipoprotein E-deficient mice.

	CTRL	Cholesterol-fe d	Cholesterol-fed/ PXS	Cholesterol-fed/ 7W	PXS	Cholesterol-fed/ atorvastatin
Body weight (Day 0)	27.0 ± 0.6	27.8 ± 0.5	26.3 ± 0.5	26.3 ± 0.4		25.7 ± 0.4
Body weight (Day 105)	30.2 ± 0.7	40.7 ± 1.8*	40.3 ± 2.1*	42.3 ± 1.1*		38.1 ± 1.3*
Fasting plasma glucose (mg/dl)	129 ± 8	197 ± 15*	156 ± 11†	157 ± 7†		139 ± 11†
Total cholesterol (mg/dl)	281 ± 21	464 ± 26*	374 ± 35†	436 ± 33*		384 ± 24†
Triglyceride (mg/dl)	116 ± 8	190 ± 13*	188 ± 15*	191 ± 12*		201 ± 24*
LDL-C (mg/dl)	234 ± 20	415 ± 25*	324 ± 37†	383 ± 33*		338 ± 24†
HDL-C (mg/dl)	47 ± 2	48 ± 2	51 ± 6	53 ± 6		46 ± 2
Creatinine (mg/dl)	0.50 ± 0.10	0.46 ± 0.05	0.49 ± 0.07	0.63 ± 0.09		0.67 ± 0.11
AST (U/L)	21 ± 2	29 ± 2	29 ± 2	30 ± 2		29 ± 3
ALT (U/L)	21 ± 5	30 ± 3	34 ± 6	34 ± 4		39 ± 6

2 Means ± SEM are shown.

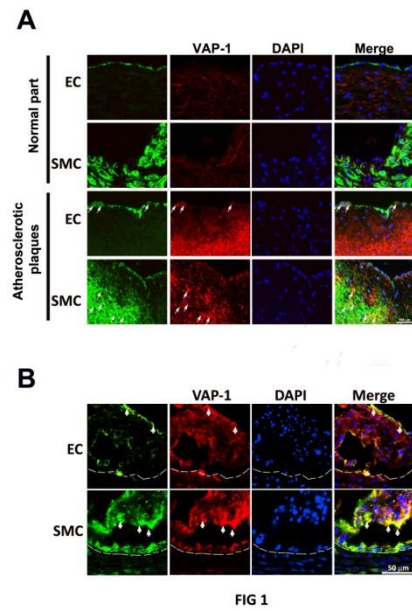
3 *p <0.05 compared with control group. †p <0.05 compared with Cholesterol-fed group.

4 Abbreviations: LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; aspartate aminotransferase, AST;
5 alanine transaminase, ALT6 CTRL, control group; Cholesterol-fed, cholesterol-enriched diet for 15 weeks; cholesterol-fed/PXS, PXS-4728A was given along with
7 cholesterol-enriched diet for 15 weeks; cholesterol-fed/PXS 7W, cholesterol-enriched diet was given for 8 weeks followed by
8 cholesterol-enriched diet plus PXS-4728A for another 7 weeks; cholesterol-fed/atorvastatin, atorvastatin was given along with
9 cholesterol-enriched diet for 15 weeks.

10

11

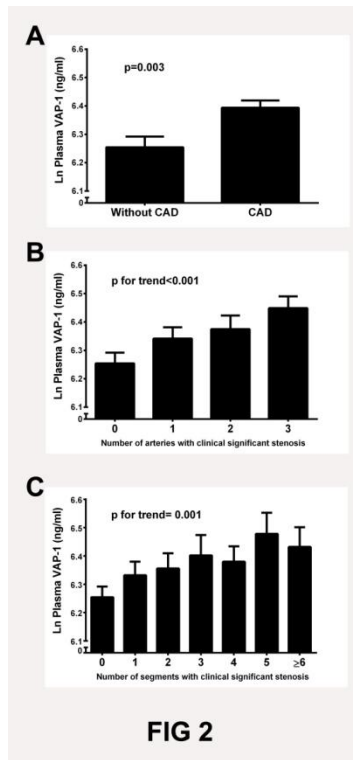
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2 FIG 1 20181010.tif

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2 Fig 2 HUMAN.tif

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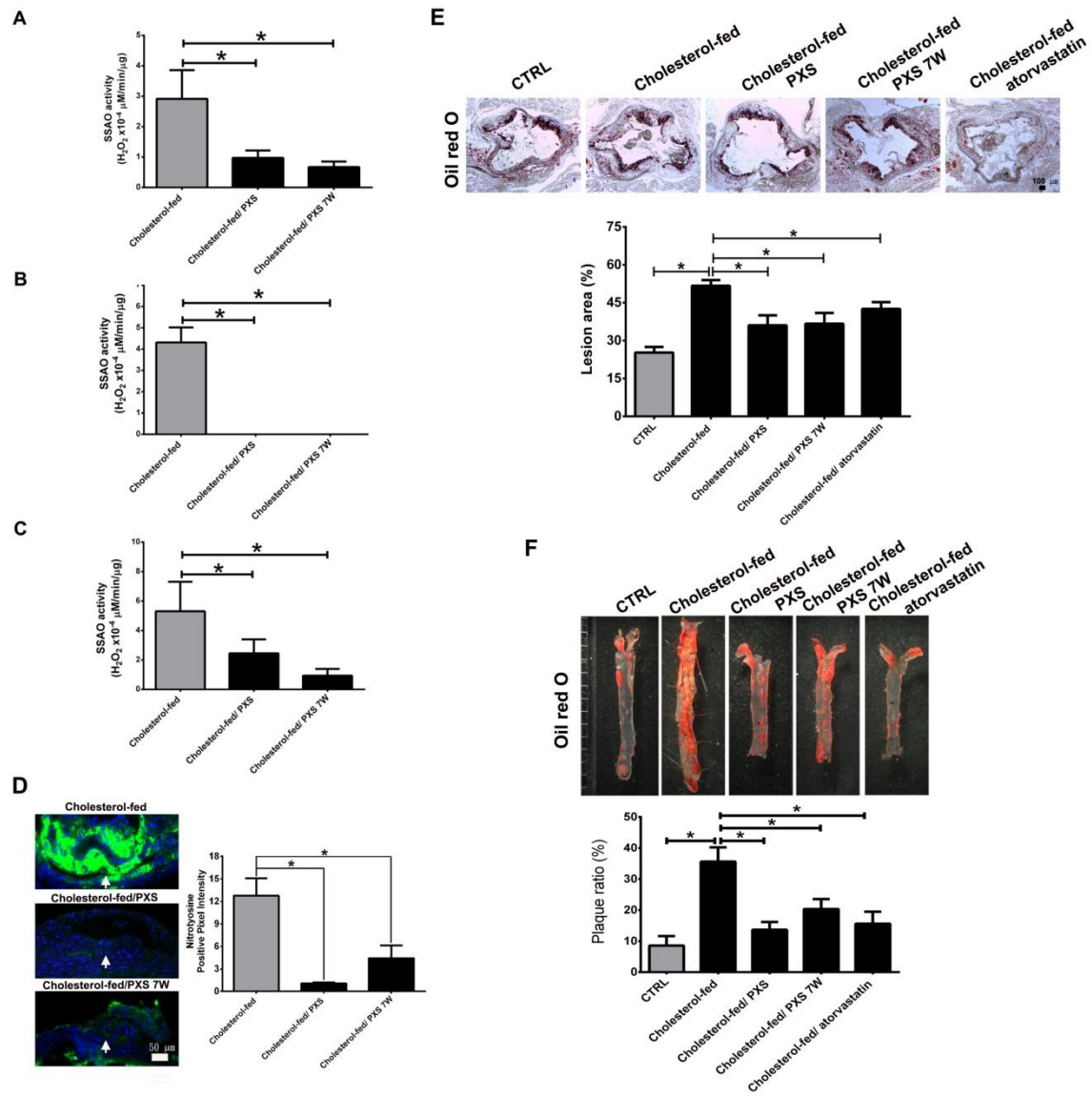


FIG 3

1 FIG 3 20180110.tif

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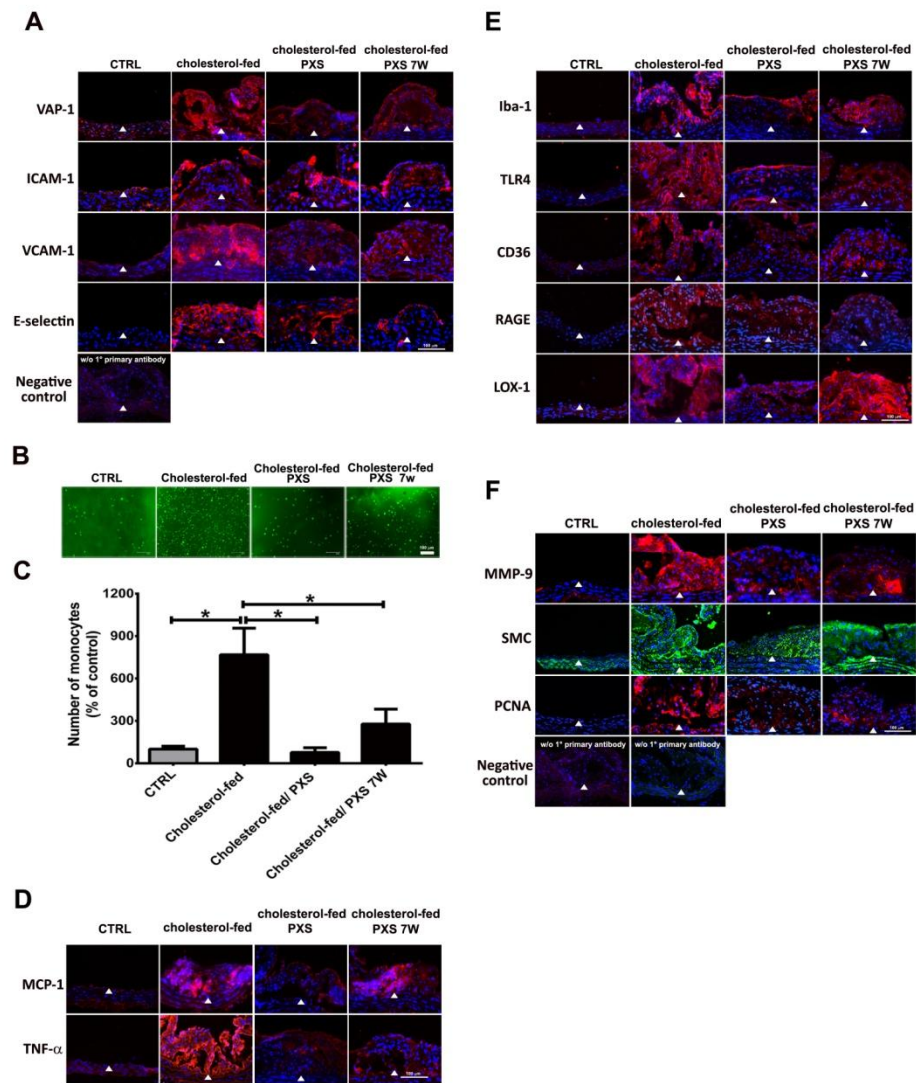


FIG 4

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1 FIG 4 20180222.tif

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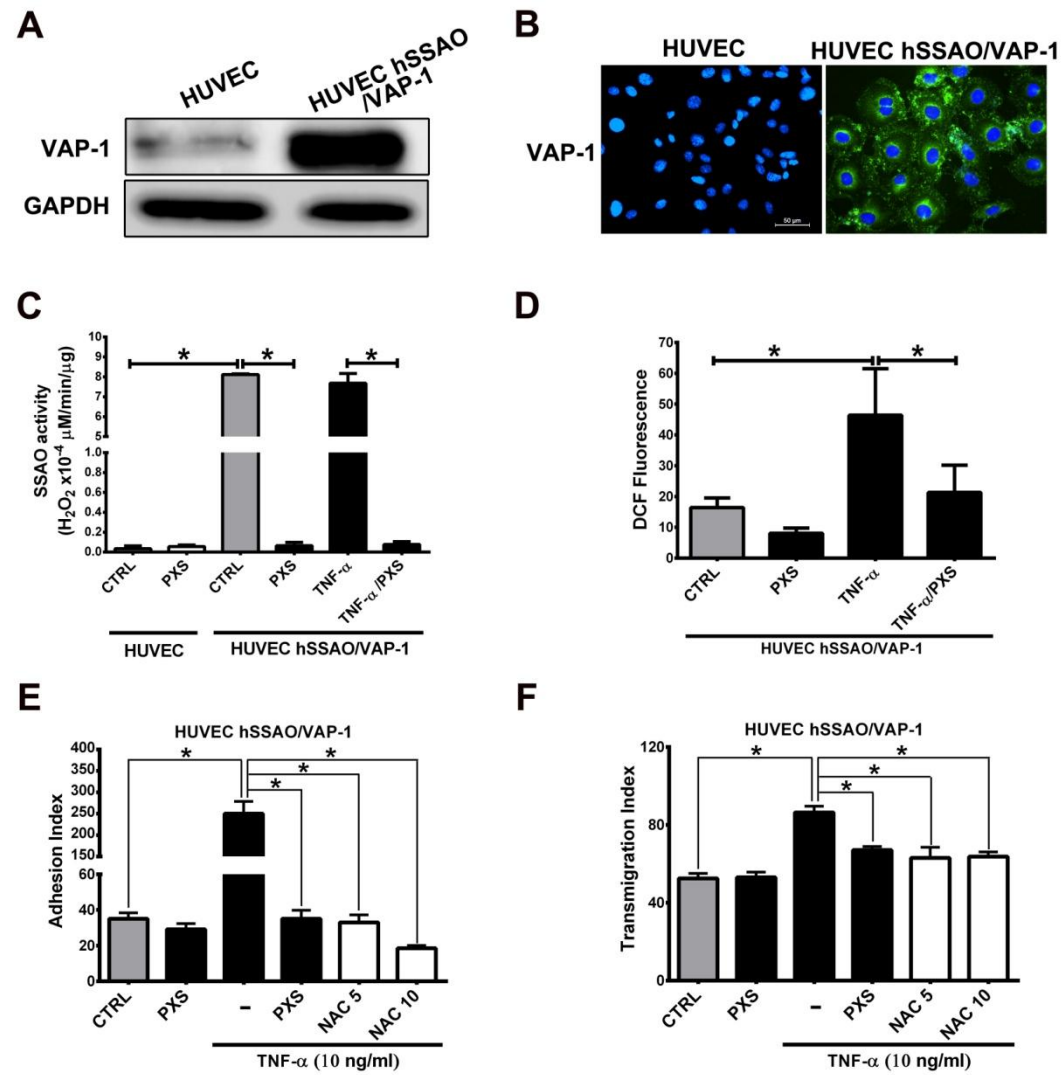


FIG 5

1 FIG 5 20180214.tif

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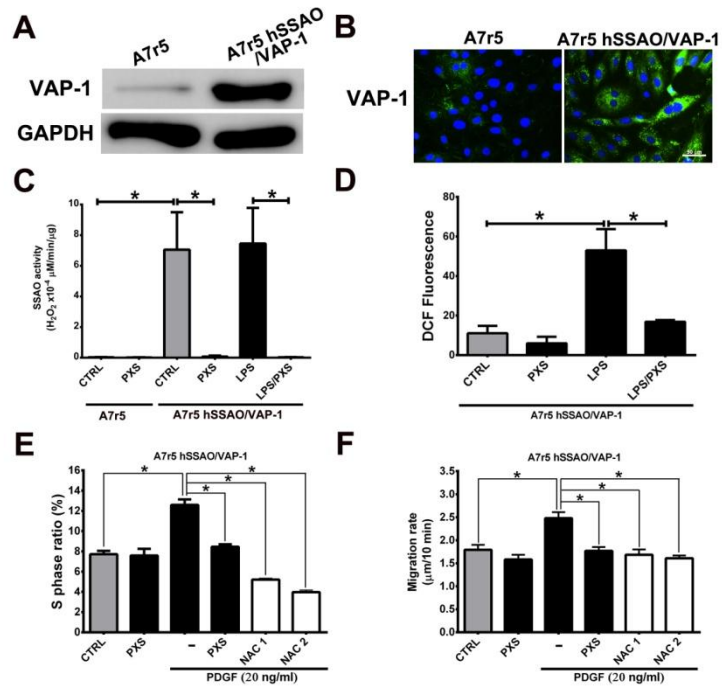


FIG 6

1

2 Fig 6 A7r5 20180214.tif