Materials and Methods

Cell culture and treatment with siRNAs

Human umbilical vein endothelial cells (HUVECs), purchased from Lonza, were cultured and maintained as described previously[1]. BMPRII siRNA (5'-UGA ACG CAA CCU GUC ACA UAA UAG GCG-3'), ActRIIa siRNA (5'-GGA CUG AUU GUG UAG AAA ATT-3') and ActRIIb siRNA (5'-GGU GUA CUU CUG CUGCUG UTT-3') were custom synthesized from MWG Biotech. Nox1 siRNA (Santa Cruz), Alk1 and Alk2 siRNA (Dharmacon), Alk6 and Alk3 siRNAs (Ambion) and non-silencing control (Invitrogen) were used at 100 nM (unless indicated otherwise) to transfect HUVECs using Oligofectamine (Invitrogen) in serum-free media for 5h as described previously[1]. Knockdown efficiency of siRNAs was confirmed by qPCR and Western blot analysis.

Neutralizing BMP9 and 10 antibodies

HUVECs transfected with BMPRII siRNA (BRII.si) or non-silencing siRNA (Non.si) for 5h were incubated in complete medium containing 10% fetal bovine serum and neutralizing antibodies for BMP9 (10 μg/ml), BMP10 (10 μg/ml) (R&D Biosystems) or control IgG as described previously[2].

BMPRII overexpression

HUVECs were treated with or without BMPRII siRNA for 5h. Cells were further infected with adenoviral constructs encoding for BMPRII-wild-type (WT) or BMPRII short form (short) lacking the c-terminal tail region (provided by Dr. Akiko Hata), for 1 day in complete medium as previously described[3]. Expression of BMPRII and endothelial inflammation was determined by Western blot analysis using antibodies to BMPRII, intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1).

Human Coronary Arterial Samples

Coronary arteries were obtained from patients undergoing heart transplant in an Institutional Review Board approved study at Emory University with written informed consent. Frozen sections were prepared and were processed from immunohistochemical staining. Atherosclerotic lesions were classified according to the American Heart Association classification[4, 5].

BMPRII heterozygotic null mice

$BMPRII^{+/-}$ mice on C57BL/6 background[6], provided by Dr. Hideyuki Beppu, were crossed with $ApoE^{-/-}$ mice to generate $BMPRII^{+/-}ApoE^{-/-}$ mice. All experiments were performed with littermate $BMPRII^{+/+}ApoE^{-/-}$ as wild-type controls.

Acute atherosclerosis model (Partial carotid ligation)

All animal studies were performed using 8-week-old male $BMPRII^{+/-}ApoE^{-/-}$ and littermate $BMPRII^{+/+}ApoE^{-/-}$ mice as control. All procedures were carried out according to the approved Institutional Animal Care and Use Committee protocol for this study by Emory University. Partial ligation of LCA was carried out under anesthesia as previously described[7] to induce low and disturbed flow in the LCA. Following carotid ligation, *d-flow* in LCA and *s-flow* in RCA was confirmed at one day post-ligation by ultrasonography using the Vevo770 system (Visualsonics, Canada)[7, 8]. RCA and LCA were isolated at indicated experimental end-points.

Chronic atherosclerosis model (High-fat diet fed model)

$BMPRII^{+/-}ApoE^{-/-}$ mice and $BMPRII^{+/+}ApoE^{-/-}$ littermate controls were used as indicated and maintained for 2 months on the Paigen's high-fat diet (HFD; Science Diets) containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid[9, 10]. Mice were provided water ad libitum. Explanted aortic tree were processed for subsequent plaque analysis and immunohistochemical analysis.

Quantitative real-time PCR (qPCR)

Total RNA was polyadenylated and reverse transcribed for use in a two-step qPCR setup using High-capacity cDNA synthesis kit (ABI) and using Brilliant II SYBR Green QPCR Master Mix (Stratagene) with custom designed primers on a Real-Time PCR System (StepOne Plus, Applied Biosystems)[8]. Fold changes between LCA and RCA were determined for all targets using the $\Delta\Delta$Ct method[11]. Sequences for primers used for mRNA expression studies have been listed in Supplementary Table 2.

Western blotting

Following treatments, cell lysates were prepared and analyzed by Western blot analysis as described previously[12, 13]. The membranes were probed with antibodies to Alk2 (1:1000, R&D), Alk3 (1:500, Santa Cruz), Alk6 (1:500, Santa Cruz), BMPRII (1:500, BD), ICAM-1 (1:1000, Santa Cruz), VCAM-1 (1:1000, Santa Cruz), Nox-1 (1:2000), FLAG (1:3000) or β-actin (1:1000, Sigma), total SMAD and phospho-SMAD antibodies (1:1000) (Cell Signaling) and appropriate secondary antibodies conjugated to alkaline phosphatase, which were detected by a chemiluminescence method.

Immunofluorescence staining

Frozen sections of human coronary arteries were fixed in ice-cold acetone for 5 min, blocked for 1 hour with 10% donkey or goat serum, and incubated with primary antibodies overnight at 4°C, followed by Rhodamine-conjugated secondary antibodies for 2 h at room temperature as described[12]. Nuclei were counter-stained with DAPI (Sigma). Primary antibodies used were specific for Alk2 (1:50, R&D systems), BMPRII (1:50, Santa Cruz), PECAM-1 (1:50, BD), CD45 (1:100, eBioscience), ICAM-1 (1:100, Santa Cruz) and VCAM-1 (1:100, BD). Twenty different human coronary artery sections, containing various stages of atherosclerosis from minimally diseased to advanced atheroma stages, from nine different patients, were examined. Images were taken using a Zeiss epi-fluorescence microscope. The semi-quantification method was used based on the blinded grading for atheroma intensity and BMPRII staining intensity in endothelium using one to five scale (1: minimally diseased, low staining intensity, 5: advanced atheroma, high staining intensity). Similar methodology was used for mouse thoracic aorta as described above in this section was used for ICAM-1 and VCAM-1 staining.

Monocyte binding assay

Following BMPRII siRNA transfection for two days and BMP4 treatment for 4h in HUVECs, monocyte binding assay was performed as described previously[12]. In some studies of HUVECs, apocynin (Calbiochem) or an NFκB inhibitor, BAY 11-7082 (Sigma) were treated for two days after 5h of transfection of BMPRII or non-silencing siRNA.

Amplex-Red assay for measuring hydrogen peroxide

Extracellular H_2O_2 was measured using a horseradish peroxidase-linked Amplex Red fluorescence assay. Briefly, after cells were transfected with non-silencing or BMPRII siRNA,

media were washed twice with Krebs Ringer Phosphate (KRP) buffer and incubated with 5 µM Amplex UltraRed (Molecular Probes) and 0.1 U/ml horseradish peroxidase type II (Sigma-Aldrich) in KRP for 40 minutes. Triplicate reading were taken in a 96-well plate using 100 µl samples of media, and fluorescence was detected via plate reader at excitation and emission of 530 nm and 580 nm, respectively. Hydrogen peroxide levels were normalized to cellular protein as measured by the Bio-Rad DC assay.

Serum Lipid Analysis

Serum lipid analysis from the mouse samples was performed by Cardiovascular Specialty Laboratories, (Atlanta, GA). All lipid determinations were performed using a Beckman CX7 biochemical analyzer and reagents from Beckman Diagnostics (Fullerton, CA) for total cholesterol, triglycerides, HDL and LDL[14].

Atherosclerotic lesion analysis

To examine atherosclerosis development in the carotid artery, aorta and carotid arteries were isolated *en bloc* as described above. Aortic trees, RCA and LCA were photographed using a CCD camera (Moticam 2500, Motic) attached to a dissection microscope at 3x magnification and the opaque area covered by plaque and total artery area of LCA were measured using NIH ImageJ software[15].

Oil-red-O staining

Oil-red-O staining was carried out using frozen sections as described[7].

Dihydroethidine (DHE) staining for measuring superoxide generation

The evaluation of basal ROS production in $BMPRII^{+/-}ApoE^{-/-}$ and $BMPRII^{+/+}ApoE^{-/-}$ were performed as described before[7, 16]. DHE hydrochloride (5 µM, Molecular Probes) was applied to the freshly cut frozen aortic sections (10 µm) for 30 min at 37°C to reveal the presence of ROS as red fluorescence (585 nm) by Zeiss (Jena, Germany) fluorescence microscope.

Statistical Analysis

Values are expressed as means±SEM unless otherwise indicated. Pairwise comparisons were performed using one-way or two-way Student T-tests. Multiple comparisons of means were performed using 1-way ANOVA followed by Tukey's Multiple Comparison tests. Differences between groups were considered significant at P values below 0.05.

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