Materials and Methods

Chemicals

Recombinant mouse Wnt3a, and Dkk1 were purchased from R&D Systems. Phenylephrine (PE), acetylcholine (Ach) and L-NAME were procured from Sigma. Antibodies were purchased from InVitrogen, Santa Cruz Biotech, Transduction Labs, Upstate Biotechnology Inc., Abcam, or Cell Signaling.

Cell culture and transfection

HUVECs were purchased from Clonetics (San Diego, CA USA) and cultured in endothelial growth medium (EGM-2, Lonza, Walkersville, MD USA) and were used until passage 10. Human embryonic kidney 293 (HEK 293) cells, L cells, and L-Wnt3a cells were purchased from American Type Culture Collection. Conditioned media was collected from L and L-Wnt3a cells. Bovine aortic endothelial cells (BAEC) were purchased from Cell Applications (San Diego, CA USA). HEK293 cells and BAECs were cultured in DMEM (Mediatech, Inc, Manassas, VA USA) supplemented with 10% FBS and 100 µg/ml streptomycin, and 100 µg/ml penicillin. Cells were transfected with plasmids, validated siRNA-p66^{Shc} or negative control siRNA using Lipofectamine 2000 (InVitrogen, Carlsbad, CA USA) per the recommendations of the manufacturer. Cells were pre-treated with Dkk1 (100 ng/ml) for 3 hrs. pcDNA3.1 (Invitrogen) was used as negative control for the transfections.

Animals and diet

p66^{Shc}RNAi transgenic and their control WT littermate mice on a C57Bl/6 background have been previously described[1]. The HFD is an adjusted calorie diet that provides 42% calories from fat (TD.88137, Harlan). Mice were placed on this diet for 16 weeks, enough to induce endothelial dysfunction. Animals were sacrificed with CO_2 inhalation and aortas rapidly harvested. All the animals were provided access to food and water *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee and were carried out according to NIH guidelines.

Kinase inhibitors

HUVECs were pre-incubated for 30 min with specific kinase inhibitors: JNK inhibitor SP600125 at 20 □M; MEK inh

then stimulated with Wnt3a for 1 hr. Phosphorylation of p66^{Shc}, total β-catenin and active (dephosphorylated) β-catenin levels were examined by standard immunoblotting.

Endothelial cell-monocyte adhesion assay

Adhesion of U937 monocytes to endothelial cells was determined as previously described[2]. Briefly, confluent HUVECs were activated by pre-incubating with Wnt3a (100ng/ml, 1 hr), U937 cells were re-suspended in EGM-2 media and 2×10^6 cells were added to each well. U937 cells were incubated with HUVECs for 30 min. and then washed with RPMI-1640 5 times. L-NAME or PEG-catalase was added to HUVECs 1 hr prior to the addition of Wnt3a. Adhesion of U937 to HUVECs monolayer was examined under inverted light microscope (20X). Number of adherent U937 cells per field (20× magnification) was determined and results represent mean of at least three independent experiments.

Aortic ring preparation and vascular tension recordings

Thoracic aortas of mice kept on either HFD or ND were used in the study. The animals were rapidly euthanized by CO_2 inhalation. The aorta was carefully dissected, rapidly removed, and placed in ice-cold oxygenated Krebs-Ringer bicarbonate solution. The vessel was carefully cleared of loose connective tissue and cut into 5-10 1.5 mm rings. Aortic rings from each mouse was suspended between two wire stirrups (150 μm) in a12.5-ml organ chambers of a four-chamber myograph system (DMT Instruments) in 5 ml Krebs-Ringer (95% O_2-5% CO_2, pH 7.4, 37°C). One stirrup was connected to a three-dimensional micromanipulator, and the other to a force transducer. The mechanical force signal was amplified, digitalized, and recorded (PowerLab 8/30). All concentration-effect curves were performed on arterial rings beginning at their optimum resting tone. This was determined by stretching arterial rings at 10 min intervals in increments of 100 mg to reach optimal tone (~500mg). One dose of KCl (60mM) was administered to verify vascular smooth muscle viability. Cumulative dose-response curve for phenylephrine (PE) (10^{-9}-10^{-5} M) was obtained by administering the drug in log doses. Endothelium-dependent and –independent vasodilatation was determined by generating dose-response curves to acetylcholine (ACh 10^{-9}-10^{-5} M) and sodium nitroprusside (SNP 10^{-9}-10^{-5} M), respectively on PE (10^{-6} M) induced pre-contracted vessel. Vasorelaxation evoked by ACh and SNP was expressed as percent relaxation, determined by calculating percentage of inhibition to the pre-constricted tension. NO bioavailability was measured physiologically by determining increase in the contractile response to NOS inhibition (L-NAME10^{-4} M) in rings pre-constricted with PE (10^{-6}M). Aortic rings were pre-incubated with recombinant Wnt3a (100 ng/ml), with or without Dkk1 (100 ng/ml), for 24 h.

Oil-Red-O (ORO) staining

ORO staining was performed as previously described[3] with some modifications. Briefly, a working solution was prepared by diluting ORO stock (3% ORO in 2-propanol) 6:4 with distilled water and filtering through 0.2-□m filter. Adventitious aortic fat tissue was carefully removed, and aortas were rinsed with 70% 2-propanol for 10 seconds and stained for 30 minutes with ORO working solution, rinsed again with 70% 2-propanol for 10 seconds and then placed in distilled water. Images of stained aorta were captured and individual aortas were kept in 100 □l chloroform/methanol (2: 1, v/v) in a 96 well plate on an orbital shaker until the stain was dissolved (5 min.). The absorbance was measured at 490 nM (Wallac 1420 Victor3 Microplate Reader, PerkinElmer, Massachusetts, USA) and dry weight of individual aortic arch was determined. The relative absorbance per mg of dry weight of aortic arch was calculated and data were expressed as % of ND.

Ex vivo adenoviral infections

Endothelium-specific gene transfer was achieved ex vivo by incubating freshly isolated aortas from mice, sutured at one end, with 3.0×10^8 pfu of the appropriate adenoviral stock, and incubated at 37°C for 4 hr. as previously described[4]. The virus was removed and the aorta was then incubated for 24 hrs before sectioning into rings. The viruses used have been previously described[5, 6].

Luciferase reporter assays

The TOP-Flash (and control FOP-Flash) luciferase reporter plasmids were a kind gift from Randall Moon. TOP-Flash measures β-catenin-mediated TCF/LEF transcriptional activity. The reporter was co-transfected with a renilla luciferase plasmid driven by a constitutive promoter reporter into cells. Firefly and renilla luciferase luminescence were measured using the Dual Luciferase reporter kit (Promega) as per manufacturer's recommendations. The firefly/renilla ratio was calculated, to normalize for variations in transfection efficiencies.

Immunoprecipitation, immunoblotting and immunohistochemistry

Immunoprecipitations were carried out by incubating 2 μg of antibody with 1 mg of cell lysate overnight, followed by 40 μl of protein A-sepharose slurry (Amersham) for 2 h. After washing, immunoprecipitates were boiled in SDS-PAGE gel loading buffer, subjected to SDS-PAGE, transferred to nitrocellulose filter, and probed with the specified primary antibody and the appropriate peroxidase-conjugated secondary antibody (Santa Cruz Biotech). Western blotting of 50 μg of whole cell lysates was similarly performed. Chemiluminescent signal was developed using Super Signal West Femto substrate (Pierce), blots imaged with a Gel Doc 2000 Chemi

Doc system (BioRad), and bands quantified using Quantity One software (BioRad). For immunohistochemical studies aortic sections were deparaffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mM). These sections were probed with appropriate primary antibodies (Wnt3a, pS36-p66^{Shc}, active-β-catenin, β-catenin, c-myc). Polyvalent biotinylated secondary antibody and streptavidin peroxidase (STV–HRP) system was used to amplify the signals, followed by detection with diaminobenzidine as a chromogen. Slides were counterstained with hematoxylin, dehydrated with alcohol and xylene and mounted in DPX.

ROS levels in HUVEC

Diffusible H_2O_2 produced by cells was measured in conditioned medium using the Amplex Red probe, as previously described[7].

In situ quantification of ROS in aortas

Aortas from wild-type mouse were isolated and infected with adenovirus encoding for either LacZ or activated non-phosphorylatable form of β-catenin (24 hrs), embedded in OCT, sectioned (6 μm), and mounted on glass slides. The sections were rinsed in PBS, and incubated in 10 μM dihydroethidium (DHE) (37°C, 30 minutes; Invitrogen). Slides were mounted, and photographed. Red channel was selected using Photoshop software from at least 5 images of each group and intensity was quantified using ImageJ software.

Statistical analysis

Statistical analysis was performed using SPSS (Version 17.0) statistical software. Significance of difference between two groups was evaluated using t-test. For multiple comparisons, one way ANOVA was used and post-hoc analysis was performed with Tukey's test. Results were expressed as Mean ± SEM and considered significant if *P* values were ≤ 0.05. All shown data is representative of at least three independent experiments.

References

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