Online Supplement

A Hypertension-causing Mutation in PPARγ Impairs Nuclear Export of NF-κB p65 in Vascular Smooth Muscle

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Supplemental Materials

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Supplemental Methods

Cell culture: Mesenteric artery smooth muscle cells (SMCs) were isolated from transgenic mice carrying WT or DN PPARγ under the control of CAG promoter as previously described.[1] Briefly, SMCs was isolated from first or secondary order mesenteric artery of male transgenic mice carrying human WT- or DN-PPARγ under the control of CAG promoter by the explant method. These cells were suspended with high-glucose DMEM supplemented with 10% FBS, 1% sodium pyruvate, 1% penicillin and streptomycin (100 units/ml) and plated to six-well plate at 37 °C in a 5% CO_2 incubator. After 3 days, nonadherent cells were removed by washing with PBS and the adherent cells were cultured until the cells reached 90% confluence, and then split for experiments. Cell passages (2-8) were used for experiments. SMCs were obtained from the transgenic mice containing a floxed STOP cassette upstream of PPARγ (either WT or DN) followed by the fluorescent protein, tdTomato. For induction of WT- or DN-PPARγ, these cells were infected with Cre adenovirus. AdGFP or AdmCherry adenovirus was used as controls. HEK293T cell were obtained from ATCC and maintained in high-glucose DMEM with 10% FBS and 1% penicillin and streptomycin at 37°C in a 5% CO_2 incubator. After reaching 70-80% confluency, cells were transfected using Lipofectamine LTX (Life Technologies) according to the manufacturer's protocol. SMC and HEK293T cells were starved overnight before stimulations, and protein or RNA lysates were collected and stored at 80°C until further analysis.

Western Blotting and Immunoprecipitation: Protein was extracted from primary cultures of SMCs or HEK cells and western blotting was performed as previously described.[2,3] Briefly, protein was isolated in a lysis buffer containing 50 mmol/L Tris Cl buffer, 0.1 mmol/L EDTA (pH 7.5), 1% w/v NA deoxycholic acid, 1% v/v NP-40 and 0.1% v/v SDS, with protease inhibitor (Roche) and phosphatase inhibitors (Roche). Samples were centrifuged (12,000 g) for 10 min at 4°C and supernatants were collected. The protein concentration of lysates was determined by Lowry assay (Biorad). For immunoprecipitation, 1 mg of total protein lysate was incubated with agarose conjugated NF-κB p65 antibody (Santa Cruz, sc-109) for 2 h at 4°C. Beads were washed four times with lysis buffer, and immunoprecipitates were eluted for 10 min at 100°C in 50 µl of 2x sample buffer. Precipitated proteins or equal amounts of cell lysates (10-1000 µg) were separated by SDS-PAGE (8-12%) and transferred to a nitrocellulose membrane (GE Healthcare). After blocking with 5% non-fat milk, membranes were incubated with primary antibodies at 4°C overnight and then visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, at RT for 1 h). PPARγ (#2435, Cell Signaling), tdTomato (GTX127897, Gene Tex), GAPDH (sc-32233, Santa Cruz), Iκ-Bα (#9242, Cell Signaling), phospho-p65 (#3033, Cell Signaling) and p65 (#3034, Cell Signaling) were used for these studies. β-actin (ab16039, Abcam)

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was utilized as a loading control.

Real-time RT-PCR: RNA was extracted from primary cultures of SMC or HEK cells and real time RT-PCR (qPCR) was performed as previously described.[2] Briefly, using oligo (dT) primers, RNaseOUT (Invitrogen), and Superscript III (Invitrogen), cDNA was synthesized from 500-1000 ng of total RNA extracted from the cells using RNeasy spin columns (RNeasy Mini Kit, QIAGEN). Each Q-PCR reaction was performed in duplicate. 10 ng of cDNA was subjected to TaqMan Gene Expression Assays using the Taqman Fast Advanced Master Mix (Applied Biosystems) and the targeted Taqman probes.   The following Taqman probes were used on Applied Biosystems StepOnePlus System to evaluate gene expression level: GAPDH (4352932-0905028) and MMP9 (Mm0044299_m1).   In some experiments, Q-PCR reactions were performed with 10 ng of cDNA and Fast SYBR Green Master Mix (Applied Biosystems) along with the targeted gene primers in a total volume of 10 µl.   The primers are mMCP1: Forward: 5'-cccaatgagtaggctggaga-3' and Reverse: 5'-tctggacccattccttcttg-3'; mVCAM1: Forward: 5'-tgcgagtcaccattgttctcat-3' and Reverse: 5'-catggtcagaacggacttgga-3'. $\Delta\Delta CT$ were calculated using GAPDH as a reference gene to determine relative mRNA expression levels.

NF-κB Promoter activity: Luciferase assays were performed using NF-κB-LUC adenovirus (gift from Dr. Steven Lentz and Dr. John F. Engelhardt, University of Iowa).[4] Mouse mesenteric SMCs from transgenic mice carrying WT-or DN-PPARγ under the control of CAG promoter reached 50% confluence were infected with NF-κB-LUC adenovirus for 72 hrs, and then were treated with TNFα for overnight.   NF-κB promoter activity was determined using a luciferase assay kit (Promega) and normalized to total cellular protein in the lysate.

Immunostaining: Cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes.   Following this incubation, the cells were rinsed with PBS three times.[5] p65 or PPARγ expression was determined using monoclonal anti-p65 (#6956 Cell Signaling) or polyclonal anti-PPARγ (#2435 Cell Signaling) diluted in 5% normal goat serum with 0.1% Triton X-100 to a final dilution of 1:250 and incubated at 4°C overnight.   The cells were then rinsed in TBS-T (TBS with 0.3% Tween 20) three times for 10 min. The secondary antibody Alexa488 (Abcam) was diluted in 5% goat serum, 0.1% Triton X-100 in TBS to a final dilution of 1:1000 and incubated at room temperature for 1 h. All incubation and rinsing steps were performed under constant agitation. The cells were mounted with Vectashield (Vector Laboratories) and imaged for the presence of green fluorescence on a Zeiss LSM710 confocal microscope. Single-plane images were collected. When comparing detection of transgene expression between samples, we kept the microscope settings, including laser power, gain, and offset, constant throughout image collection. Final images were processed

using ImageJ software (version 1.48, National Institutes of Health; Java 1.6.0–20, 64-bit) to make adjustments to image size or linear parameters such as brightness and contrast. All adjustments were kept consistent across samples.

Flow cytometry: Mice were injected with TNFα (66.7 µg/kg/day) intraperitoneally for three consecutive days and were then sacrificed on the fourth day.   Single cell suspensions were prepared from aortas as previously described.[6]   Briefly, the entire aorta with surrounding perivascular fat was minced with fine scissors and digested with 1 mg/mL collagenase A, 1 mg/ml collagenase B, and 100 µg/ml DNAase I in phenol-free RPMI 1640 medium with 5% FBS for 30 min at 37°C, with intermittent agitation.   Fc receptors were blocked with anti-mouse CD16/CD32 (BD Biosciences, clone 2.4G2) for 20 min at 4°C prior to the staining of surface markers.   The antibodies used were: Alexa Fluor 488 anti-mouse CD45, APC-Cy7 anti-mouse CD3, PE anti-mouse CD4, PE-Cy7 anti-mouse CD8, and APC anti-mouse F4/80.   One million aortic cells were incubated in 100 µl of FACS buffer containing 1.5 µl of each antibody for 35 minutes.   The cells were then washed twice with FACS buffer and immediately analyzed on an LSR II flow cytometer with DIVA software (BD Biosciences).   Dead cells were eliminated from analysis using Hoechst 33528 (Sigma-Aldrich). For each experiment, we performed flow minus one (FMO) controls for each fluorophore to establish gates. Data analysis was performed using FlowJo 10.2 software (Tree Star, Inc.).

Bioluminescence imaging: Luciferase assay was performed using NF-κB-LUC mice as described previously.[2]   NF-κB-LUC mice were bred with S-WT or S-DN mice.[7,8]   After treatment of isolated aorta and carotid artery with TNFα (0-500 µM) for 16-24 hr, sample was washed using ice-cold Dulbecco's phosphate-buffered saline (dPBS) and incubated with dPBS including 1.5 mg/ml D-luciferin (Gold Biotechnology).[9]   Bioluminescence imaging was performed on a Xenogen IVIS-200 System.   Luminescence was quantitated where peak of the luminescent signal occurred.

Chemicals: Mouse TNFα (T7539) was from Sigma (St. Louis, MO). We used an NF-κB p65 inhibitor (NBP2-29321) from Novus Biologicals (Littleton, CO).   Pioglitazone and GW9662 were from Cayman Chemical (Ann Arbor, MI) and were dissolved in DMSO according to the manufacturer's instructions.

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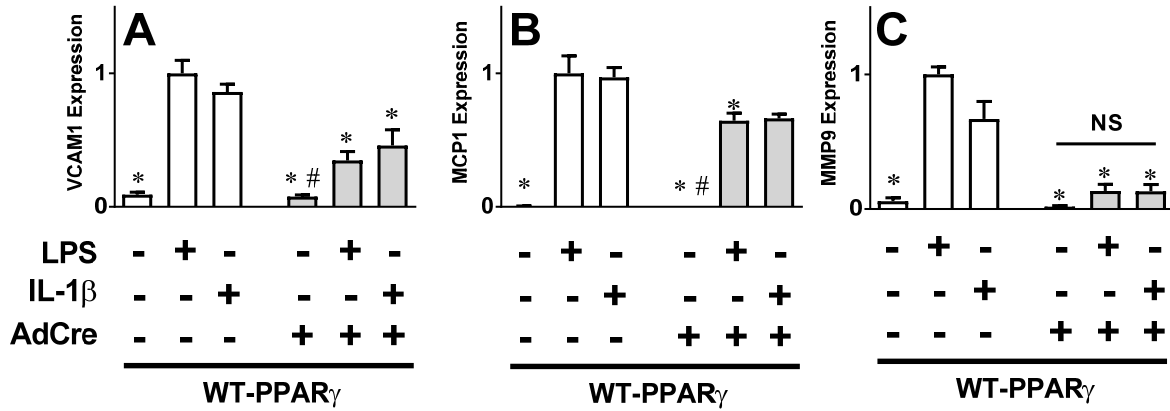
Figure S1. *NF-κB Target Gene Expression and Promoter Activity*

A-C) Relative mRNA expression of mouse vascular cell adhesion molecule 1 (VCAM-1) (A), monocyte chemotactic and activating factor 1 (MCP1) (B), and Matrix metallopeptidase 9 (MMP9) (C) were determined by quantitative real-time RT-PCR in interleukin 1β (IL-1β, 5 ng/ml)- or lipopolysaccharide (LPS, 1 μg/ml)-treated primary mesenteric SMC from transgenic mice with inducible expression of WT- or DN-PPARγ infected with AdGFP or AdCre.   Data were normalized to the control value, which was set to 1.0. All data are means ± SEM.   *p<0.05, vs. Control LPS or vs. Control IL-1β, #p<0.05, vs. Cre LPS or vs. Cre IL-1β.
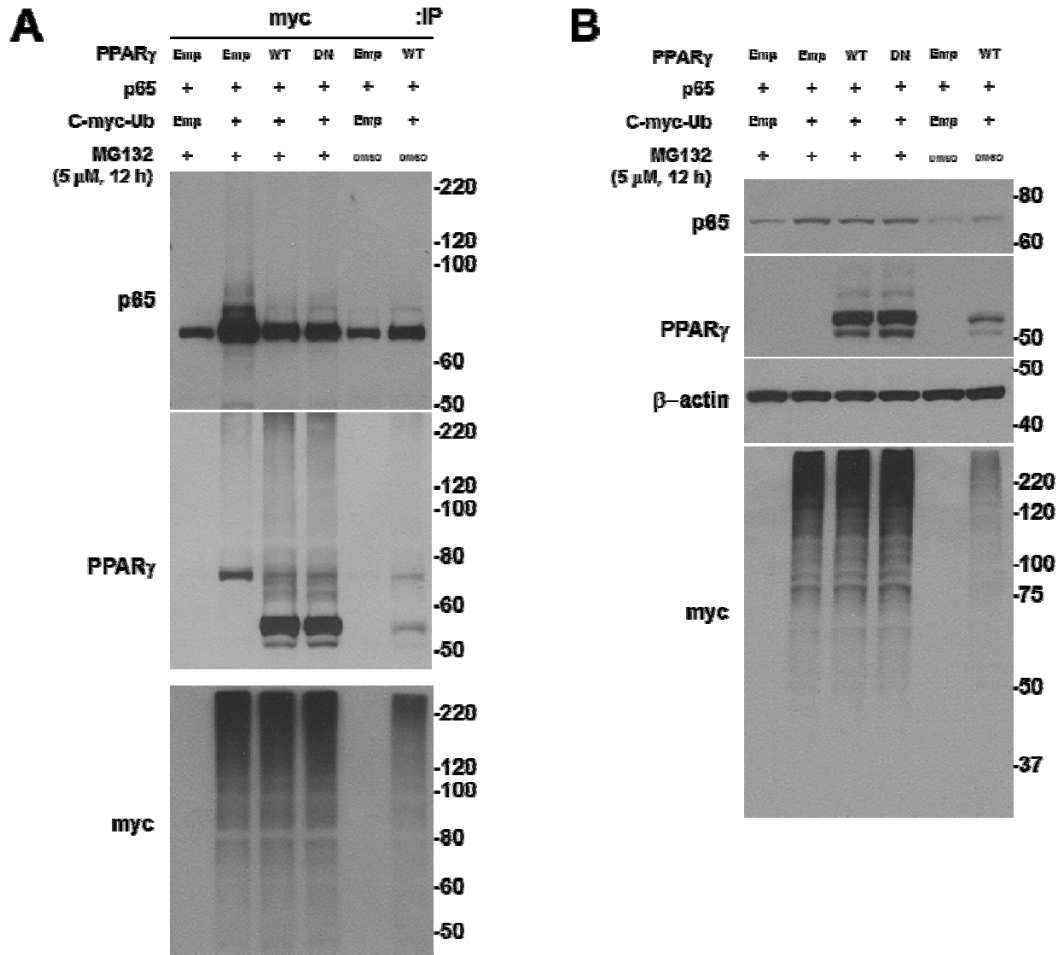
Figure S2. *Ubiquitination of p65*

HEK293T cells were transfected with constructs expressing p65, WT-PPARγ, DN-PPARγ, and myc-tagged ubiquitin (C-myc-Ub), as indicated. Cells were treated with either MG132 (5 µM) or dimethyl sulfoxide for 12 hr as indicated. A) Proteins were immunoprecipitated with myc antibodies and Western blotted for the indicated protein. B) Cell lysates were Western blotted with the indicated antibodies. Size markers transferred from the blots are shown.
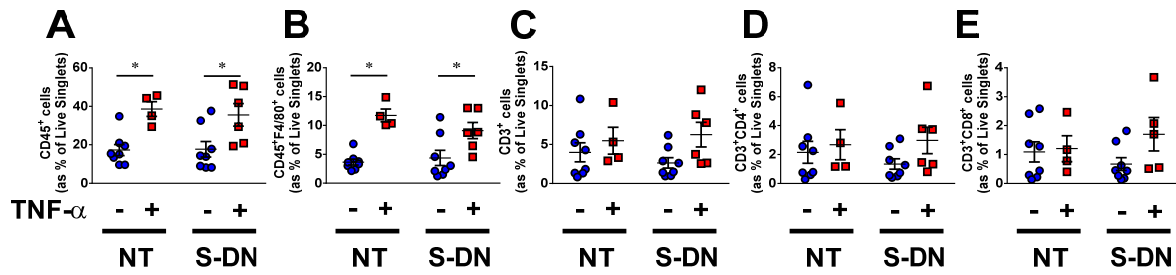
Figure S3. *Inflammatory Cell Infiltration in Aorta*

Quantification of flow cytometry for total leukocytes (CD45+ cells, A), monocytes/macrophages (CD45+F4/80+ cells, B), total T lymphocytes (CD45+CD3+ cells, C), T helper cells (CD3+CD4+ cells, D) and cytotoxic T cell (CD3+CD8+, E) in thoracic aortas of NT or S-DN mice infused with vehicle (circles) or TNFα (66.7 µg/kg/day, square) for 3 consecutive days.   *p<0.05, vehicle vs. TNFα.