Supplemental Methods and Data

Regulation of PPARγ by Angiotensin II via TGF-β1 Activated p38 MAP Kinase in Aortic Smooth Muscle Cells

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Running Title - AngII regulation of PPARγ in SMC

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MATERIALS AND METHODS

Isolation and culture of VSMCs

Adult male C57BL/6J mice (10-12 weeks old; stock # 000664) were obtained from the Jackson Laboratory (Bar Harbor, ME). Aortas were excised and dissected free of adventitia and fat.[1] Aortas were sectioned into thoracic (from the left subclavian to the last intercostal), suprarenal abdominal regions (from the last intercostal to the left renal branch), and infrarenal abdominal regions (from the left renal branch to the bifurcation). VSMCs were isolated as described previously[2] from mouse aortas with cells from 4 pooled mice being plated per 6 well plate and grown in DMEM with FBS (20%) and penicillin and streptomycin (1%) in 5% CO_2 at 37°C. VSMC phenotype was determined by immunostaining cells for alpha-actin (Clone 1A4, Sigma-Aldrich, St. Louis, MO). Cells were grown to 80-90% confluence and serum-starved for 24 hours before use. All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Cell culture

VSMCs were incubated with either saline or AngII (1 μM) for 24 hours to measure PPARγ expression and transcriptional activity, or 0 - 60 minutes to measure MAPK activity, NF-kB p65, and TGF-β1 expression. Losartan (10 μM; AT1 receptor blocker), SP600125 (10 μM; inhibitor of JNKs), SB203580 (10 μM; inhibitor of p38), PD98059 (20 μM; inhibitor of ERK), a TGF-β1 antibody (2 μg/ml) were added 30 minutes prior to addition of AngII. AngII, SP600125 and SB203580 were purchased from Sigma-Aldrich, St. Louis, MO. Losartan was obtained from Merck, NJ. An antibody to TGF-β1, recombinant TGF-β1, and recombinant active p38 were purchased from R&D Systems (Minneapolis, MN). All other inhibitors were purchased from Calbiochem, La Jolla, CA. Incubation with recombinant TGF-β1 (10 ng/ml) for 30 minutes or 24 hours was used to measure MAPKs activity or PPARγ expression. Incubation with recombinant active p38 (100 nM) for 24 hours was also performed to measure PPARγ expression and transcriptional activity.

Western blot analyses

Total cell lysates were prepared in RIPA lysis buffer and protein content was measured using the Bradford assays (Bio-Rad Laboratories, Hercules, CA). Equal amounts of proteins (10-20 μg) were resolved by 10% SDS-PAGE and transferred electrophoretically to PVDF membranes. After blocking, the following antibodies against the following proteins were used to probe the membranes: PPARγ (Biomol Research laboratories, Plymouth, PA and Affinity Bioreagents, Rockford, IL), TGF-β1 (Torrey Pines Biolabs, East Orange, NJ), phospho-p38, p38, pSMAD2, phospho-ERK, ERK, phospho-JNK, JNK, phospho-BCR kinase, phospho-NF-kB p65, NF-kB p65 (Cell Signaling Technology, Danvers, MA), BCR kinase (Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (Sigma-Aldrich, St. Louis, MO). Membranes were then incubated with appropriate secondary antibodies, and immune complexes were visualized by chemiluminescence (Pierce) and quantified using a Kodak Imager.

Real time PCR

Total RNA was extracted from cells using the SV total RNA isolation system (Promega, Madison, WI) according to the manufacturer's instructions. Reverse transcription and real time PCR were performed using an iCycler (Bio-Rad).[3] RNA (100 ng) was reverse transcribed using the i-script cDNA synthesis kit (Bio-Rad). Primer sequences for PPARγ real-time RT-PCR amplification were as follows: PPARγ, 5'-AGCATCAGGCTTCCACTATG-3' and 5'-ATCCGGCAGTTAAGATCACA-3', and 18S, 5'-CTCTGTTCCGCCTAGTCCTG-3' and 5'-AATGAGCCATTCGCAGTTTC-3'. Real-time PCR product accumulation was monitored using the intercalating dye SYBR Green. mRNA abundance was calculated by using the ΔΔCt method with normalization to 18S.

Transient Transfection Assays

A PPARγ1 promoter construct was kindly provided by Dr. Janardan K Reddy (Northwestern University Medical School, Chicago).[4] Mouse aortic VSMCs (70-80 % confluence) plated in a 12 well plate were transfected for 8 hours with a PPARγ promoter luciferase reporter plasmid (0.5 μg/well) in OptiMEM using Lipofectamine 2000 (Invitrogen). After transfection, cells were cultured overnight in FBS (0.1%) followed by incubation with vehicle, AngII, or recombinant active p38 protein for 24 hours. Luciferase activities were analyzed using a Dual-Luciferase Reporter Assay system (Promega, Madison, WI). Transfection efficiency was normalized to renilla luciferase activities generated by co-transfection of 0.1 μg pGL4.74[hRluc/TK] (pRL-Tk, Promega) per well.[5]

RNA Silencing Experiments

For small interfering RNA (siRNA) experiments, VSMCs plated in a 6 well plates. were placed in serum medium (10% v/v) without antibiotics for 3 hours and then transfected with 100 nmol / L siRNA oligonucletides or a scrambled control (Dharmacon, Lafayette, CO) using Xtreme Gene SiRNa transfection reagent (Roche Diagnostics, Indianapolis, IN) for 48 hours. SiRNA experiments were performed using the On-target plus SMARTpool technology, which provides a mixture of four different siRNAs specific to one of the following genes: TGF-β1, P38, BCR kinase and HDAC3. After transfection, cells were maintained under serum deprivation for 24 hours and then subjected to incubation with either AngII or recombinant TGF-β1 / p38 protein.

Statistical Analyses

All data are reported as means ± SEM. Statistical analyses were performed using Student's *t*-test or one-way ANOVA with Holm-Sidak post hoc tests, as appropriate, using Sigmastat (SPSS inc). $P < 0.05$ was considered statistically significant.

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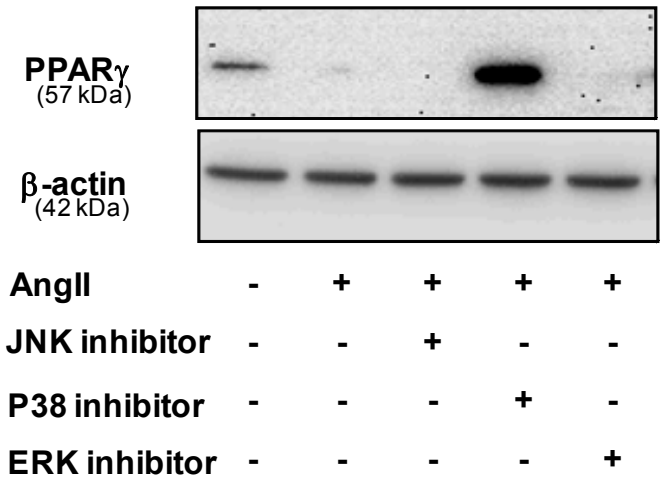

Figure I. **Blockade of JNK, and ERK MAPK did not affect AngII-induced PPARγ downregulation.** VSMCs were incubated with vehicle (DMSO) or AngII (1 μM) and inhibitors of JNK (SP-600125; 25 μM), ERK (PD-98059; 20 μM) and p38 (SB-203580; 10 μM) for 24 hours. Total cell lysates were analyzed by Western blotting using an anti-PPARγ antibody. β-actin was used as an internal control (n = 3). Representative blots show blockade of p38 MAPK rescued PPARγ in VSMCs incubated with AngII.

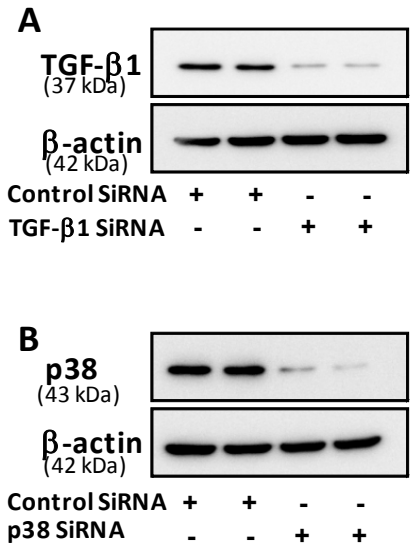

Figure II. **SiRNA mediated silencing of TGF-β1or p38 in VSMCs.** VSMCs were transfected with either control, TGF-β1, or p38 SiRNA for 48 hours and total cell lysates were analyzed by Western blot using antibodies to TGF-β1**(A)** or p38 **(B)**.

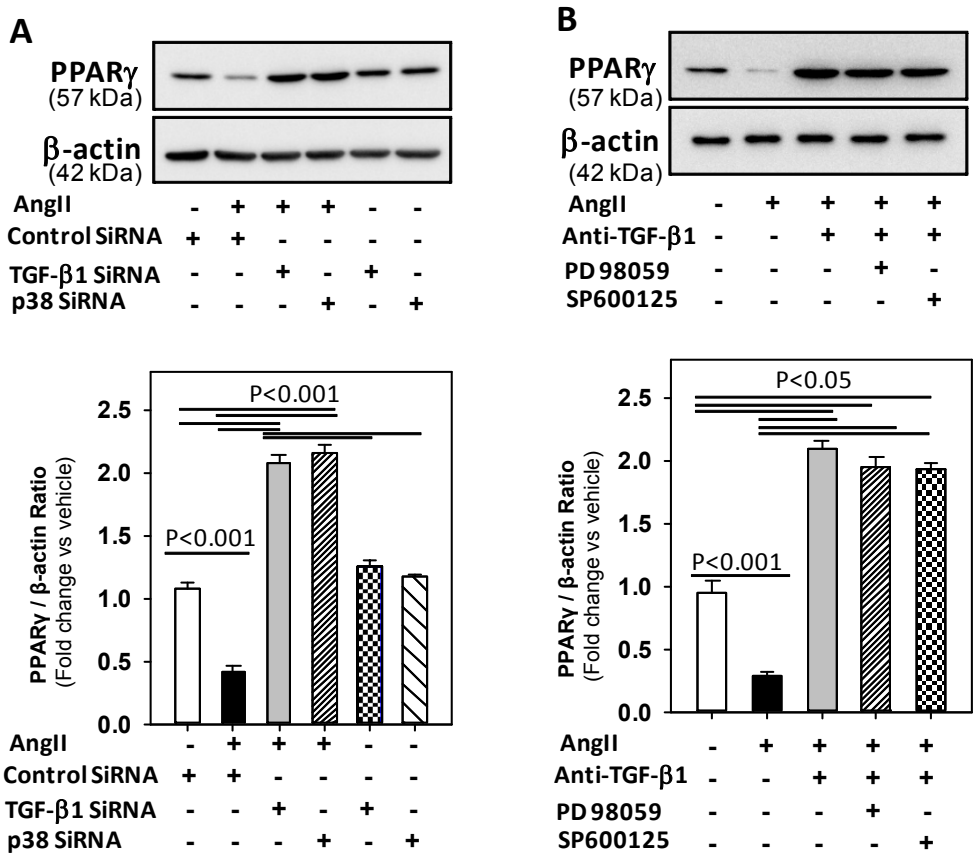

Figure III. **A: SiRNA mediated silencing of TGF-β1 or p38 attenuated AngII-induced PPARγ reduction in VSMCs.** VSMCs were transfected with either control, TGF-β1, or p38 SiRNA for 48 hours, followed by incubation with either vehicle (saline) or AngII (1 μM) for 24 hours. Total cell lysates were analyzed by Western blot using antibodies to PPARγ (n = 4). **B: Super-induction of PPARγ post TGF-β1 blockade is not mediated by ERK or JNK MAPKs.** VSMCs were pre-incubated with anti-TGF-β1 antibody (2 μg/ml) + PD98059 (10 μM) / SP600125 (20 μM) for 30 minutes followed by AngII incubation for 24 hours. Total cell lysates were analyzed by Western blot using antibodies to PPARγ (n = 4). Results are represented as means ± SEMs; Data were analyzed by one-way ANOVA with a Holm-Sidak multiple comparison post-hoc test.

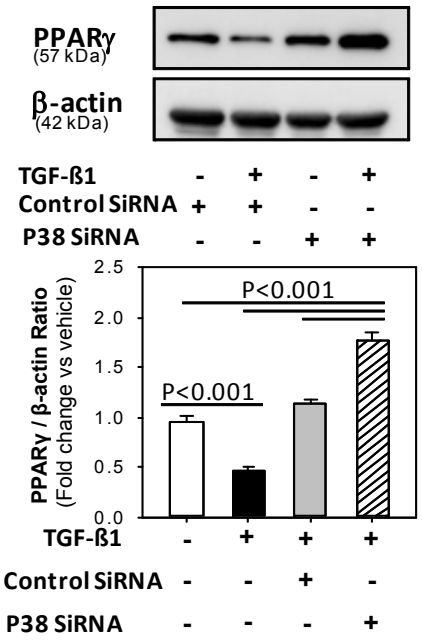

Figure IV. **SiRNA mediated silencing of p38 attenuated TGF-β1-induced PPARγ reduction in VSMCs.** VSMCs were transfected with either control or p38 SiRNA for 48 hours, followed by incubation with either vehicle (DMSO) or TGF-β1 (10 ng/ml) for 24 hours. Total cell lysates were analyzed by Western blot using antibodies to PPARγ (n = 3). Results are represented as means ± SEMs; Data were analyzed by one-way ANOVA with a Holm-Sidak multiple comparison post-hoc test.

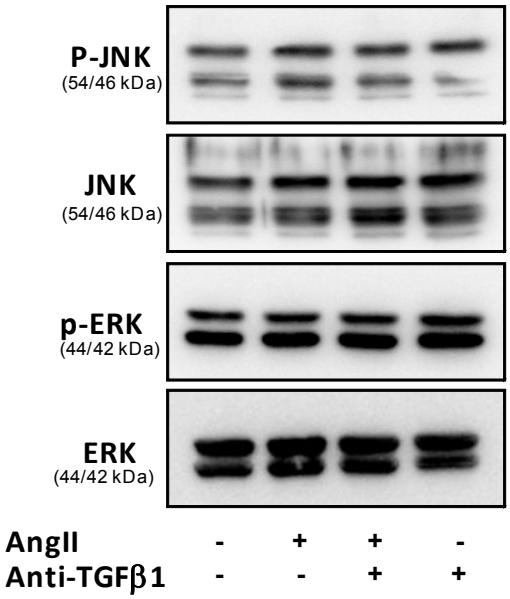

Figure V. **Neutralization of TGF-β1 did not effect AngII-induced JNK, or ERK phosphorylation.** VSMCs were incubated with either vehicle (saline), AngII (1 μM) or AngII + anti-TGF-β1 antibody (2 μg/ml) for 30 minutes. Total cell lysates were analyzed by Western blotting using antibodies to p-JNK, JNK, p-ERK and ERK (n = 3).

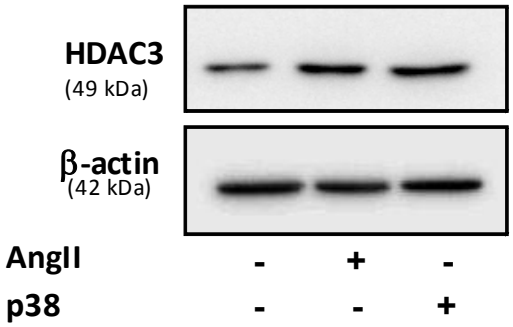

Figure VI. **AngII / active p38 increased HDAC3 protein in VSMCs.** VSMCs were incubated with either vehicle (DMSO), AngII (1 μM) or recombinant active p38 (100 nM) for 24 hours. Total cell lysates were analyzed by Western blotting using antibodies to HDAC3 (n = 3).

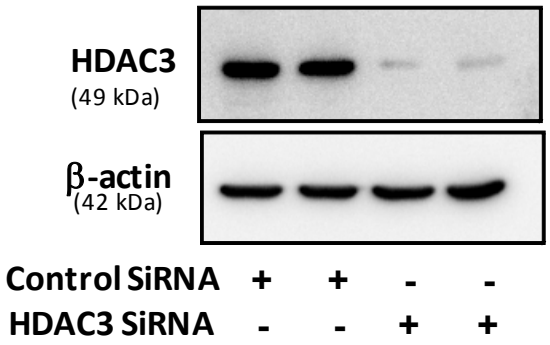

Figure VII. **SiRNA mediated silencing of HDAC3 in VSMCs.** VSMCs were transfected with either control, or HDAC3 siRNA for 48 hours and total cell lysates were analyzed by Western blot using antibodies to HDAC3.

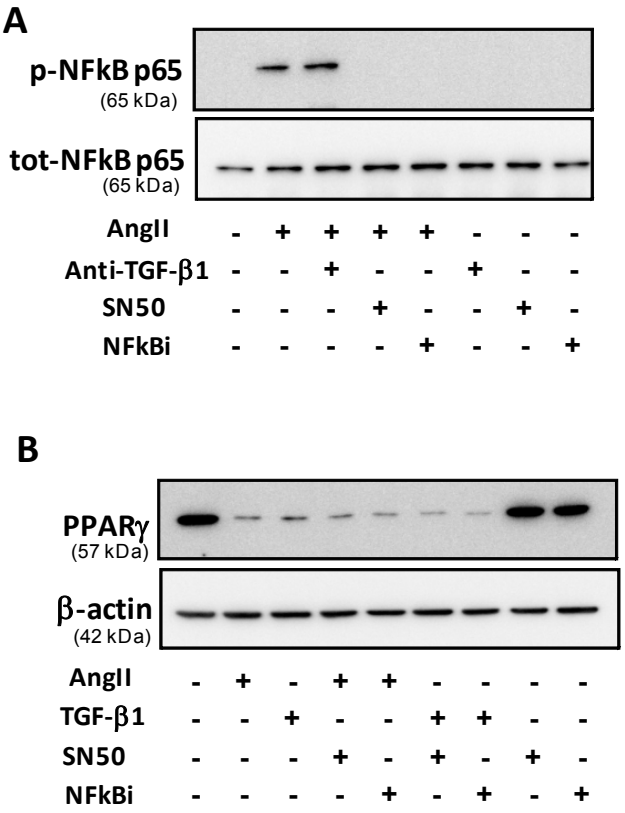

Figure VIII. **NF-kB inhibition had no effect on either AngII- or TGF-β1- induced reduction in PPARγ protein. A:** VSMCs were pre-incubated with anti-TGF-β1 (2 mg/ml) / SN50 (10 μM) / NF-kB activation inhibitor (NF-kBi 10 μM) for 30 minutes followed by incubation with either vehicle (saline) or AngII for 24 hours. Total cell lysates were analyzed by Western blot using antibodies to phospho-NF-kB p65 / NF-kB p65 (n=3). **B:** VSMCs were pre-incubated with SN50 (10 μM) / NF-kB activation inhibitor (NF-kBi 10 μM) for 30 minutes followed by incubation with either vehicle (DMSO) or AngII / recombinant TGF-β1 for 24 hours. Total cell lysates were analyzed by Western blot using antibodies to PPARγ (n = 4). β-actin was used as an internal control.

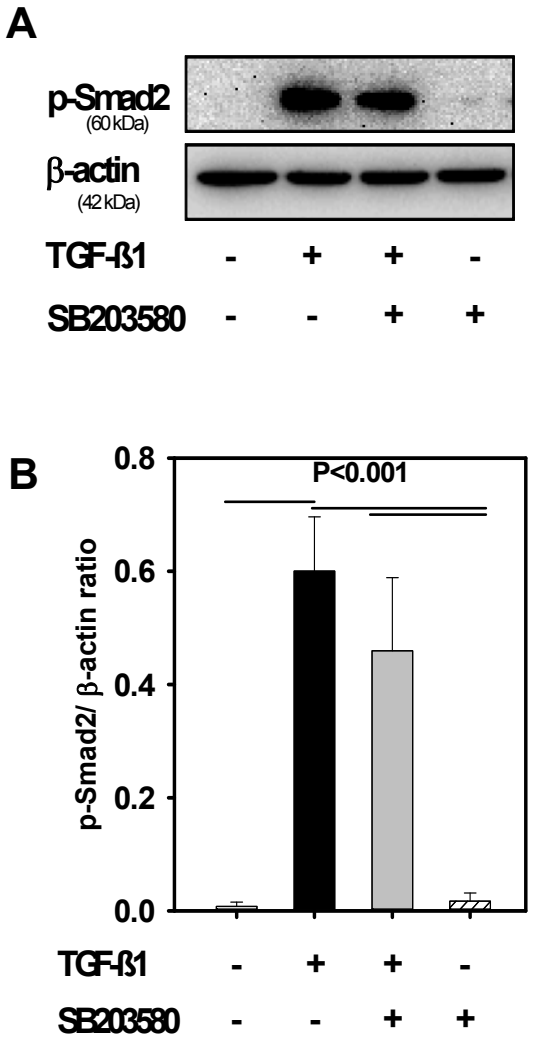

Figure IX. **Inhibition of p38 did not effect TGF-β1-induced Smad-2 activation.** VSMCs were incubated with vehicle, TGF-β1 (10 ng/ml) or TGF-β1 + SB-203580 (10 μM) for 24 hours. Total cell lysates were analyzed by Western blot using anti-pSmad-2 antibodies (n=3-5). Results are represented as means ± SEMs. Data were analyzed by one-way ANOVA with a Holm-Sidak multiple comparison post-hoc test.