Angiotensin-(1-7) Modulates Renal Vascular Resistance Through Inhibition Of P38 Mitogen-Activated Protein Kinase In Apolipoprotein E Deficient Mice

Sebastian A. Potthoff¹, Michael Fähling², Tilman Clasen¹, Susanne Mende¹, Bassam Ishak¹, Tatsiana Suvorava³, Stefanie Stamer¹, Manuel Thieme¹, Sema H. Sivritas¹, Georg Kojda³, Andreas Patzak², Lars C. Rump¹, Johannes Stegbauer¹

¹Department of Nephrology, Medical Faculty, Heinrich-Heine University, Düsseldorf, Germany
²Institute of Vegetative Physiology, Charité – Universitaetsmedizin Berlin, Berlin, Germany
³Institute of Pharmacology and Clinical Pharmacology, Heinrich-Heine-University, Düsseldorf, Germany

Supplement – Materials and Methods

Animal treatment

In this study, apoE (-/-) mice were fed a high fat diet in order to accelerate the progression of endothelial dysfunction due to progressing atherosclerosis. WT animals fed a high fat diet served as healthy controls. Ang-(1-7) treatment was introduced 6 weeks after the start of the high fat diet as an interventional treatment. The rational of this study design was to treat after occurrence of vascular injury.

At age 6 weeks, all groups were set on a high fat “western diet” (Sniff, Soest, Germany) (42 % fat, 0.15 % cholesterol) for 12 weeks.

Group 1: WT untreated for 6 weeks and treated with saline for consecutive 6 weeks (osmotic minipump, Alzet Model 1004)

Group 2: apoE (-/-) untreated for 6 weeks and treated with saline for consecutive 6 weeks (osmotic minipump, Alzet Model 1004)

Group 3: apoE (-/-) untreated for 6 weeks and treated with Ang-(1-7) (82 µg/kg/hr) for consecutive 6 weeks (osmotic minipump, Alzet Model 1004)

Group 4: apoE (-/-) treated for 12 weeks with an oral p38-inhibitor (BIRB796, 50 mg/kg/day, high fat diet preparation containing the inhibitor), after 6 weeks additional treatment with saline for consecutive 6 weeks (osmotic minipump, Alzet Model 1004).

Group 5: Mas (-/-) / apoE (-/-) untreated for 6 weeks and treated with saline for consecutive 6 weeks (osmotic minipump, Alzet Model 1004)

Group 6: Mas (-/-) / apoE (-/-) untreated for 6 weeks and treated with Ang-(1-7) (82 µg/kg/hr) for consecutive 6 weeks (osmotic minipump, Alzet Model 1004)

All animals were sacrificed at the age of 18 weeks.

Isolated perfused mouse kidney

Mice were anesthetized by i.p. injection with Ketamin (100 mg/kg) and Xylazin (5 mg/kg). Kidneys were isolated microscopically (Olympus CO11) and perfused with Krebs-Henseleit buffer according to an amended method described previously.¹ Changes in perfusion pressure reflected changes in vascular resistance of renal resistance vessels immediately after preparation, a bolus injection of 60 mM KCl was
delivered to test the viability of the preparation followed by a stabilization period of 30 min. After the stabilization period, renal vasoconstriction was induced by increasing concentrations of Angiotensin II (Sigma-Aldrich) in the presence or absence of the specific p38 MAPK inhibitor SB203580 (5 µM; Sigma-Aldrich), the ERK1/2 inhibitor PD98059 (5 µM; Sigma-Aldrich) or Ang-(1-7) (0.1 µM; Bachem). Increase in pressor response was measured in mmHg.

Acute infusion experiments

18 weeks old apoE (-/-) (group 2 and 4) mice were anesthetized by i.p. injection with Ketamin (100 mg/kg) and Xylazin (5 mg/kg). Mice were placed on a heating plate to ensure constant body temperature at approx. 36 °C. Left carotid artery was cannulated for continuous measurement of mean arterial pressure (MAP). Left jugular vein was cannulated for application of increasing doses of Ang II (bolus of 0.1, 1, 10 µM/kgBW). Change in mean arterial pressure (MAP) was measured for each concentration of Ang II.

Systolic blood pressure measurement

Systolic blood pressure (BP) was measured non-invasively by tail-cuff sphygmomanometer using a BP-98A device (Softron, Japan). Mice were trained for 4 days prior to evaluation of BP. BP was measured for 5 days at the end of the observational period (week 18).

Isolation of preglomerular vessels

Preglomerular vessels, containing mainly interlobular arteries and afferent arterioles, were isolated by a modified iron oxide-sieving technique as described previously. The kidneys were perfused via cannulation of the aorta, smaller needles (G20, G23) and pores sieves (100 µm) were used for tissue separation and separation of renal particles, respectively.

Quantification of urinary 8-isoprostane concentration

24-h urine samples were collected in metabolic cages at the end of the experimental period. Urinary concentrations of 8-isoprostane were measured using a colorimetric-assay kit (Cayman Chemical Company) and normalized to urinary creatinine concentration.

Immunoblotting for p38 MAPK and phospho-p38 MAPK

Renal cortex tissue of pre-glomerular vessels were placed into ice-cold 1 % Triton lysis buffer (containing a protease inhibitor cocktail (Sigma Aldrich)) and were immediately homogenized. Lysates were centrifuged at 15,000 x g for 10 min at 4 °C. Protein concentrations from the supernatant were measured using a Bradford assay (Bioassay Systems). After dithiothreitol treatment (100 mM) and denaturation (5 min at 95 °C), 30 µg of total protein were loaded onto 10 % SDS-PAGE gels and then transferred to nitrocellulose membranes according to manufacturer's instructions (X-Cell Blot Module, Invitrogen). Membranes were treated with blocking buffer (5 % BSA, and 0.1 % tween 20 in PBS) for 1h at room temperature and then incubated either with primary monoclonal rabbit anti-p38 antibody (1:750) (Cell Signalling Technology) or primary monoclonal rabbit anti-phospho-p38 antibody (1:1300) (Cell Signalling Technology), and rabbit anti-β-actin (1:2000), (Santa Cruz Biotechnology) over-night. Bound primary antibody was detected with anti-rabbit HRP conjugated
secondary antibody (1:10000) (Dako, Germany) by 60 min incubation at room temperature. Antibody labelling was visualized by the addition of a chemiluminescence reagent. Chemiluminescence was visualized using a FluorChem FC2 Imager (Alpha Innotec, USA). Immunoblots from each tissue were performed in triplicates.

**Immunoblotting for phospho-MLC\textsubscript{20}**

Pre-glomerular vessel tissue was lysed and processed as described above. Polyclonal rabbit anti-phospho-MLC\textsubscript{20} antibody was used as primary antibody (1:500) (Acris Antibodies GmbH). Bound primary antibody was detected with anti-rabbit HRP conjugated secondary antibody (1:50000) (Dako, Germany) by 60 min incubation at room temperature. Assessment of chemiluminescence was performed as described above. Reference protein was alpha-actin (1:2000).

**Immunoblotting for p47phox (renal cortex)**

Renal cortex tissue was lysed and processed as described above. Polyclonal rabbit anti-p47phox antibody was used as primary antibody (1:200) (Santa Cruz Biotechnology). Bound primary antibody was detected with anti-rabbit HRP conjugated secondary antibody (1:10000) (Dako, Germany) by 60 min incubation at room temperature. Assessment of chemiluminescence was performed as described above.

**Quantitative real time RT-PCR (qPCR)**

Kidney cortex samples were analyzed for relative expression levels (mRNA) of AT1a-receptor (AT1a), AT2-receptor (AT2), angiotensin converting enzyme 2 (ACE2) and Mas-receptor (MasR). After homogenization of tissue with a Tissue Ruptor (Qiagen, Germany), total RNA was isolated using a RNA Micro Kit (Qiagen, Germany) according to the manufacturer's instructions. Quantitative real time RT-PCR was performed with an ABI PRISM 7300 (Applied Biosystem, Germany) and the SYBR Green master mix (Qiagen, Germany). The PCR reaction was performed in a total volume of 20 µl with 1 µl cDNA corresponding to 50 ng RNA as template.

The PCR conditions were 15 min at 95 °C, followed by 40 cycles (denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s, extension at 72 °C for 34 s, detection at 79 °C for 34 s). Experiments were performed in duplicates. 18S ribosomal RNA was chosen as the endogenous control (housekeeping gene). The levels of targeted genes were normalized to 18S rRNA expression. Data was analyzed using the DeltaCT-method. Statistical analysis was performed with a one-way ANOVA for repeated measurements followed by Bonferroni correction post-hoc test.

The following primer sequences were used: 18S ribosomal RNA (QuantiTect Primer Assays, Qiagene, Cat-N° QT00324940), MasR: forward: TTGTGGGAGCACGTAAAGAAGA, reverse: ATGGATACAGTGTCGCTTG. ACE2: forward: TCTGGGCAAATCTGCTGGA, reverse: TGATGGGCTGTCAGAAGATTG. AT2: forward: ACCTGAGTGGGTCGCTTAGGT, reverse: CTGACATCCCACAAATTTAATTG. AT1a: forward: GCTTGTGGGTATGTCGCTCC, reverse: GGGCGAGATTTGAGAGC.

**Lucigenin superoxide detection**
Lucigenin-enhanced chemiluminescent detection of superoxide production in kidney cortex of apoE (-/-) on western diet treated with or without Ang-(1-7) (group 2 and 3) was performed as described previously. Briefly, freshly cleaned and harvested thoracic aortic and kidney cortex segments were equilibrated in Krebs-HEPES buffer for 30 min at 37 °C. Aortic and kidney segments were transferred to vials containing albumin buffer enriched with 5 µM lucigenin, and chemiluminescence was recorded every 2 min for 20 min using Packard Luminometer Analyzer (Picolite A6112, Packard, Downers Grove, IL, USA). Background readings were subtracted from sample reading and results are expressed as counts/min/mg tissue dry weight (mean ± SEM).

Statistics

Data are expressed as mean ± SEM (n=number of animals). Student's t-test was used to compare means of two groups with Gaussian distribution. Multiple comparison of more than two groups with Gaussian distribution were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison post-hoc test. Statistical analyses of data of two groups in which Gaussian distribution was not normal (or could not be assumed) were analysed by the Mann-Whitney-U-Test. Statistical analyses of data of more than two groups in which Gaussian distribution was not normal (or could not be assumed) were analysed by the Kruskal-Wallis-Test followed by Dunn’s multiple comparison post-hoc test. Differences between dose-response curves were analysed by two-way ANOVA for repeated measurements followed by Bonferroni correction post-hoc test.

Probability levels of p < 0.05 were considered statistically significant. If applicable, a higher level of statistical significance is stated (p < 0.01, p < 0.001). The number of experiments (n) refers to the number of mice or the number of individual samples.
Supplement – References


S1: Ang II-induced pressor response in isolated perfused kidney from WT mice was not significantly different if treated chronically with Ang-(1-7) (82 µg/kg/hr) (WT: n= 5; WT + Ang-(1-7): n=7) (p=NS). Two-way ANOVA for repeated measurements followed by Bonferroni correction post-hoc test.

S2: Acute Ang-(1-7) (0.1µM) administration did not influence Ang II-induced pressor response in isolated perfused kidney of non-treated apoE (-/-) mice significantly (n=6; each group) (p=NS). Two-way ANOVA for repeated measurements followed by Bonferroni correction post-hoc test.
S3: Comparison of relative gene expression (mRNA level) of RAS-related genes in WT vs. apoE (-/-) vs. apoE (-/-) + Ang-(1-7). (n=4; each group) There was no significant difference in gene expression. (real time PCR data for AT1aR, AT2R, ACE2, MasR. deltaCT to 18s expression normalized to WT expression). p=NS. One-way ANOVA for repeated measurements followed by Bonferroni correction post-hoc test.
S4: Ang II-induced pressor response is not influenced by MEK 1/2-inhibition neither in kidneys of WT (A; n=11 each group) nor apoE (-/-) (B; n=5 each group) mice. P=NS for apoE (-/-) versus apoE (-/-) + PD98059. P=NS for WT versus WT + PD98059. Two-way ANOVA for repeated measurements followed by Bonferroni correction post-test.