Online Supplement

INCREASED ANGIOTENSIN II INDUCED HYPERTENSION AND INFLAMMATORY CYTOKINES IN MICE LACKING ANGIOTENSIN CONVERTING ENZYME N DOMAIN ACTIVITY

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Detailed Methods

Materials. LPS (Escherichia coli 055:B5) was from Sigma-Aldrich (St. Louis, MO). The prolyl oligopeptidase inhibitor S-17092 was a generous gift from Servier (Suresnes, France). Anti-mouse IL-10 functional grade purified antibody was from eBioscience (San Diego, CA). Murine tumor necrosis factor α (TNFα), IL-10 and IL-12/p40 ELISA kits were purchased from eBioscience (San Diego, CA) and used according to manufacturer's instructions.

Mice. The generation of mice expressing site-inactivated ACE was previously described in detail.[1,2] Briefly, N-KO mice express a full length ACE protein where the N-terminal catalytic site was inactivated by mutating the two zinc-binding histidines (395H and 399H) to lysines. In the C-KO strain, 993H and 997H, which are responsible for the zinc binding and catalytic activity of the C-terminal enzymatic site, were mutated to lysine. During the back breeding of mice to C57BL/6, animals were selected for mating so that all mice possess only a single renin gene (Ren-1c). 8 to 12-week old N-KO, C-KO or wild-type littermates were used. Animal breeding and experimental procedures were approved by Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.

Peptide measurement. Mice were anesthetized and blood was collected from the inferior vena cava directly into a syringe containing 5 ml of 4 mol/l guanidine thiocyanate using a 25-gauge needle. The left kidney was then rapidly removed and homogenized in 5 ml of 4 4 mol/l guanidine thiocyanate. The blood and tissue homogenates were frozen and stored at -80°C until shipped on dry ice to St. Vincent's Institute of Medical Research, where peptide measurements were performed. Peptides were measured using HPLC-based radioimmunoassays, as previously described.[3]

Blood pressure. Systolic blood pressures were measured as previously described.[4] Briefly, blood pressure was measured in conscious mice using a Visitech Systems BP2000-automated tail-cuff system. Mice were trained for several days before data aquisition. On each day that blood pressure was determined, 20 measurements were collected and averaged to calculate the pressure.

Angiotensin II administration. Angiotensin II (Bachem AG) at the dose of 980 ng/kg/min was infused via osmotic mini-pumps (Alzet model 1002). The mini-pumps were implanted subcutaneously between the scapulas following manufacturer's instructions. Angiotensin II was infused for either 14 or 21 day. At the end of the experiment, the mice were sacrificed by CO_2 inhalation and cervical dislocation.

Flow cytometry and cytokine determination. To measure cytokine expression, peritoneal cells were collected and cultured for 5 hrs with 5 μg/ml brefeldin A. Cells were surface-stained with FITC-conjugated anti-F4/80 (BioLegend) followed by intracellular staining of APC-conjugated anti-TNFα (eBioscience) with fixation and permeabilization buffer (eBioscience). The stained samples were analyzed on a Beckman Coulter CyAn ADP and data were analyzed with FlowJo software.

Peritoneal macrophage extraction. Thioglycollate-elicited peritoneal exudates were collected via peritoneal lavage 4 days after a 2 ml intraperitoneal injection of 3% thioglycollate broth. $1x10^6$/ml cells were cultured at 37°C, 5% CO2 in RPMI 1640 medium enriched with 10% fetal calf serum, 50 μM 2-ME, 0.5 mM sodium pyruvate, 10 mM HEPES buffer, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2mM L-glutamine. For purification of macrophages, peritoneal exudates were allowed to adhere for 2 h, after which

non-adherent cells were removed with phosphate buffered saline (PBS) to achieve a >95% purity of macrophages. For flow cytometry assay, peritoneal exudates were cultured in Costar low adherence culture plates. Then, the macrophages were cultured as above with or without lipopolysaccharide (LPS, 1 mg/ml) for 18 h. The concentration of TNFα, IL-10, and IL-12/p40 in the supernatant was determined by ELISA. In Fig. 6a, daily intraperitoneal S-17092 (40 mg/kg) was given was given for 4 days.

POP inhibition with S-17092. The specific POP inhibitor S-17092 was administered daily to mice IP at a dose of 10 mg/kg in normal saline from a freshly prepared solution. For *in vitro* experiments, injection of S-17092 began 4 days before the collection of thioglycollate-induced peritoneal macrophages. The macrophages were carefully counted to verify that the yield of macrophages was not affected by S-17092. For *in vivo* inhibition of POP, S-17092 administration began four days before angiotensin II mini-pump implantation and continued daily until the mice were sacrificed.

***In vivo* LPS challenge**. Mice were injected intraperitoneally with 45 mg/kg of LPS in sterile PBS, and blood was collected 60 min, 90 min, and 120 min after injection. TNFα concentration in sera was measured by ELISA.

Statistics. All results are expressed as mean ± SEM, with $p<0.05$ considered statistically significant. Unless otherwise stated, one-way ANOVA analysis with a Tukey correction was used to analyze significance between groups. For analysis of angiotensin 1-7 levels, data was analyzed using two-way ANOVA taking into account the interaction between genotype and time. Where indicated, we use a two tailed Student's t-test.

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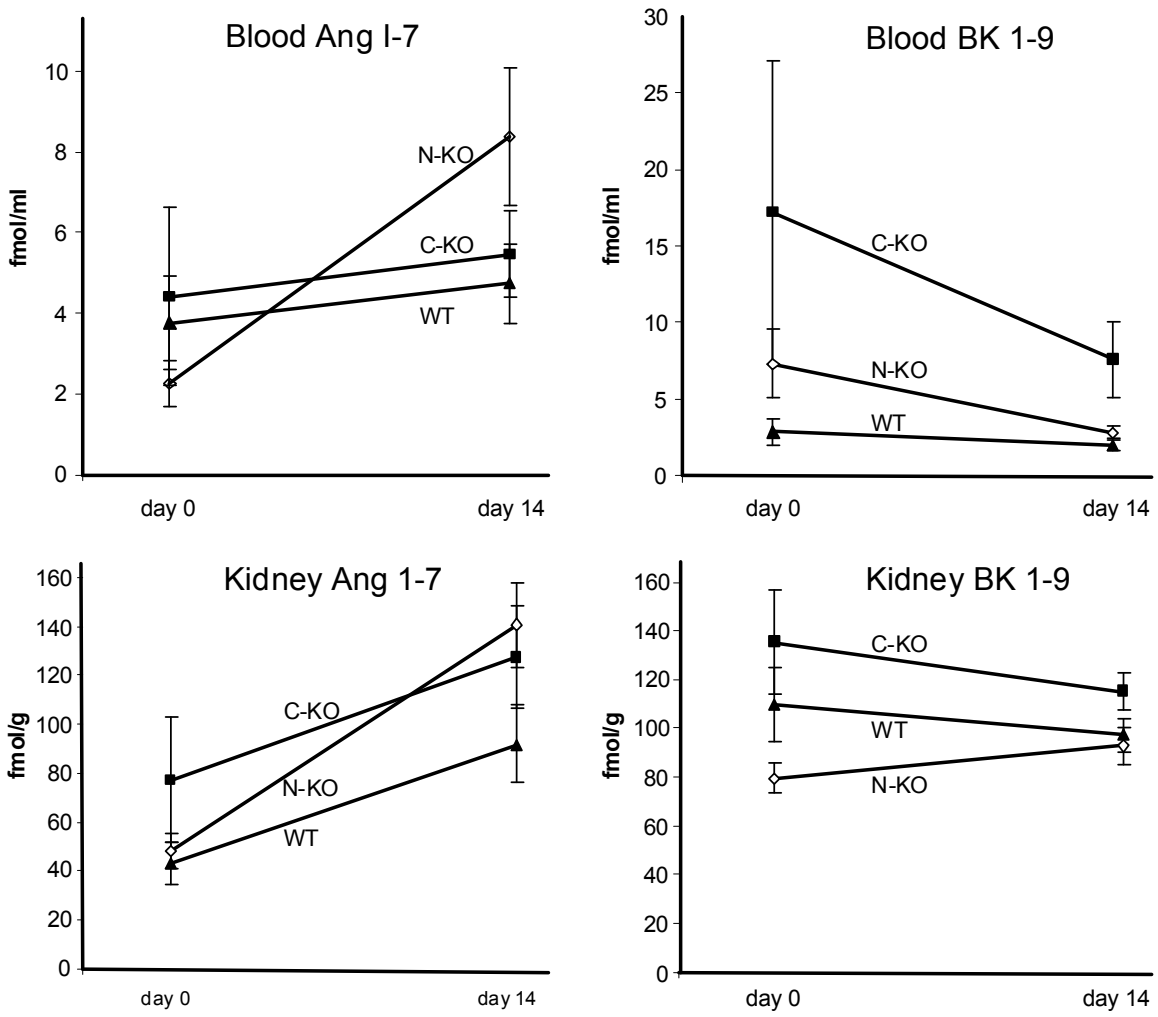
1. Fuchs S, Xiao HD, Adams JW, Frenzel K, Keshelava G, Capecchi MR, Corvol P, Bernstein KE. Role of the N-terminal catalytic domain of ACE investigated by targeted inactivation in mice. *J Biol Chem*. 2004;279:15946-15953.
2. Fuchs S, Xiao HD, Michaud A, Campbell DJ, Hubert C, Capecchi MR, Corvol P, Bernstein KE. The ACE C-terminal catalytic domain is the main site of angiotensin I cleavage in vivo. *Hypertension*. 2008;51:267-274.
3. Campbell, DJ, Kladis, A, Duncan, A-M, Lawrence AC, Strategies for measurement of angiotensin and bradykinin peptides and their metabolites in central nervous system and other tissues. *Methods in Neurosciences*. 1995;23:328-343.
4. Krege JH, Hodgin JB, Hagaman JR, Smithies O. A noninvasive computerized tail-cuff system for measuring blood pressure in mice. *Hypertension*. 1995;25:1111-1115.

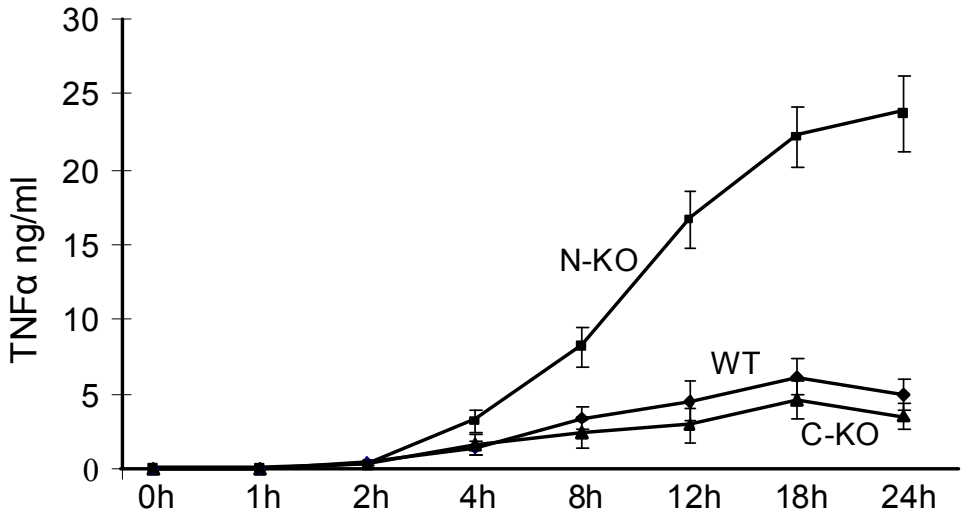
S1. Angiotensin 1-7 and bradykinin peptide levels.



S1. Angiotensin I-7 and bradykinin were measured in the blood and kidney of N-KO, C-KO and WT mice before and after infusion of angiotensin II for 14 days.

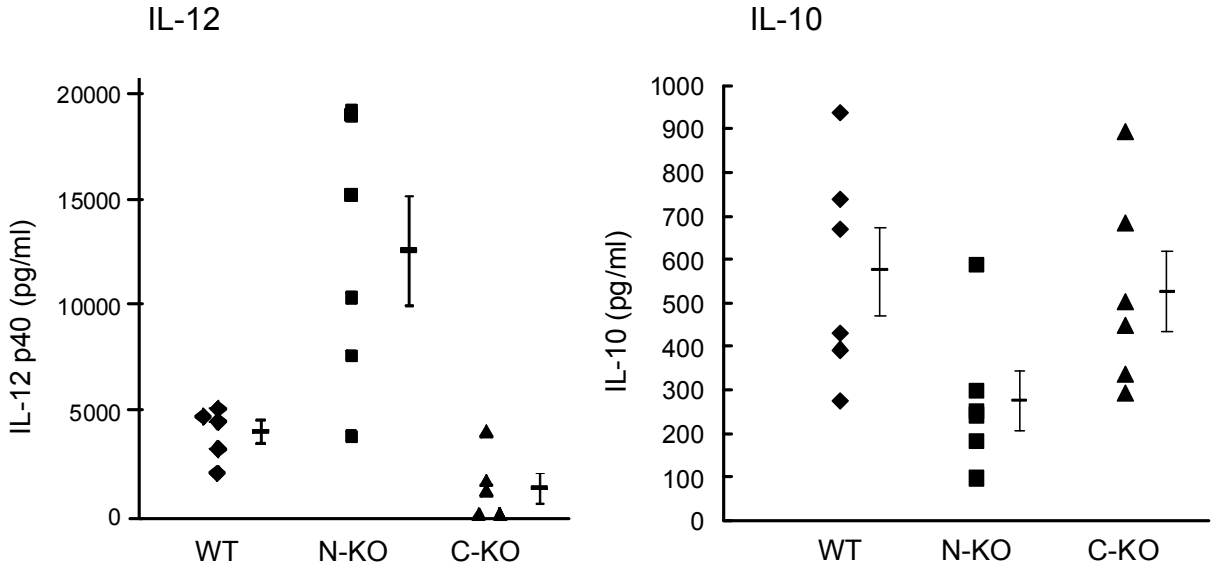
On day 0, no significant differences in blood or renal angiotensin 1-7 were found in WT, N-KO or C-KO mice. With the infusion of angiotensin II, there was a rise in angiotensin 1-7 levels, with the slope of the rise being greatest for the N-KO group. However, by 2-way ANOVA, taking into account the interaction of time and genotype, there were no significant differences between the groups. Also, on day 14, there were no significant differences by 1-way ANOVA. There was no significant difference in blood or kidney bradykinin levels between the 3 groups of mice. For both angiotensin 1-7 and bradykinin, the number of mice studied on day 0 was 12, 6 and 12 for N-KO, C-KO and WT, while for day 14 the number of mice studied was 17, 12 and 15 for N-KO, C-KO and WT. Blood levels of peptide are fmol/ml, while kidney levels are fmol/g.

S2. Time course of TNFα expression.



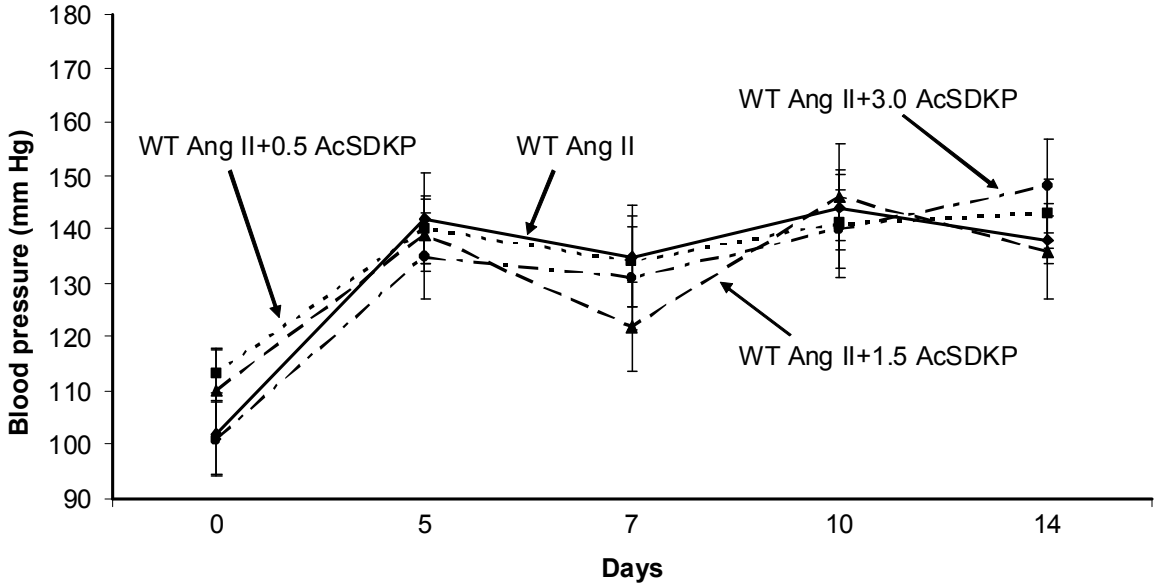
S2. Peritoneal macrophages from N-KO, C-KO and WT mice were collected after thioglycollate injection and cultured with LPS. At the indicated times after LPS addition, the concentration of TNFα was measured by ELISA (n=3 per point).

S3. Macrophage cytokine response to LPS



S3. Peritoneal macrophages from N-KO, C-KO and WT mice were collected after thioglycollate injection. After purification, the macrophages were cultured with LPS for 18 hrs. The concentration of IL-12 (**left**) or IL-10 (**right**) was then determined. Cells from N-KO mice make significantly more IL-12 than from the other groups ($p<0.02$). N-KO cells make less of the inhibitory IL-10. While p values by Student's t test were significant (N-KO vs WT: 0.03; N-KO vs C-KO: 0.05), these values were not significant by one way ANOVA with the Tukey correction (N-KO vs WT: 0.07; N-KO vs C-KO: 0.14).

S4. Effect of AcSDKP on blood pressure in WT mice treated with angiotensin II



S4. WT mice were treated with angiotensin II (♦), angiotensin II + 0.5 mg/kg/day AcSDKP (■), angiotensin II + 1.5 mg/kg/day AcSDKP (▲), and angiotensin II + 3.0 mg/kg/day AcSDKP (●) via minipump. Blood pressure was measured on days 5, 7, 10, and 14 after implantation. The figure shows the group means and SEM. No significant differences were observed. n=10 per group.