Online Supplement

The Cys18-Cys137 Disulfide Bond in Mouse Angiotensinogen

Does Not Affect AngII-dependent Functions In Vivo

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Materials and Methods

Hepatocyte-specific AGT Deficient Mice

AGT floxed mice were developed under a contract with InGenious Targeting Laboratory (Stony Brook, NY, USA) using a construct containing mouse AGT sequence with insertions of 3 loxP and 2 FRT sites, and one neo cassette as described previously.[1,2] This construct was electroporated into 129 embryonic stem cells that were subsequently bred in C57BL/6 mice. The breeding strategy to develop mice with hepatocyte-specific deficiency of AGT included 3 steps: (1) AGT floxed mice were bred to FLPe mice (B6;SJL-Tg(ACTFLPe)9205Dym/J, Stock # 003800, N2 to C57BL/6 strain, The Jackson Laboratory) to remove the neo cassette inserted in intron 2 of mouse AGT gene. (2) After removal of the neo cassette (termed "AGT F/F" in this manuscript), these mice were bred to LDL receptor-/- mice (B6.129S7-Ldlr^{tm1Her}/J, Stock # 002207, N13 to C57BL/6 strain, The Jackson Laboratory) to generate a LDL receptor -/- background. Male mice expressing Cre recombinase under the control of a hepatocyte-specific albumin promoter (B6.Cg-Tg(Alb-cre)21Mgn/J, Stock # 003574, N7 to C57BL/6 strain, The Jackson laboratory) were bred to LDL receptor -/- mice to generate male albumin-Cre+/- mice in the LDL receptor -/- background. (3) AGT F/F x LDL receptor-/- females were bred to albumin-Cre+/- x LDL receptor-/- males to generate wild type (hepAGT+/+) and hepatocyte-specific AGT deficient (hepAGT-/-) mice in LDL receptor-/- background. hepAGT+/+ and -/- littermates were used for experiments described in this manuscript.

All mouse experiments reported in this manuscript were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee (University of Kentucky IACUC protocol number: 2006-0009).

Production and Injection of Adeno-associated Viral (AAV) Vectors

AAV vectors (serotype 2/8) driven by a hepatocyte-specific thyroxine-binding globulin (TBG) promoter were produced by the Viral Vector Core at the University of Pennsylvania. These AAV vectors contained inserts expressing either wild-type mouse AGT or mouse AGT with mutations at Cys18 and Cys137 (cysteines in mouse AGT were replaced by serines). Empty AAV vector (null AAV) was used as control.

AAV vectors were diluted in sterile PBS (200 µl per mouse) and injected intraperitoneally. In a preliminary study, two concentrations (1×10^{10} or 3×10^{10} genome copies for each mouse) of AAVs containing wild type AGT were tested in hepAGT -/- mice. Since 3×10^{10} genome copies of AAV containing wild-type AGT in hepAGT-/- mice resulted in comparable plasma AGT concentrations to hepAGT+/+ mice after prolonged infections (Figure S1), this dosage was used in mice of the present study. hepAGT+/+ mice were injected with null AAV. hepAGT-/- mice were randomized to 3 groups receiving injections of AAVs containing a null insert, wild-type AGT, or Cys18Ser, Cys137Ser (C18S, C137S) mutated AGT.

Mouse Housing Condition and Diets

Male adult mice were maintained in individually vented cages (maximally 5 mice/cage) on a light : dark cycle of 14 : 10 hours. Cage bedding was Teklad Sani-Chip bedding (Cat # 7090A, Harlan Teklad). Mice were fed a normal rodent laboratory diet (Diet # 2918, Harlan Teklad) and provided with drinking water from a reverse osmosis system ad libitum. Two weeks after AAV injections, all mice were fed a diet supplemented with saturated fat (milk fat 21% wt/wt; Diet # TD.88137, Harlan Teklad) for 12 weeks. This diet was developed in 1988 in a collaboration between Harlan Teklad and researchers at Rockefeller University to mimic the composition of a food chain in western countries.[3] This diet is referred to as "Western" diet in this manuscript.

Systolic Blood Pressure Measurements

Systolic blood pressure was measured on conscious mice using a non-invasive tail-cuff system (Coda 8, Kent Scientific Corporation) following a standardized protocol described previously.[4] Briefly, systolic blood pressure measurements were performed at the same time every day prior to AAV injections (baseline), and 2 and 8 weeks after AAV injections. Twenty computerized cycles were run every day for each mouse and mean values were used for data analysis. Data from four consecutive days were collected at each time point for data analysis. This tail-cuff system uses a volume-pressure recording (VPR) method, which provides good agreements on systolic blood pressure measurements, with tail-cuff measurements being 0.25 mm Hg lower than radiotelemetry measurements on average within a range of 110 - 180 mm Hg.[5] However, this method is less accurate, compared to radiotelemetry, when systolic blood pressures are below 110 mm Hg or above 180 mm Hg.

Measurements of Plasma Samples

Mouse blood samples were collected with EDTA (final concentration: 1.8 mg/ml) and centrifuged at 400 g x 20 minutes, 4 °C to separate plasma. For determination of plasma AGT concentrations, blood samples were collected using submandibular bleeding on conscious mice. Plasma AGT concentrations were determined using an ELISA kit (Code # 27413, Immuno-Biological Laboratories Co., Ltd).

At termination, blood samples were collected using right ventricular puncture after anesthesia (a mixture of ketamine 100 mg/kg and xylene 10 mg/kg). For determination of plasma AngII concentrations, aprotinin (0.6 TIU per 1 ml of blood; Cat # RK-APRO, Phoenix Pharmaceuticals, Inc.) was added to blood samples.

Plasma cholesterol concentrations were measured using an enzymatic kit (Cat # 439-17501; Wako Chemicals USA).[6] Lipoprotein distribution of cholesterol in plasma was determined using size exclusion gel chromatography followed by the enzymatic measurement.

Plasma renin concentrations were measured by quantifying AngI generated in

mouse plasma. Briefly, plasma samples (8 µl) harvested with EDTA were incubated in an assay buffer (Na_2HPO_4 0.1 M, EDTA 0.02 M, maleate buffer pH 6.5, phenylmethyl-sulfonyl fluoride 2 µl; total volume of 250 µl) with an excess of rat AGT at 37 °C for 30 minutes. Rat AGT was obtained through partial purification of nephrectomized rat plasma. The reaction was terminated by placing samples at 100 °C for 5 minutes. AngI generated in each sample was quantified by radioimmunoassay using a commercially available kit (Cat # 1553; DiaSorin).

After extraction of plasma samples using Sep-Pak C18 classic cartridges (Cat # WAT051910; Waters Corp.), AngII concentrations were measured using radioimmunoassay. Antibody used for this assay was a rabbit anti-AngII antibody (Cat # T-4005; Bachem) as described previously.[7]

Western Blotting to Determine Disulfide-bridged versus Nonbridged Forms of Mouse AGT

To determine the abundance of disulfide-bridged versus nonbridged form of AGT in mouse plasma, plasma samples were incubated with methoxypolyethylene glycol maleimide (mPEG; Product # 63187; Sigma-Aldrich) as described by Zhou et al.[8] Briefly, mouse plasmas (5 µl) were incubated with mPEG (10 µl of 20 mM) in reaction buffer (5 µl, 100 mM Tris-HCl with pH 8.0, 5 mM EDTA, and 0.15 M NaCl) at 37 °C for 3 hours. Subsequently, reducing loading buffer (Cat # 39000; Thermo Scientific) was added to each sample, and samples were heated at 98 °C for 5 minutes. Proteins (volume of plasma loaded/sample was 0.1 µl) were resolved by SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membranes (Cat # IPVH09120; EMD Millipore). After blocking in fat-free milk buffer (5% wt/vol), membranes containing transferred proteins from mouse plasma were incubated with a chicken anti-mouse AGT antibody (1 µg/ml; 1 hour at room temperature) developed by our laboratory and produced in Aves Labs (Tigard). The sequence of the peptide antigen used to develop this antibody is EEEQPTTSVQQPGSP E (403 aa - 418 aa), which recognizes both full length AGT and its renin cleaved form (des(AngI)AGT). This antibody also recognizes both oxidized and reduced forms of AGT. The specificity of this antibody to mouse AGT was confirmed by the absence of an immunoreactive band in plasma of AGT deficient mice. The secondary antibody used was HRP-conjugated rabbit anti-chicken IgY (Cat # 303-035-003; Jackson ImmunoResearch Laboratories, Inc.) at a concentration of 0.5 µg/ml for 1 hour at room temperature. Immunoreactive bands were visualized by exposing membranes on a Kodak Image Station 4000R Pro after incubation with Pierce enhanced chemiluminescence (ECL) Western blotting substrate.

AngII Measurements in Mouse Kidneys

Mouse renal AngII concentrations were measured as described previously.[9] In brief, kidney samples from the study mice were weighed and homogenized in 10 volumes of ice-cold buffer containing HCl (0.1N), ethanol (80%), o-phenanthroline (0.5 mM), pepstatin (0.1 mM), and captopril (10 µM). Homogenates were centrifuged at 20,000 *g* for 20 min at 4 °C. Supernatants were incubated, centrifuged, and diluted

(1:1) with orthophosphoric acid (0.02%). Angiotensin peptides in each sample were purified using C18 mini-columns and eluted with methanol. Eluate was vacuum-evaporated and reconstituted in the assay buffer. Radioimmunoassay was performed using a rabbit anti-AngII antibody (Cat# T-4005; Bachem/Peninsula Laboratories) to determine AngII concentrations. Data were normalized using kidney sample weights.

Quantification of Atherosclerosis

Atherosclerosis was quantified on the aortic intima including the ascending region, aortic arch and 3 mm of the descending region using an en face method with ImagePro software as described previously.[10,11]

Statistical Analyses

Data are represented as means ± standard error of means (SEM). SigmaPlot version 12.0 (SYSTAT Software Inc.) was used for statistical analyses. To compare multiple-group data, one way ANOVA was used for normally distributed variables and Kruskal-Wallis one way ANOVA on Ranks was used for non-normally distributed variables. Post-hoc analysis for these two analyses were Holm-Sidak method and Dunn's method, respectively, except for plasma renin concentrations and renal AngII concentrations in which post-hoc analysis used Tukey-Kramer adjustment and Benjamini-Hochberg adjustment, respectively. Plasma AGT concentrations (after square root transformation) were compared using two way repeated measures ANOVA and Holm-Sidak post-hoc method. $P < 0.05$ was considered statistically significant.

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Figure S1

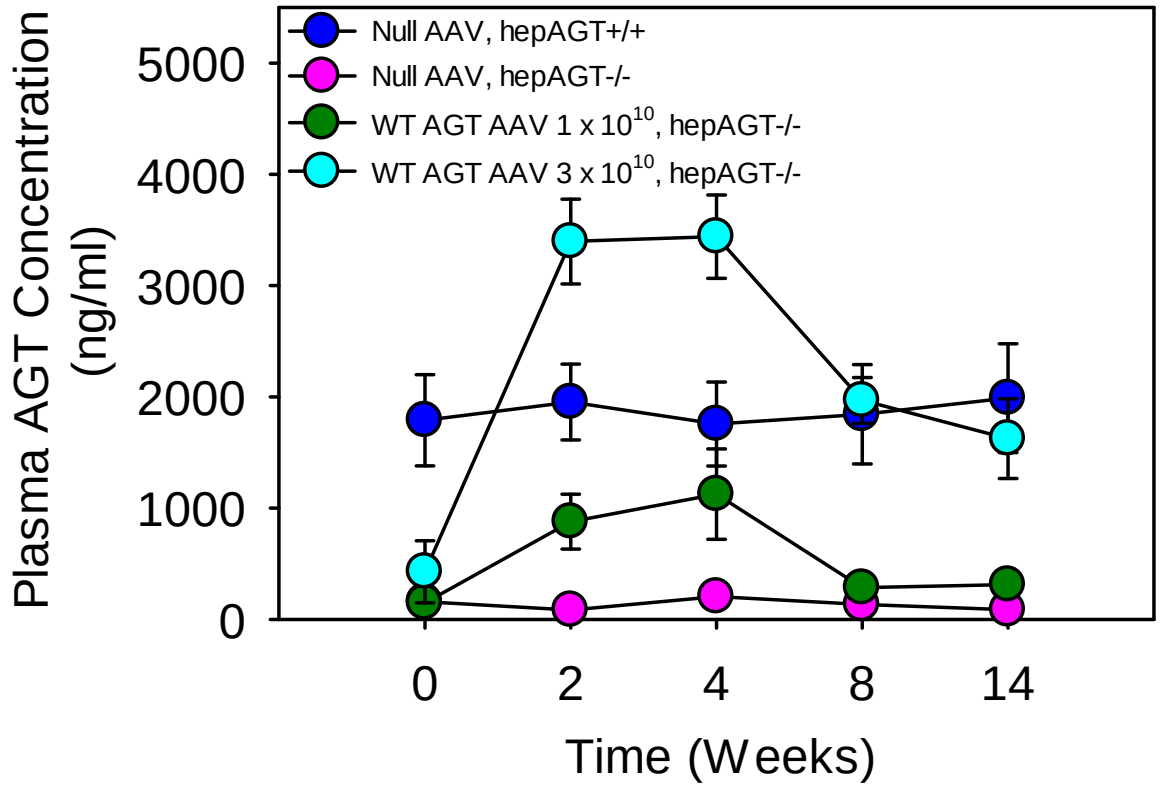

Figure S1. Plasma total AGT concentrations in mice injected with AAVs containing a null insert or wild type AGT. Plasma total AGT concentrations were measured using an ELISA kit at baseline, and 2, 4, 8, and 14 weeks after AAV injection (N = 4 /group). WT: wild-type.

Figure S2

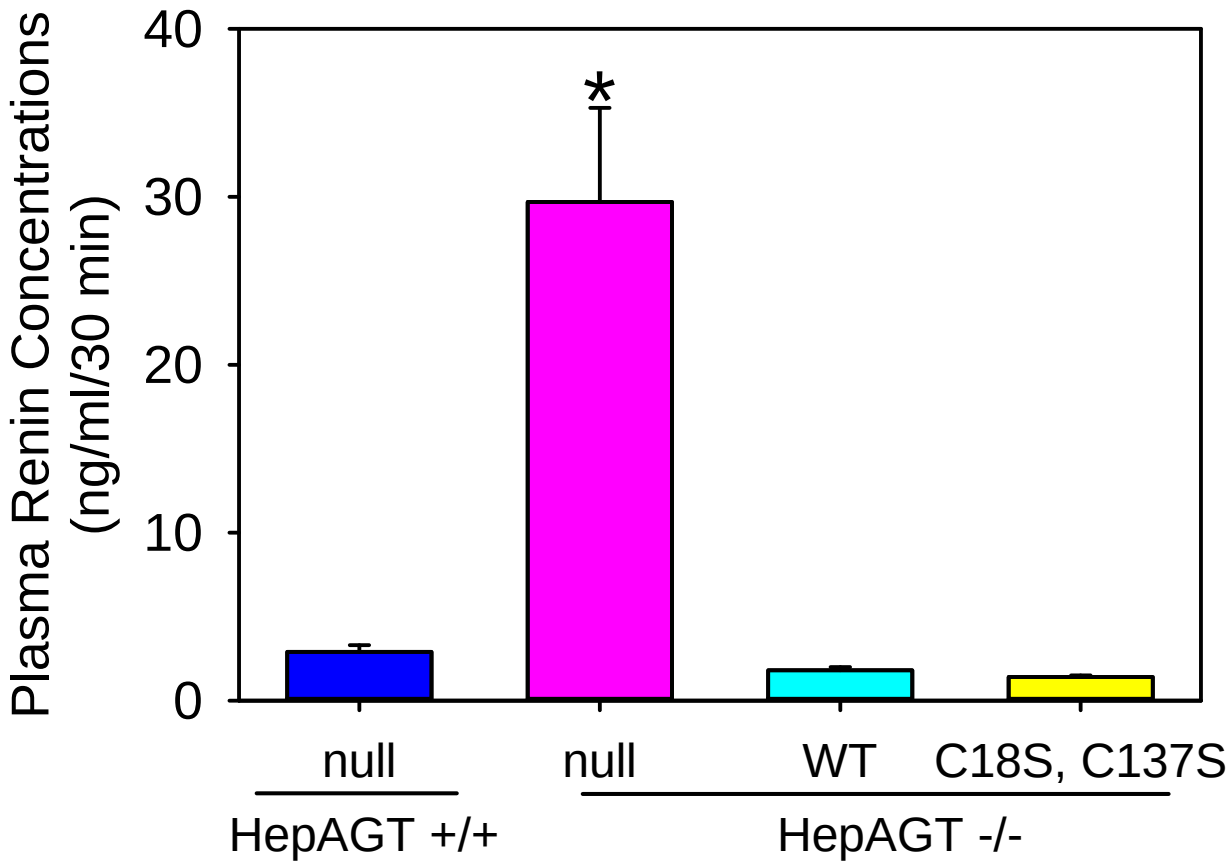

Figure S2. Deletion of Cys18-Cys137 disulfide bond of AGT did not affect plasma renin concentrations in mice. Plasma renin concentrations at 2 weeks after AAV injections were measured using radioimmunoassay. N = 5 - 6/group. * $P < 0.001$ versus the other 3 groups individually by one way ANOVA with Holm-Sidak method.

Figure S3

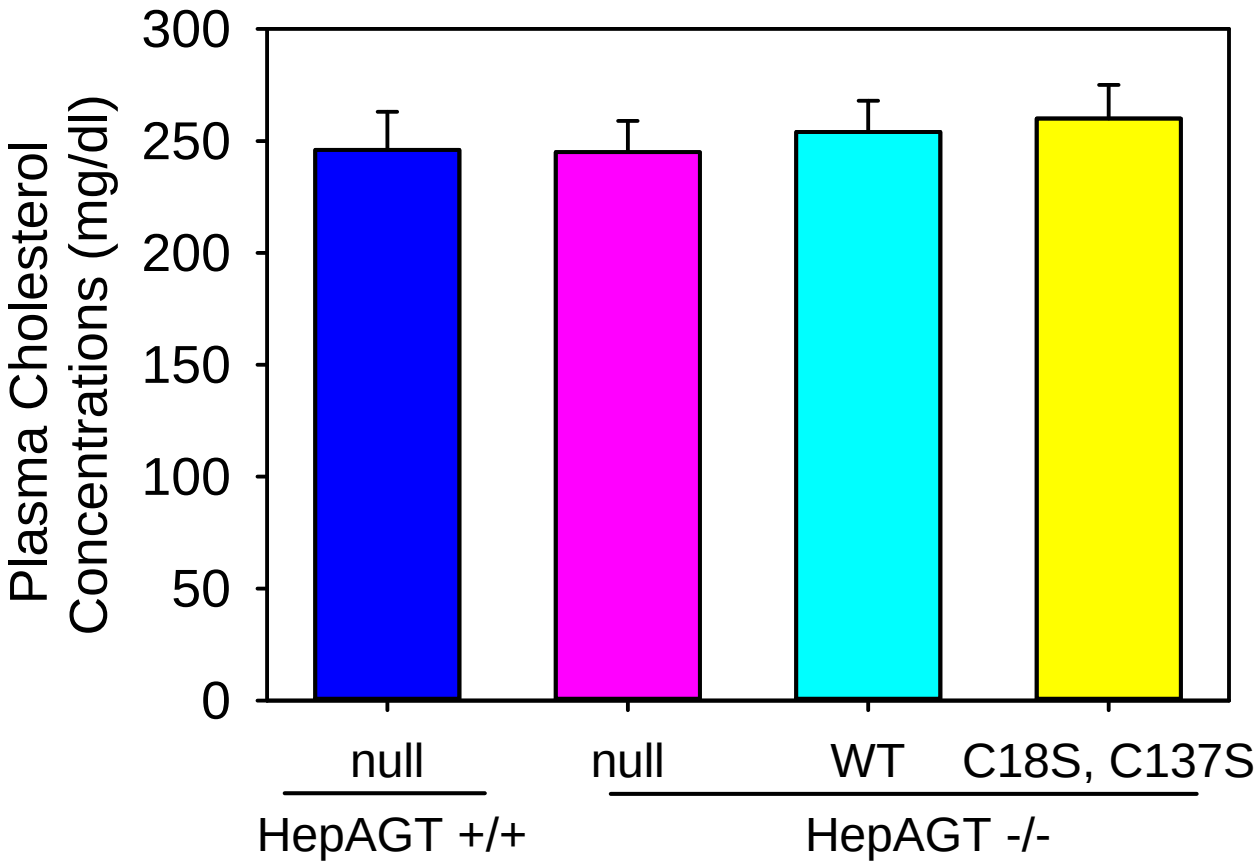

Figure S3. Deletion of Cys18-Cys137 disulfide bond of AGT did not affect plasma cholesterol concentrations. Blood was collected via submandibular bleeding with EDTA 2 weeks after AAV injections. Plasma total cholesterol concentrations were measured using an enzymatic kit. Histobars are mean and error bars are SEM. N = 7 - 10/group. P = 0.89 by one way ANOVA .

Figure S4

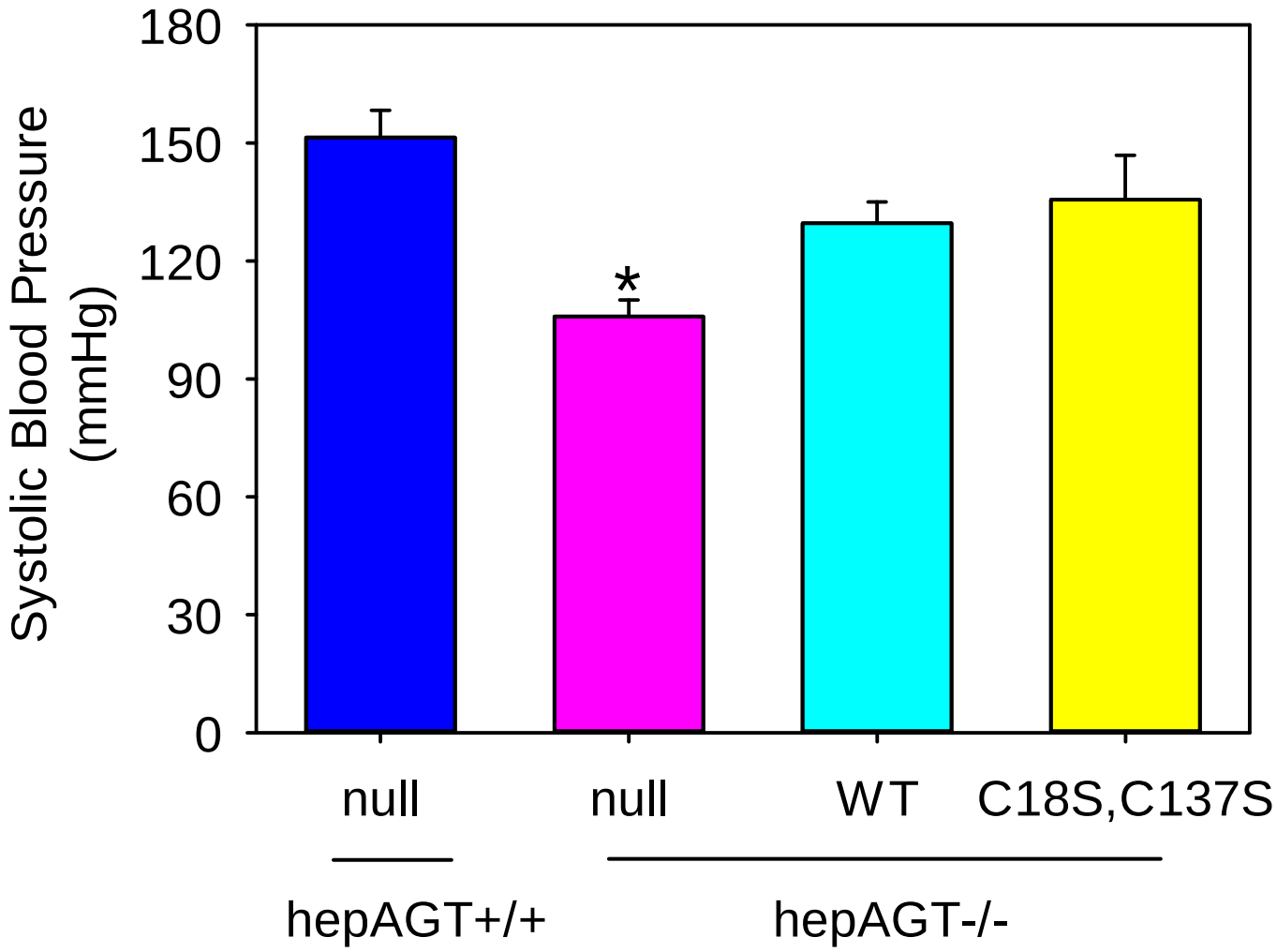

Figure S4. Deletion of Cys18-Cys137 disulfide bond of AGT did not affect systolic blood pressure. Systolic blood pressures were measured using a non-invasive tail-cuff system 2 weeks after AAV injection. N = 4 - 6/group. * P = 0.002 versus hepAGT+/+ mice injected with null AAV by one way ANOVA with Holm-Sidak method.