Cys18-Cys137 Disulfide Bond in Mouse Angiotensinogen Does Not Affect AngII-Dependent Functions In Vivo

Congqing Wu,* Yinchuan Xu,* Hong Lu, Deborah A. Howatt, Anju Balakrishnan, Jessica J. Moorleghen, Craig W. Vander Kooi, Lisa A. Cassis, Jian-an Wang, Alan Daugherty

Abstract—Renin cleavage of angiotensinogen (AGT) releases angiotensin I (AngI) in the initial step of producing all angiotensin peptides. It has been suggested recently that redox regulation of a disulfide bond in AGT involving Cys18-Cys137 may be important to its renin cleavage efficiency in vivo. The purpose of this study was to test this prediction in a mouse model by comparing AngII production and AngII-dependent functions in mice expressing wild-type AGT versus a mutated form of AGT lacking the disulfide bond. Wild-type (hepAGT+/+) and hepatocyte-specific AGT-deficient (hepAGT−/−) littermates were developed in an low-density lipoprotein receptor −/− background. hepAGT+/+ mice were injected intraperitoneally with adeno-associated viral (AAV) vector containing a null insert. hepAGT−/− mice were injected with AAV containing a null insert, wild-type AGT or Cys18Ser and Cys137Ser mutated AGT. Two weeks after AAV injection, mice were fed a Western diet for 12 weeks. Administration of AAV containing either form of AGT led to similar plasma AGT concentrations in hepAGT+/+ mice. High plasma renin concentrations in hepAGT−/− mice were suppressed equally by both forms of AGT, which were accompanied by comparable increases in plasma AngII concentrations similar to hepAGT+/+ mice. AAV-driven expression of both forms of AGT led to equivalent increases of systolic blood pressure and augmentation of atherosclerotic lesion size in hepAGT−/− mice. These measurements were comparable to systolic blood pressure and atherosclerotic lesions in hepAGT+/+ mice. These data indicate that the Cys18-Cys137 disulfide bond in AGT is dispensable for AngII production and AngII-dependent functions in mice. (Hypertension. 2015;65:800-805. DOI: 10.1161/HYPERTENSIONAHA.115.05166.) • Online Data Supplement

Key Words: angiotensin | angiotensinogen | atherosclerosis | blood pressure
modeling-predicted difference in the in vivo conversion of these 2 forms of AGT to angiotensin peptides that are coupled to well established measures of AngII-induced pathophysiological effects.

AngII regulates blood pressure and promotes atherosclerosis.\textsuperscript{6–11} In the present study, we determined effects of the presence versus absence of the AGT disulfide bond on these 2 AngII-dependent effects. We used AGT floxed mice that we have described previously,\textsuperscript{12} in which plasma AGT concentrations were severely depleted by hepatocyte-specific expression of Albumin Cre recombinase. In these mice that had diminutive plasma AGT concentrations, adeno-associated viral (AAV) vectors were injected to populate, in a hepatocyte-specific manner, either wild-type AGT or AGT containing Cys18Ser and Cys137Ser (C18S, C137S) mutants that were unable to form the disulfide bond. In wild-type mice, plasma AGT was present in the completely oxidized form to provide a marked contrast to the mutant AGT that totally lacked the disulfide bridge. This study feature enabled a comparison of AGT proteins that were at either end of the oxidation and reduction spectrum. Comparison of these forms of AGT was unable to discern any differential effects on plasma AngII concentrations or AngII-dependent effects in mice.

**Materials and Methods**

**Animals**

All mouse experiments reported in this article were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee (University of Kentucky IACUC protocol number: 2006-0009). A detailed Methods section is available in the online-only Data Supplement.

**Results**

**Predominance of Disulfide-Bridged AGT in Mouse Plasma**

Western blotting of AGT from wild-type mouse plasma revealed a single band with molecular weight of \(\approx 50 \text{kDa}\) (Figure 1A). Antibody specificity against AGT was demonstrated by the lack of a discernable band in plasma harvested from hepAGT\textsuperscript{−/−} mice (Figure 1A). Incubation of plasma from wild-type mice with mPEG alone increased molecular weight of AGT by 10 kDa, indicating that 2 of the 4 AGT cysteine residues are reactive to mPEG (Figure 1B). Next, mice were injected with AAV encoding either wild-type or mutated (C18S, C137S) AGT. Two weeks post AAV injection, plasmas were collected from all groups and analyzed using Western blotting after preincubation with mPEG. Western blotting of plasma AGT failed to detect any immunoreactive band in hepAGT\textsuperscript{−/−} mice to mPEG (Figure 1B). Next, mice were injected with AAV encoding either wild-type or mutated (C18S, C137S) AGT. Two weeks post AAV injection, plasmas were collected from all groups and analyzed using Western blotting after preincubation with mPEG. Western blotting of plasma AGT failed to detect any immunoreactive band in hepAGT\textsuperscript{−/−} mice injected with AAV containing a null insert (Figure 1C). In contrast, a 60 kDa immunoreactive band was detected clearly in each hepAGT\textsuperscript{+/−} mouse repopulated with wild-type AGT, demonstrating that AAV-derived wild-type AGT was completely oxidized (Figure 1C). Plasma in mice repopulated with mutated (C18S, C137S) AGT showed a 60 kDa band caused by interaction of mPEG with the remaining 2 cysteine residues.

These data are consistent with mPEG labeling the 2 cysteines at positions 300 and 316 on the non-renin interacting face, but being unable to interact with vicinal cysteines at position 18 and 137 because of the disulfide linkage. These results are in contrast to human AGT, which shows labeling of either 2 or 4 cysteines,\textsuperscript{3} indicating a difference between species with a stable C18S, C137S disulfide bond predominant in mouse plasma.

**AAV Vector Infection Generated Equivalent Plasma Concentrations of Disulfide- and Non–Disulfide Bridged Forms of AGT**

To evaluate expression efficiency and stability of AAV vectors, we measured plasma AGT concentrations before AAV injection and at multiple intervals after AAV injection. At baseline (week 0), hepAGT\textsuperscript{−/−} mice lacking AGT expression in hepatocytes had barely detectable plasma AGT concentrations.
The low plasma AGT concentrations remained unchanged in hepAGT−/− mice receiving AA V vector containing the null insert (Figure 2A). Plasma AGT concentrations in hepAGT−/− mice receiving AA V vector expressing either wild-type or mutated AGT peaked 2 weeks postinjection and remained at concentrations comparable to hepAGT+/+ littermates from 6 to 14 weeks after AA V injections. At study end point, there was no difference in plasma AGT concentrations except for hepAGT−/− mice administered AA V vector containing the null insert (Figure 2A).

**Disulfide- and Non–Disulfide Bridged Forms of AGT Had Equivalent Effects on Plasma Renin and Plasma and Renal AngII Concentrations**

Plasma renin concentrations were increased significantly in hepAGT−/− mice infected with the null AA V vector as determined 2 weeks after AA V injections (Figure S2 in the online-only Data Supplement) or at termination (Figure 2B), which were caused by diminished negative feedback attributable to low concentrations of plasma AngII (Figure 2C).13 This contrasted with the renin and AngII concentrations in hepAGT+/+ mice receiving the null AA V vector (Figure 2B and 2C).

HepAGT−/− mice replenished with wild-type AGT showed no significant differences in plasma renin and AngII concentrations compared with hepAGT+/+ littermates. Plasma renin and AngII concentrations in hepAGT−/− mice infected with mutated AGT AA V were not significantly different from those in hepAGT−/− mice administered AA V expressing wild-type AGT (Figure 2B and 2C).

Kidney is the major source of renin and has abundant prorenin receptors. To determine the effects of disulfide bond of AGT in local production of AngII, we measured AngII concentrations in kidney tissues of the study mice. As expected, renal AngII concentrations were lower in hepAGT−/− mice than in hepAGT+/+ mice receiving the null AA V vector (Figure 2B and 2C). HepAGT−/− mice replenished with wild-type AGT showed no significant differences in plasma renin and AngII concentrations compared with hepAGT+/+ littermates. Plasma renin and AngII concentrations in hepAGT−/− mice infected with mutated AGT AA V were not significantly different from those in hepAGT−/− mice administered AA V expressing wild-type AGT (Figure 2B and 2C).

**Disulfide- and Non–Disulfide Bridged Forms of AGT Had Equivalent Effects on AngII-Mediated Effects**

Two weeks after AA V injections before Western diet feeding, plasma cholesterol concentrations were equivalently low in all study groups (Figure S3). Consistent with our previous findings,6,14,15 low-density lipoprotein (LDL) receptor−/− mice fed...
Western diet for 12 weeks exhibited severe hypercholesterolemia that were attributed to increases of very low-density lipoproteins and LDL as demonstrated by plasma total cholesterol measurements and lipoprotein distribution analyses (Figure 4A and 4B). Mouse genotype or AA V infection had no effect on plasma cholesterol concentrations and lipoprotein distribution.

In agreement with the low plasma AngII concentrations, administration of AA V containing the null vector to hepAGT−/− mice had lower systolic blood pressure both at 2 weeks post AA V injection (Figure S4) and the end point (Figure 5A), compared with their hepAGT+/+ littermates. Administration of AAV vectors containing either wild-type or the mutated form of AGT increased systolic blood pressure in hepAGT−/− mice to levels equivalent to hepAGT+/+ littermates. However, systolic blood pressures were not significantly different between mice expressing wild-type and mutated AGT (Figure S4; Figure 5A).

LDL receptor−/− mice develop profound atherosclerotic lesions in the aortic arch region after 12 weeks of Western diet feeding.7,14,16 hepAGT−/− mice administered AAV containing the null vector had significantly smaller lesions compared with hepAGT+/+ littermates, as measured by percent lesion area in the aortic arch region. In contrast, hepAGT−/− mice receiving either wild-type or mutated AGT developed pronounced atherosclerotic lesions, and percent lesion areas were not different between these 2 groups, as well as compared with hepAGT+/+ mice (Figure 5B).

**Discussion**

In the present study, we used a mouse model with nearly undetectable plasma AGT concentrations as a result of hepatocyte-specific disruption of the mouse Agt gene to determine whether the presence of the disulfide bond in AGT is critical in determining AngII production and AngII-dependent effects in mice. Plasma AGT was repopulated in these mice by injection with AAVs expressing wild-type or a mutated form of AGT in a hepatocyte-specific manner. The mutated form of AGT lacked the disulfide bond between Cys18 and Cys137 because of their substitutions by serines. Because plasma AGT in mice exists exclusively in a disulfide-bridged form, this system provides 2 extreme conditions of AGT, namely, completely disulfide-bridged form versus unbridged form, to determine its functional consequences. Using this in vivo system, the combined findings of multiple parameters demonstrate that the absence of Cys18-Cys137 disulfide link has no discernable effects on plasma and local AngII concentrations and AngII-dependent functions in mice. Therefore, although all AGT in mouse plasma has the disulfide bridge, this fact did not negate the equivalent capability of mouse renin to cleave non-disulfide

![Figure 4](image-url) Deletion of Cys18-Cys137 disulfide bond of angiotensinogen (AGT) did not affect plasma cholesterol concentrations. **A**, Plasma total cholesterol concentrations at termination were measured using an enzymatic kit. Histobars are mean and error bars are SEM. N=7 to 10/group. *P<0.05 by Kruskal–Wallis 1-way ANOVA on Ranks. **B**, Plasma lipoprotein distributions were resolved by size exclusion chromatography. Circles and error bars are means±SEM. WT represents wild-type AGT adeno-associated viral (AAV) and C18S, C137S represents AGT in AAV vector with Cys to Ser mutation at 18 and 137 residues.

![Figure 5](image-url) Deletion of Cys18-Cys137 disulfide bond of angiotensinogen (AGT) did not affect systolic blood pressure or atherosclerosis. **A**, Systolic blood pressures were measured using a noninvasive tail-cuff system 8 weeks after adeno-associated viral (AAV) injection. N=4 to 6/group. *P<0.01 versus the other 3 groups by 1-way ANOVA with Holm–Sidak method. **B**, Atherosclerotic lesion areas were measured on the intimal surface of the aortic arch region using an en face method. Percent lesion area=lesion area/the entire intimal area×100%. Triangles represent values of individual mice (N=8–11/group), circles represent means, and error bars are SEM. *P<0.001 versus the other 3 groups by 1-way ANOVA with Holm–Sidak method. WT represents wild-type AGT AAV and C18S, C137S represents AGT in AAV vector with Cys to Ser mutations at 18 and 137 residues.
bonded AGT, implicating that disulfide bond is not necessary for renin to get access to the cleavage site of AGT as proposed by Zhou et al.3

Cys18 and Cys138 disulfide linkage (Cys18-Cys137 in mouse) of AGT is conserved across species.1 In vitro studies of disulfide-bridged versus unbridged form of AGT by Zhou et al3 revealed minor changes of the $K_m$ and $K_{cat}$ when incubated with renin alone. Although no statistical significance was stated, differences in reaction kinetics of these 2 forms of AGT with renin appeared to be enhanced in the presence of pro-renin receptors and accompanied by increases in AngI release.3 Currently, a role of pro-renin receptors in AngI release in vivo has not been determined,17 although the high abundance of pro-renin receptors in the kidney is consistent with a possible role in blood pressure regulation. It has been demonstrated recently that hepatocyte-derived AGT is required for blood pressure maintenance via a kidney-based production of AngII.18 Therefore, the system used in the present study provided an opportunity for both forms of AGT to engage with renal pro-renin receptors to regulate blood pressure through an AngII-dependent mechanism. Because kidney is the major source of renin and has abundant presence of pro-renin receptors, we determined whether the disulfide bond of AGT would affect local AngII productions as proposed by Zhou et al.3 Renal AngII concentrations were not diminished by removal of the disulfide bond in AGT, providing compelling evidence that the presence of this disulfide bond does not enhance the effectiveness of AGT cleavage by renin and pro-renin receptor interaction.

Development of atherosclerosis in LDL receptor−/− mice fed Western diet is profoundly influenced by AngII interacting with AT1a receptors.6,7 Therefore, the effects of AGT manipulations on atherosclerosis are attributable to generation of AngII. Unlike blood pressure responses, there has been no inference of a role for pro-renin receptors on the local response of atherogenic AngII. However, the reduced lesion size in LDL receptor−/− mice with renin-deficient macrophages implies a local production of this AngII.14 Therefore, the effect of AGT on atherosclerosis seems to be caused by its cleavage at a cellular level.5 Altogether, the system designed in this study provides an in vivo approach to test effects of the 2 forms of AGT on 2 major AngII-dependent end points, blood pressure, and atherosclerosis. Neither was affected by the presence versus absence of the disulfide bridge in AGT.

The extent of the disulfide bond formation between Cys18 and Cys137 of AGT has been proposed as a novel mechanism of blood pressure regulation in humans.3,39 Our data indicate that this is not the case in mice. There are several differences of AGT between mouse and human. These differences provided an optimal environment to determine whether the disulfide bond of AGT plays a critical role in the generation of AngII. First, as demonstrated in the present study, only the disulfide-bridged form of AGT is present in mouse plasma. In contrast, humans have both disulfide-bridged and non-bridged forms in a ratio that is ≈60:40.3 The exclusively disulfide bonded form of AGT in mouse plasma did not negate the ability of mouse renin to cleave non–disulfide bonded AGT applied exogenously, implicating that the disulfide bond does not facilitate renin access to the cleavage site of AGT as proposed by Zhou et al.3 Second, plasma AGT concentrations in humans are ≈1 μM,20,21 which is close to the Michaelis–Menten constant of human renin in plasma (1.25 μM).22 Plasma AGT concentrations in mice are ≈40 nmol/L,23 which is 25x lower than its concentrations in humans. Therefore, AGT is the rate-limiting factor for the generation of AngI in mouse plasma,23 whereas renin is the rate-limiting enzyme to cleave AGT in humans. A shortcoming of existing methodologies is that these data represent measurements of total AGT concentrations and do not discriminate between intact and renin-cleaved des(AngI) AGT forms. Hence, concentrations of intact AGT that serves as the renin substrate are unknown in either species. More importantly, in contrast to what was hypothesized by the study of Zhou et al,3 local production of AngII in kidney tissues was equivalent between mice injected with wild-type and Cys18Ser- and Cys137Ser-mutated AGT AAVs. We conclude that the disulfide bond of mouse AGT does not alter AGT–renin interactions and cleavage, either in circulation or local tissues, such as kidney.

In summary, we sought to directly determine the role of Cys18-Cys137 disulfide bridge of AGT in vivo on AngII-dependent functions. Using a system in which AGT was present at either end of the oxidation-reduction spectrum, we did not distinguish any differences between the 2 forms on plasma AngII production and AngII-dependent functions in mice. This does not negate a role for the AGT disulfide bridge in humans where further research is needed to determine the contribution of this disulfide bridge to blood pressure regulation. The provocative hypothesis that liability of the disulfide bridge of AGT is a modulator of blood pressure was primarily based on a cross sectional study that determined the ratio of oxidized to reduced AGT in 12 women with preeclampsia compared with 12 normotensive pregnant women.3 Confirmation in a larger cohort study would be enhanced by measurements of absolute concentrations of oxidized forms of AGT, coupled with plasma concentrations of AngII.

Perspectives

Using an in vivo mouse system, we demonstrated that structural modulation by formation or removal of the disulfide bridge between Cys18 and Cys137 did not influence AngII production and AngII-dependent blood pressure regulation and atherosclerosis. Because multiple divergences exist between humans and mice, future studies are necessary to determine the importance/relevance of this structural modulation of AGT in regulation of AngII production and functions in human subjects.

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AGT Disulfide Bond and AngII Functions

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Novelty and Significance

What Is New?

• This article reports a new mouse model of hepatocyte-specific deficiency of angiotensinogen (AGT) in a hypercholesterolemic background that provides an excellent experimental approach for studying AGT-related physiological and pathophysiological functions.

• This is the first study revealing that AGT in mouse plasma is mainly disulfide-bridged.

• This is the first study that directly determined effects of the disulfide bond of AGT in local production of angiotensin (Ang II).

• This is the first study providing in vivo evidence that the disulfide bridging of AGT does not influence AngII production and AngII-dependent regulation of blood pressure and atherosclerosis in mice.

What Is Relevant?

• AGT is the only known precursor of the renin-angiotensin system, a critical hormonal system in blood pressure regulation and the development of atherosclerosis.

• The pathophysiological role of the disulfide bond of AGT has been highlighted as the linchpin of a recent study published in the Nature by Zhou et al. This published prediction has become a debate in multiple reviews whether this is applicable to hypertension or other Ang-mediated conditions in humans.

Summary

In mice with hepatic-specific deficiency of AGT, repopulation of AGT that contains (Cys18-Cys137) or does not contain (Ser18-Cys137) the disulfide bond has equivalent effects on renin regulation, AngII production, blood pressure regulation, and the development of atherosclerosis.
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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<sup>tm1Her</sup>/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 x 10<sup>10</sup> or 3 x 10<sup>10</sup> genome copies for each mouse) of AAVs containing wild type AGT were tested in hepAGT-/- mice. Since 3 x 10<sup>10</sup> 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. 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. 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. 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). 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 (Na₂HPO₄ 0.1 M, EDTA 0.02 M, maleate buffer pH 6.5, phenylmethylsulfonyl 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 EEEEQPTTSVQQPGSE (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.\textsuperscript{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.
References


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. 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. 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. 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.