Strong and Sustained Antihypertensive Effect of Small Interfering RNA Targeting Liver Angiotensinogen

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Abstract—Small interfering RNAs (siRNAs) targeting hepatic angiotensinogen (*Agt*) may provide long-lasting antihypertensive effects, but the optimal approach remains unclear. Here, we assessed the efficacy of a novel AGT siRNA in spontaneously hypertensive rats. Rats were treated with vehicle, siRNA (10 mg/kg fortnightly; subcutaneous), valsartan (31 mg/kg per day; oral), captopril (100 mg/kg per day; oral), valsartan+siRNA, or captopril+valsartan for 4 weeks (all groups, n=8). Mean arterial pressure (recorded via radiotelemetry) was lowered the most by valsartan+siRNA (−68±4 mm Hg), followed by captopril+valsartan (−54±4 mm Hg), captopril (−23±2 mm Hg), siRNA (−14±2 mm Hg), and valsartan (−10±2 mm Hg). siRNA and captopril monotherapies improved cardiac hypertrophy equally, but less than the dual therapies, which also lowered NT-proBNP (N-terminal pro-B-type natriuretic peptide). Glomerular filtration rate, urinary NGAL (neutrophil gelatinase-associated lipocalin), and albuminuria were unaffected by treatment. siRNA lowered circulating AGT by 97.9±1.0%, and by 99.8±0.1% in combination with valsartan. Although siRNA greatly reduced renal Ang (angiotensin) I, only valsartan+siRNA suppressed circulating and renal Ang II. This coincided with decreased renal sodium hydrogen exchanger type 3 and phosphorylated sodium chloride cotransporter abundances. Renin and plasma K^+ increased with every treatment, but especially during valsartan+siRNA; no effects on aldosterone were observed. Collectively, these data indicate that Ang II elimination requires >99% suppression of circulating AGT. Maximal blockade of the renin-angiotensin system, achieved by valsartan+siRNA, yielded the greatest reduction in blood pressure and cardiac hypertrophy, whereas AGT lowering alone was as effective as conventional renin-angiotensin system inhibitors. Given its stable and sustained efficacy, lasting weeks, RNA interference may offer a unique approach to improving therapy adherence and treating hypertension. (*Hypertension*. 2019;73:1249-1257. DOI: 10.1161/HYPERTENSIONAHA.119.12703.) ● Online Data Supplement

Key Words: acute kidney injury ■ hypertrophy, left ventricular ■ hypertension ■ renin-angiotensin system ■ RNA, small interfering ■ RNAi therapeutics

Treatment of hypertension generally requires multiple antihypertensive drugs, but is hampered by reduced adherence with every drug added to the treatment regimen.[1,2] The management of hypertension is further complicated by the upregulation of counterbalancing mechanisms, for example, the rise in renin during blockade of the renin-angiotensin system (RAS). As a consequence, Ang II (angiotensin II) levels are often restored to their original, pretreatment levels during the chronic treatment phase.[3–5] All angiotensin stems from AGT (angiotensinogen), and blockade of the RAS at this level may ultimately annihilate RAS activity. Indeed, subcutaneously delivered *Agt*-directed antisense oligonucleotides and intravenous injection of lipid nanoparticles containing small interfering RNAs (siRNAs) targeting *Agt* effectively lower blood pressure in hypertensive rats.[6,7] Still, these approaches typically require weekly doses and may have a less-ideal safety profile.[8] Ongoing efforts to optimize siRNA designs have now yielded hepatocyte-directed, N-acetylgalactosamine (GalNAc)-conjugated molecules with long-term, single-dose efficacy. In fact, stable suppression of PCSK9 (proprotein convertase subtilisin/kexin type 9) has been achieved in a phase 2 clinical trial, employing biannual dosing of one such siRNA.[9] In the current study, we set out to investigate the efficacy of a GalNAc-conjugated siRNA targeting *Agt* in spontaneously hypertensive rats (SHRs), making a detailed comparison with the angiotensin-converting enzyme inhibitor (ACE inhibitor) captopril and the angiotensin receptor blocker valsartan, as well as with dual RAS blockade (AGT siRNA+valsartan and captopril+valsartan). We focused not only on blood pressure, but also unraveled the underlying RAS biochemistry

and monitored the renal effects of these treatments, given the well-known side effects of RAS blockade, that is, hypotension, renal dysfunction, and hyperkalemia.[10] Using this approach, we demonstrate a sustained antihypertensive and cardioprotective effect of AGT siRNA.

Methods

All supporting data are available within the article and in the online-only Data Supplement.

Animal Studies

All studies were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC (protocol number 16-511-01). Male, 12-week-old SHRs, receiving a standard sodium diet, were treated for 4 weeks with vehicle, valsartan (31 mg/kg per day dissolved in drinking water), captopril (100 mg/kg per day dissolved in drinking water), captopril+valsartan, AGT siRNA (10 mg/kg fortnightly; subcutaneous injection; provided by Alnylam Pharmaceuticals, Cambridge, MA), or valsartan+siRNA (all groups, n=8). Conjugation of siRNA to triantennary GalNAc ensures selective delivery to hepatocytes. Although single and bi-weekly dosing provided similar AGT suppression over a 4-week period (Table S1 in the online-only Data Supplement), fortnightly dosing of 10 mg/kg AGT siRNA was selected to ensure maximal target silencing. For valsartan and captopril, water intake was checked every other day, and drug concentrations were adjusted to achieve the described daily doses. Blood pressure, heart rate, and activity were recorded continuously via radiotelemetry. Oligonucleotide synthesis and complete procedures are described in the online-only Data Supplement.

Biochemical Measurements, Quantitative Polymerase Chain Reaction, siRNA Quantification, and Western Blotting

In plasma, AGT and active plasma renin concentration were measured by enzyme kinetic assays. In the cases that measurements were at or below the lower limit of detection, this limit was applied to allow for statistical analysis. Urinary AGT was measured by direct AGT radioimmunoassay and plasma aldosterone by solid-phase radioimmunoassay. Angiotensin and bradykinin (BK) metabolites in blood and renal tissue were measured by liquid chromatography-tandem mass spectrometry analysis as described before. Procedures and lower limits of detection are described in the online-only Data Supplement.

Total RNA was isolated from selected tissues and reverse transcribed into cDNA. Gene expression was quantified using primers designed with NCBI (Primer-BLAST; Table S2). A gene-specific TaqMan assay was used for *Agt* mRNA quantification. The ΔΔCt method was used for relative quantification of mRNA expression levels.

siRNA quantification was performed as described previously. Antisense levels in siRNA standard curve dilutions, kidney and liver samples were quantified by stem-loop reverse transcription followed by quantitative polymerase chain reaction. Primer and probe sequences are described in the online-only Data Supplement. Ct values derived from samples were interpolated onto the standard curve, adjusted for sample dilution, and expressed as micrograms of antisense strand per gram of tissue.

Whole kidney protein extract was separated by electrophoresis, transferred to a membrane, and incubated overnight at 4°C with primary antibodies against renal transporters described in the online-only Data Supplement . Signals were detected by chemiluminescence and quantified using ImageQuant. Samples (n=8 per group) were
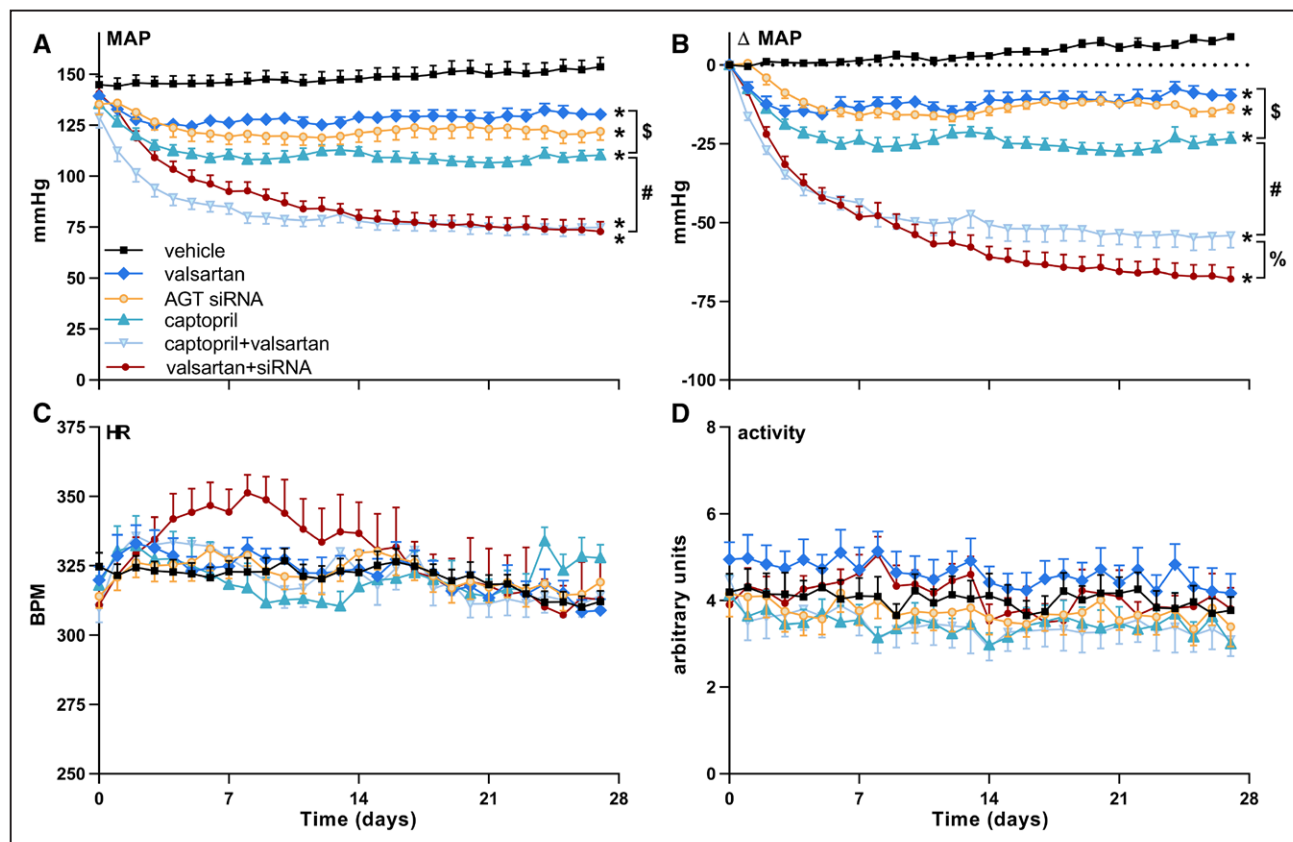
Figure 1. Mean arterial pressure (MAP), heart rate (HR), and locomotor activity. Spontaneously hypertensive rats were treated for 28 d with vehicle, valsartan, AGT (angiotensinogen) small interfering RNAs (siRNAs), captopril, valsartan+siRNA, or captopril+valsartan (all groups, n=8). **A**, MAP, (**B**) change from baseline MAP (ΔMAP), (**C**) HR, and (**D**) locomotor activity were recorded via radiotelemetry. Data, represented as means±SEM, were analyzed using 2-way ANOVA and post hoc Bonferroni (*$P \leq 0.0001$ vs vehicle; $P \leq 0.01$; #$P \leq 0.0001$; %$P \leq 0.05$). BPM indicates beats per minute.
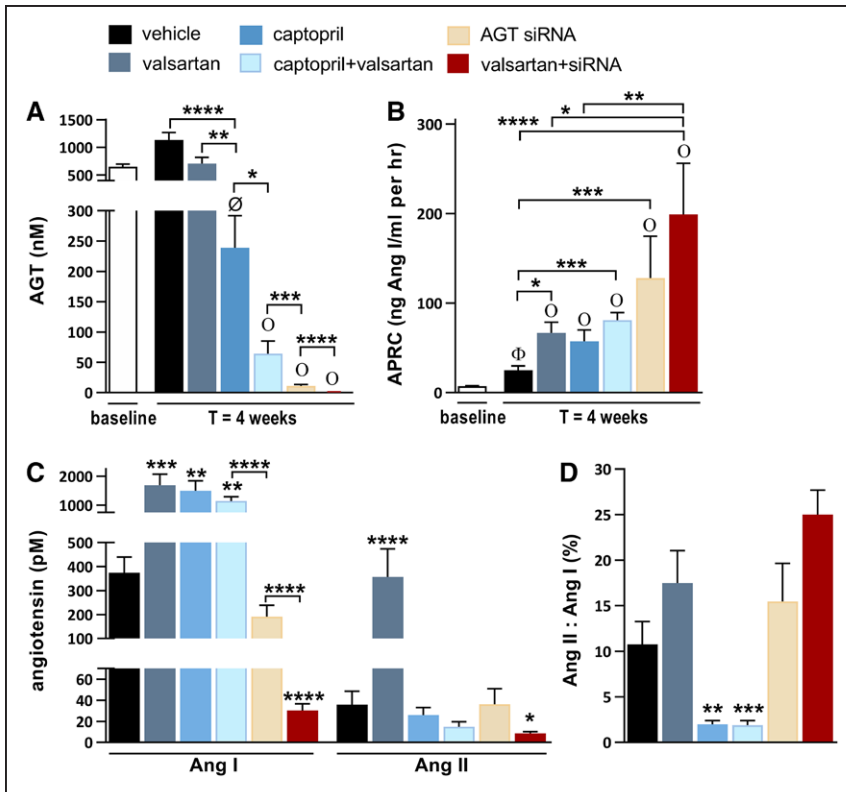
Figure 2. Renin-angiotensin system parameters in plasma. Plasma levels of (**A**) AGT (angiotensinogen) and (**B**) active plasma renin concentration (APRC) of spontaneously hypertensive rats before treatment (baseline) and after 4 wk of vehicle, valsartan, captopril, AGT small interfering RNAs (siRNA), captopril+valsartan, or valsartan+siRNA treatment (all groups, n=8). Plasma (**C**) Ang (angiotensin) levels and (**D**) Ang II/Ang I ratio after 4 wk of treatment. Data are represented as means±SEM. Natural log-transformed data were analyzed using repeated measures or 1-way ANOVA and post hoc Bonferroni (øP≤0.05, ΦP≤0.01, OP≤0.0001 vs baseline; *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 vs vehicle or otherwise indicated in graph).

divided over 2 blots (n=4 per group per blot). To allow for combined statistical analysis, samples were first normalized to the loading control actin, followed by normalization to the average of the vehicle-treated rats from the respective immunoblot.

Kidney Function

Glomerular filtration rate (GFR) in awake rats was measured before treatment (baseline), and after 2 and 4 weeks of treatment via transcutaneous measurement of fluorescein isothiocyanate (FITC)-labeled sinistrin, administered via the tail vein. A noninvasive clearance kidney fluorescent detection device together with partner software generates the elimination kinetics curve of FITC-sinistrin. Excretion half-life ($t_{1/2}$) is used to derive GFR using a conversion factor and formula validated for rats:

GFR (mL/min per 100 g body weight (BW))=31.26 (mL/100 g BW)/$t_{1/2}$ FITC-sinistrin (minutes).
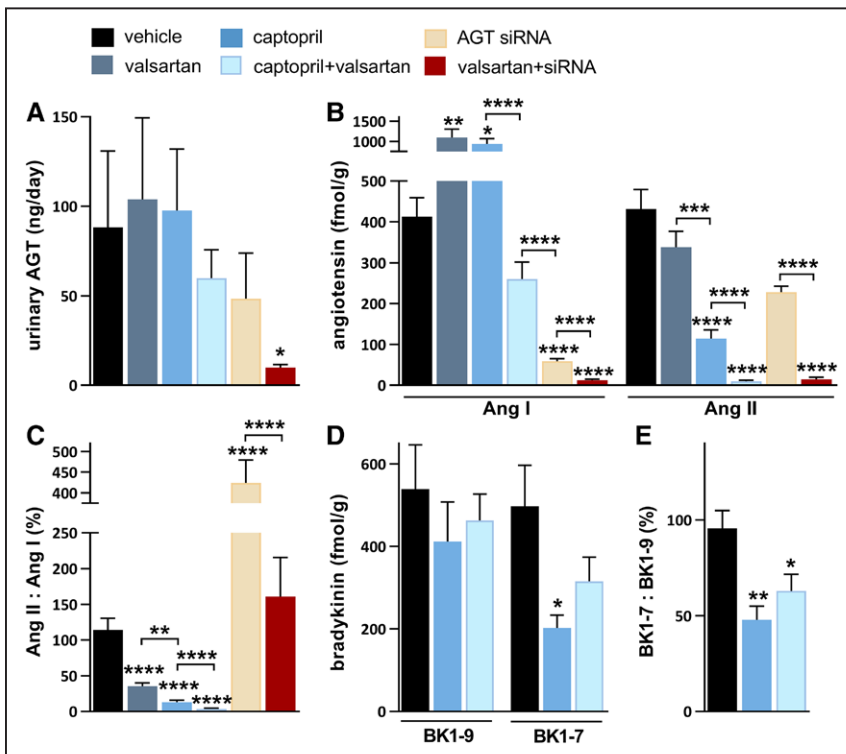
Figure 3. Renal renin-angiotensin system and bradykinin (BK) levels. **A**, Urinary AGT (angiotensinogen) and renal (**B**) Ang (angiotensin) levels, (**C**) Ang II/Ang I ratio, (**D**) BK levels, and (**E**) BK 1–7/1–9 ratio of spontaneously hypertensive rats after 4 wk of vehicle, valsartan, captopril, AGT small interfering RNAs (siRNA), captopril+valsartan, or valsartan+siRNA treatment (all groups, n=8). Data are represented as means±SEM. Natural log-transformed data were analyzed using ANOVA and post hoc Bonferroni (*P≤0.05, **P≤0.01, ***P≤0.0001 vs vehicle or otherwise indicated in graph).

Histology

Cardiomyocyte size was measured after Gomori silver staining of sections (5 µm) of the left ventricle of the heart. Sirius red staining was applied to visualize collagen as a measure of cardiac fibrosis.

Myograph Studies

Responses of iliac arteries were measured in a Mulvany myograph as changes in isometric force. Exposure to 100 mmol/L potassium chloride determined the maximum contractile response. Concentration-response curves were constructed to ET (endothelin)-1 in the absence or presence of ET_A or ET_B receptor antagonists. Iliac arteries were preconstricted with U46619 to construct concentration-response curves to the endothelium-dependent vasodilator acetylcholine, in the absence or presence of the NO synthase inhibitor L-NAME, the small conductance Ca^{2+}-activated K^+ channel inhibitor apamin and the intermediate conductance Ca^{2+}-activated K^+ channel inhibitor 1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM34).

Statistical Analysis

Data are expressed as mean values±SEM. Data were analyzed with a 1-way ANOVA (for data obtained at a single point in time) or a repeated measures ANOVA (for data obtained at multiple points in time) using Prism v7 (Graphpad Software Inc, La Jolla). Post hoc correction according to Bonferroni was performed in case of multiple comparisons. To conform to normality, nonparametric data were transformed to natural logarithms before statistical testing (AGT, renin, angiotensin metabolites, and albuminuria). Univariate linear associations were assessed by calculation of Pearson coefficient of correlation. Two-tailed P values <0.05 were considered statistically significant.

Results

AGT siRNA Causes a Similar Antihypertensive Effect as Valsartan or Captopril

Mean arterial pressure (MAP) at baseline was 137±2 mm Hg and progressively increased over time in vehicle-treated rats (9±1 mm Hg; $P≤0.001$ versus baseline; Figure 1A and 1B). After 4 weeks, valsartan+siRNA lowered MAP the most (−68±4 mm Hg; $P≤0.05$ versus captopril+valsartan), followed by combined treatment with captopril+valsartan (−54±4 mm Hg; $P≤0.0001$ versus captopril; Figure 1B). Monotherapy with either captopril, AGT siRNA or valsartan lowered MAP similarly (−23±2, −14±2, and −10±2 mm Hg, respectively; $P=0.12$ captopril versus siRNA; $P>0.99$ siRNA versus valsartan; all $P≤0.0001$ versus vehicle). None of the treatments affected heart rate (Figure 1C) or locomotor activity (Figure 1D).

Near-Complete AGT Depletion Is Required for Lowering Plasma Ang II

Plasma AGT levels amounted to 651±50 pmol/mL at baseline. They were lowered by 97.9±1.0% after 4 weeks of treatment with AGT siRNA, and this increased to 99.8±0.1% in combination with valsartan ($P≤0.0001$ versus siRNA; Figure 2A). Captopril with or without valsartan reduced plasma AGT levels by 86±4% and 44±13%, respectively (both $P≤0.05$ versus baseline), whereas valsartan alone had no effect on AGT. Reductions in AGT concentrations were paralleled by increases in active plasma renin concentration in all treatment groups, the highest rise occurring after valsartan+siRNA (Figure 2B). In agreement with previous observations,[11,12] active plasma renin concentration exhibited a negative correlation with both circulating AGT and MAP ($P<0.001$; Figure

S1A and S1B). The well-known correlation between active plasma renin concentration and Ang I ($r=0.80$; $P<0.0001$ in non-siRNA–exposed rats, data not shown) disappeared when including siRNA-treated rats in the analysis (Figure S1C). In fact, now AGT correlated with Ang I (Figure S1D). This was anticipated, since at very low AGT levels an increase in renin no longer results in a parallel rise in Ang I. Accordingly, circulating Ang I levels were elevated after 4 weeks of treatment with valsartan, captopril, and captopril+valsartan (all $P≤0.01$ versus vehicle; Figure 2C). Valsartan alone also increased Ang II ($P≤0.0001$ versus vehicle; Figure 2C). Captopril, with or without valsartan, did not lower Ang II levels, although, consistent with ACE inhibition, it did diminish the Ang II/Ang I ratio (Figure 2C and 2D). Remarkably, only valsartan+siRNA lowered plasma Ang II (−76±5%; $P≤0.05$ versus vehicle; Figure 2C). No treatment altered plasma aldosterone after 4 weeks (Table S3).

## AGT siRNA Reduces Renal Ang I, but Requires Valsartan for Simultaneous Ang II Lowering

After 4 weeks, hepatic siRNA levels amounted to 36±13 µg/g tissue, versus 3.7±0.5 µg/g in the kidneys of rats treated with siRNA with or without valsartan. No siRNA could be detected in control rats. Given average liver and kidney weights of 12±1 and 2.3±0.1 g, this translates to a ≈50-fold siRNA enrichment in liver versus kidney. In agreement with this, siRNA lowered hepatic *Agt* mRNA levels to 0.7±0.1% of control, whereas renal *Agt* mRNA levels remained unaffected (88±8% of control; Figure S2A). Of note, *Agt* mRNA levels in the liver of control animals were ≈170-fold higher than in the kidneys of these animals, based on *Gapdh*-normalized *Agt* ΔCt values (kidney ΔCt 8.2±0.5 versus liver ΔCt 0.8±0.3).

Although liver-targeted *Agt* silencing did not affect renal *Agt* expression, dual blockade with valsartan+siRNA did lower urinary AGT by 89±2% (Figure 3A). Valsartan and captopril increased renal Ang I (both $P<0.01$ versus vehicle), whereas captopril+valsartan did not affect renal Ang I (Figure 3B). Only siRNA with or without valsartan lowered renal Ang I by 97±1% and 86±1%, respectively (both $P<0.0001$ versus vehicle). Yet, valsartan did not affect renal Ang II, siRNA and captopril modestly lowered renal Ang II ($P=$nonsignificant for siRNA), and only dual blockade greatly reduced renal Ang II ($P<0.0001$; Figure 3B). As a consequence, the renal Ang II/Ang I ratio was increased 4-fold after siRNA ($P<0.0001$), reduced by >70% after valsartan, captopril, and captopril+valsartan (all $P<0.0001$) and unaltered after siRNA+valsartan (Figure 3C). Renal Ang-(1–7) was increased after valsartan and captopril, unchanged after captopril+valsartan, and reduced after siRNA with or without valsartan (Table S3). Captopril with or without valsartan impaired the renal degradation of BK-(1–9) into BK-(1–7), as reflected by reduced BK-(1–7)/BK-(1–9) ratios, without increasing BK-(1–9) levels (Figure 3D and 3E). Systemic BK levels were all below the lower limit of detection.

Dual RAS Blockade Synergistically Improves Cardiac Hypertrophy

Cardiac weight (indexed to tibia length) exhibited a strong positive correlation to MAP ($r=0.84$; $P≤0.0001$; Figure 4A).
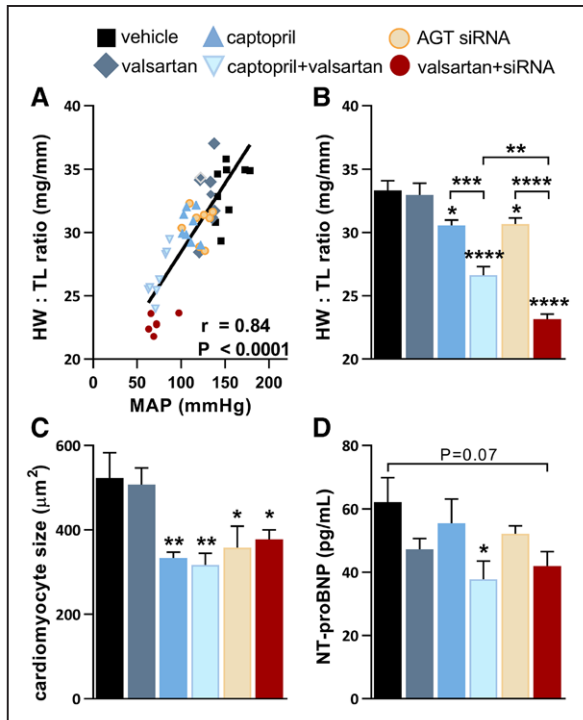
Figure 4. Indices of cardiac hypertrophy. **A**, Relationship between heart weight (HW) indexed for tibia length (TL) and mean arterial pressure (MAP), (B) HW:TL ratio, (**C**) cardiomyocyte surface area, and (**D**) plasma NT-proBNP (N-terminal pro-B-type natriuretic peptide) levels in spontaneously hypertensive rats after 4 wk of vehicle, valsartan, captopril, AGT (angiotensinogen) small interfering RNAs (siRNA), captopril+valsartan or valsartan+siRNA treatment (all groups, n=8). *r* denotes Pearson correlation coefficient. Data, represented as means±SEM, were analyzed using a 1-way ANOVA and post hoc Bonferroni (*P≤0.05, **P≤0.01, ****P≤0.0001 vs vehicle or otherwise indicated in graph).

Proportional to the magnitude of blood pressure reductions achieved, AGT siRNA and captopril monotherapies improved the cardiac weight/tibia length ratio equally, but less than dual RAS blockade (Figure 4B). Whereas

valsartan+siRNA lowered this ratio the most (*P≤0.01 versus captopril+valsartan), valsartan monotherapy had no effect. Likewise, all treatments, except for valsartan, decreased cardiomyocyte surface area (Figure 4C). Between-group differences were no longer detectable. Only captopril+valsartan lowered plasma NT-proBNP (N-terminal pro-B-type natriuretic peptide) levels (*P≤0.05 versus vehicle), and a similar trend was observed for valsartan+siRNA (*P*=0.07 versus vehicle; Figure 4D). At this stage, cardiac fibrosis had yet to develop (left ventricular collagen content 0.3±0.1% in vehicle-treated rats). Hence, this could not be improved by any treatment (data not shown).

None of the Treatments Caused Acute Kidney Injury

GFR at baseline was 1.3±0.1 mL/min per 100 g body weight and remained stable throughout the 4-week treatment period in all groups (*P≥0.65 versus baseline; Figure 5A). Daily urine production was 4-fold higher after captopril and doubled after valsartan+siRNA (*P≤0.0001 versus captopril; both *P≤0.0001 versus baseline; Figure 5B). This was due to equivalent increases in water intake (Table S4). None of the treatments decreased urine output or increased urinary NGAL (neutrophil gelatinase-associated lipocalin) levels, a biomarker for early manifestations of kidney damage which precedes renal function decline in acute kidney injury (Figure 5C). Preexisting chronic kidney disease was negated by very low levels of albuminuria at baseline (0.6±0.1 mg/day), which could not be improved further by any treatment (Figure 5D). None of the treatments affected natriuresis (Figure 5E). Plasma potassium levels tended to increase in all groups (*P≤0.001 for linear trend), significance being reached only with valsartan+siRNA (*P≤0.01 versus vehicle; Figure 5F). Both types of dual blockade attenuated normal growth (as indicated by body weight) from the second week of treatment onwards, without altering food intake (Table S4).
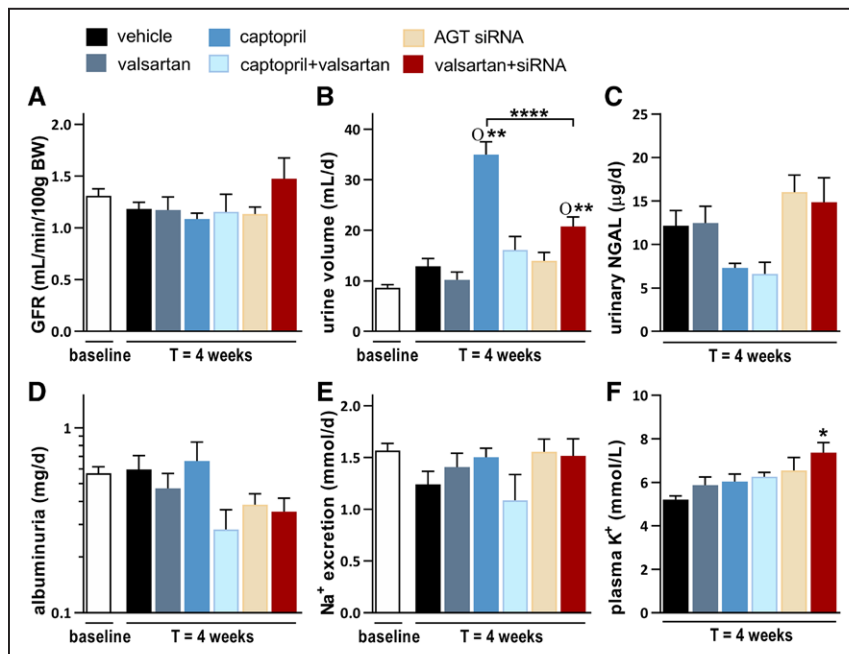
Figure 5. Indices of kidney damage. **A**, Glomerular filtration rate (GFR), determined by transcutaneous measurement of FITC-sinistrin clearance curves, (**B**) 24-h urine production, (**C**) urinary NGAL (neutrophil gelatinase-associated lipocalin) levels, (**D**) 24-h albuminuria, and (**E**) 24-h sodium (Na⁺) excretion of spontaneously hypertensive rats before treatment (baseline) and after 4 wk of vehicle, valsartan, captopril, AGT (angiotensinogen) small interfering RNAs (siRNA), captopril+valsartan, or valsartan+siRNA treatment (all groups, n=8). **F**, Plasma potassium (K⁺) levels after 4 wk of treatment. Data, represented as means±SEM, were analyzed using repeated measures or 1-way ANOVA and post hoc Bonferroni (O*P≤0.0001 vs baseline; *P≤0.01, **P≤0.0001 vs vehicle or otherwise indicated in graph).

Treatment Did Not Alter Renal *AT1* or *ACE* Expression, but Reduced NHE3, pNCC, and γ-ENaC

AT_{1a} receptor expression in cortex or medulla was not affected by any treatment (Figure S2). AT_{1b} receptor expression in the cortex was reduced after valsartan+siRNA ($P \le 0.01$ versus control), and a similar trend was found for captopril+valsartan ($P=0.07$ versus control). All treatments decreased AT_2 receptor expression in the medulla. Captopril+valsartan increased AT_2 and *ACE* expression in the cortex. None of the treatments affected *ACE2* or (pro)renin receptor expression. As anticipated, all treatments increased renin expression in the renal cortex (paralleling the changes in plasma renin), whereas dual blockade additionally increased medullary renin expression.

All treatments reduced NHE3 (sodium-hydrogen exchanger type 3) abundance ($P<0.01$; Figure 6), although the reduction was nonsignificant in the case of valsartan+siRNA ($P=0.1$). Significant reductions in pNCC (sodium chloride cotransporter phosphorylated at threonine 58) were seen in the groups with the largest blood pressure decreases (captopril and the 2 dual-blockade groups with modest nonsignificant changes in the other groups). NCC abundance changes followed this pattern. Valsartan+siRNA increased the abundance of NKCC2 (sodium potassium chloride cotransporter 2; $P=0.07$ versus control; $P<0.01$ versus AGT siRNA). Only siRNA with or without valsartan decreased the γ-subunit of ENaC (epithelial sodium channel; $P<0.05$ versus control). None of the treatments affected aquaporin-2, neural precursor cell expressed, developmentally downregulated protein 4-2, serum- and glucocorticoid-regulated kinase 1, or α-ENaC. In summary, these data confirm that blood pressure lowering during RAS blockade is accompanied by NHE3 and NCC inhibition, whereas the observed potassium rise may have occurred secondary to decreased ENaC activity.

## Endothelial Function and ET-1 Responsiveness Were Not Affected by Any Treatment

Acetylcholine relaxed preconstricted iliac arteries of vehicle-treated rats by 89±7% (Figure S3A). Blockade of both NO and endothelium-derived hyperpolarizing factor was required to prevent this effect. None of the treatments altered this outcome. ET-1 potently constricted iliac arteries of control SHR (pEC_{50} [the negative logarithm of the half-maximal effective concentration], 8.2±0.6). ET_A receptor blockade (pEC_{50}, 7.6±0.1; $P \le 0.5$), but not ET_B receptor blockade (pEC_{50}, 8.2±0.2), prevented this effect in control SHR. Single RAS blockade did not alter this outcome, whereas after dual RAS blockade ET_B receptor blockade resulted in a leftward shift, suggesting ET_B receptor-mediated vasodilation, with the rightward shift after ET_A receptor blockade being significant only when compared with the ET-1 curve in the presence of BQ788 (Figure S3B).

Discussion

This is the first study comparing liver-directed AGT siRNA treatment with single and dual RAS blockade in a well-established hypertension animal model, the SHR. The results show that AGT siRNA is as effective in lowering blood pressure as single RAS blockade, whereas impressive synergy occurs in combination with a second RAS blocker, valsartan (Figure S4). Remarkably, the data also reveal that siRNA-induced suppression of hepatic *Agt* mRNA, which reduced circulating AGT by almost 98%, did not significantly lower plasma or renal Ang II levels. This is reminiscent of clinical observations after chronic ACE inhibition, which also did not result in chronic Ang II suppression, despite an acute initial decrease in Ang II.[4,13] This Ang II escape can be explained by the rise in renin following treatment. Previous studies in humans, applying high renin inhibitor doses, demonstrated that the capacity of the body to upregulate renin expression is almost unlimited, with elevations of several 100-fold possible.[11] In fact, a low AGT/high renin condition naturally occurs in mice.[14] Their AGT levels are in the same range as those in SHR after AGT siRNA, whereas their renin levels are 2–3 orders of magnitude above those in rats, pigs, and humans.[15] Yet, mouse Ang II levels are identical to those in the latter species.[16,17] Thus, the body has a tremendous capacity to restore Ang II levels, simply by upregulating renin. Nevertheless, at a very high degree of RAS blockade (obtained in this study by combining AGT siRNA+valsartan), the limit of this mechanism is reached: the high renin levels then essentially deplete any remaining AGT, and thus Ang II levels fall. As a consequence, blood pressure decreased sharply, resulting in the largest reduction in cardiac hypertrophy of all treatment groups. Such strong antihypertensive effects in rodents have been described previously during dual RAS blockade, particularly under low-salt conditions, and were accompanied by a similar degree of weight loss as observed in the present study as well as renal insufficiency.[18,19] Indeed, dual RAS blockade with conventional RAS blockers is known to result in renal dysfunction, both in animals and humans.[10] Renal dysfunction was not apparent in the present study, most likely because renal function (reflected by GFR, albuminuria, and NGAL levels) was normal at the start of the study. Yet, siRNA+valsartan did raise potassium, possibly because low Ang II levels reduced ENaC activity. Plasma aldosterone levels were not suppressed after 4 weeks of treatment, suggestive for its well-known restoration in an Ang II–independent manner after prolonged RAS blockade.[13,20]

A question raised by these data is why AGT suppression lowered blood pressure in the absence of changes in plasma Ang II. Such observations are typically attributed to alterations in tissue angiotensin levels, for example, in the kidney. *Agt* mRNA is present in renal tissue, as confirmed in the present study. Yet, based on the ΔCt values, these levels are ≈170-fold lower than the hepatic *Agt* mRNA levels and were unaffected by liver-targeted AGT siRNA. Nevertheless, AGT siRNA did lower renal Ang I levels by >85% (single treatment) or >95% (combined treatment with valsartan), and under the latter conditions, urinary AGT virtually disappeared. Therefore, as has been concluded before in renal and hepatic *Agt* KO mice,[21,22] renal angiotensin generation entirely depends on hepatic AGT, which suggests that renal AGT has no function, even when some of it is released into urine. Second, given the fact that renal Ang I levels already dropped during single AGT siRNA treatment, whereas circulating Ang I levels still remained intact, it appears that tissue angiotensin generation is even more sensitive to decreases in hepatic AGT synthesis. This has been
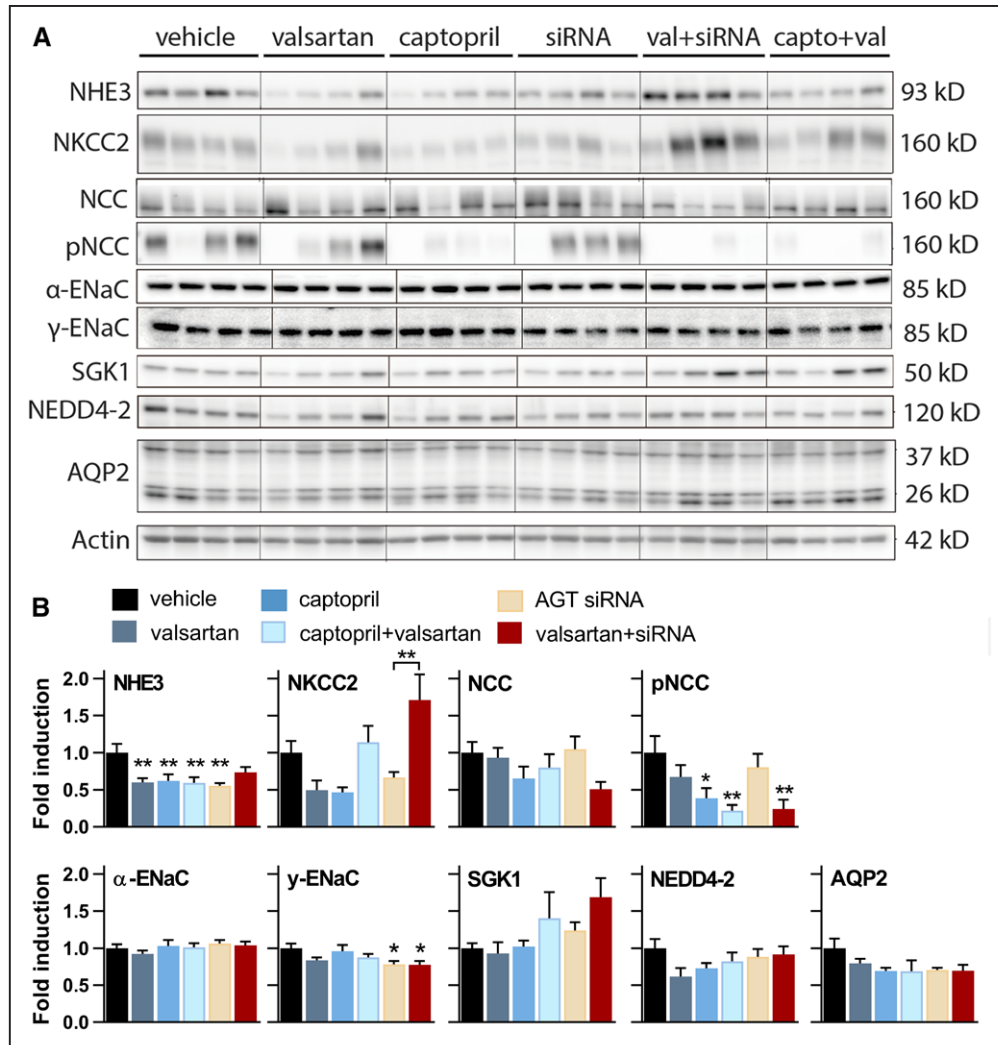
Figure 6. Renal sodium transporter protein abundance. **A**, Representative immunoblots showing protein abundances of renal sodium transporters: NHE3 (sodium-hydrogen exchanger type 3), NKCC2 (sodium potassium chloride cotransporter 2), NCC (sodium chloride cotransporter), pNCC (NCC phosphorylated at Thr58), α- and γ-subunit of ENaC (epithelial sodium channel), SGK1 (serum and glucocorticoid-regulated kinase 1), NEDD4-2 (neural precursor cell expressed developmentally downregulated protein 4-2), AQP2 (aquaporin 2), and β-actin in whole kidney homogenates after 4 wk of vehicle, valsartan, captopril, AGT (angiotensinogen) small interfering RNAs (siRNA), captopril+valsartan, or valsartan+siRNA treatment. **B**, Data, expressed as fold induction relative to vehicle-treated control (means±SEM; all groups, n=8), were analyzed using 1-way ANOVA and post hoc Bonferroni (*$P \leq 0.05$, **$P \leq 0.01$ vs vehicle or otherwise indicated in graph).

observed before in cardiac tissue of heart failure patients,[23] and most likely reflects the difficulty of circulating AGT diffusing into the tissue interstitium.[24] Despite the substantial reduction in renal Ang I production, AGT siRNA alone did not significantly reduce renal Ang II levels. Here, it is important to realize that tissue Ang II is bound to AT_1 receptors, that is, it is located either on the cell surface or internalized.[16,25,26] An increase in AT_1 receptor density will, therefore, result in a rise in tissue Ang II levels.[25] Alternatively, *ACE* upregulation might overcome the reduction in Ang II formation.[27] Quantification of AT_{1a} receptor, AT_{1b} receptor, and *ACE* expression in the kidney ruled out these possibilities. Most likely, therefore, factors affecting Ang II internalization (eg, AT_1 receptor-associated protein[28]) are responsible for maintaining near-normal renal Ang II levels, even when renal Ang I generation is diminished. Preventing internalization should then lower tissue Ang II levels. This is indeed what was observed during AT_1 receptor blockade with valsartan coadministered with AGT

siRNA: renal Ang II now decreased in parallel with Ang I, and the renal Ang II/I ratio was no longer different from control.

Ang II is a well-known regulator of both NHE3[29] and NCC, thus determining sodium balance.[30,31] In the present study, changes in pNCC fully paralleled the changes in blood pressure, that is, the largest pNCC reductions were seen in the rats with the largest blood pressure reduction. Alterations in renal Ang II synthesis and internalization (not necessarily reflected by reduced renal Ang II levels) may underlie these pNCC reductions, which then in combination with the RAS blockade-induced suppression of NHE3, may explain, at least in part, the antihypertensive (circulating RAS-independent) effect of RAS blockade. An additional explanation is that the higher plasma potassium concentrations inhibited pNCC.[32]

We did not quantify tissue Ang II levels in the heart, vascular wall, or adrenal gland, but given their dependency on hepatic AGT,[15,33,34] it is reasonable to assume that alterations in their Ang II content paralleled those in the kidney. Such

alterations, as for example reflected by the occurrence of ET_B receptor-mediated vasodilation, may have contributed to the blood pressure-lowering effect. The virtual disappearance of Ang-(1–7) after AGT siRNA suggests that a contribution of this metabolite is unlikely. Similarly, the lack of BK-(1–9) accumulation during ACE inhibition in renal tissue argues against a major role for BK-(1–9), although the BK-(1–7)/BK-(1–9) ratio did decrease during this treatment. Together with the reduction in Ang II/Ang I ratio, this latter observation at least confirms that ACE inhibition was achieved at the tissue level.

Perspectives

In SHRs, near-elimination of hepatocyte-derived AGT with siRNA+valsartan depleted angiotensin, causing a synergistic reduction in blood pressure and cardiac hypertrophy, greater than that induced by captopril+valsartan. Yet, renal function was preserved. AGT siRNA as monotherapy provided similar antihypertensive and cardioprotective efficacy as conventional small-molecule RAS inhibitors. Unique to AGT siRNA is the long-term effectiveness of a single subcutaneous injection. Indeed, preliminary studies in cynomolgus monkeys support AGT suppression for >100 days after a single siRNA injection of 10 mg/kg (n=3; unpublished observations). As medication nonadherence is associated with greater cardiovascular risk, long-acting agents have the potential to significantly improve outcomes. Such efforts are already underway for the management of hypercholesterolemia. Inclisiran, a GalNAc-conjugated siRNA targeting *PCSK9* now in phase 3 trials, requires only biannual dosing. Future studies should better define the relationship between AGT suppression and blood pressure to establish optimal dosing, as well as safety and efficacy in the context of common comorbidities, such as heart failure and chronic kidney disease, and in combination with other RAS blockers or a low-salt diet. Such studies should also address whether the massive renin rise in the absence of AGT involves recruitment of renin-secreting cells outside the juxtaglomerular apparatus and juxtaglomerular hypertrophy.[35]

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Novelty and Significance

What Is New?

- Small interfering RNA (siRNA) targeting liver AGT (angiotensinogen) provides antihypertensive and cardioprotective efficacy comparable to that of single renin-angiotensin system blockade.
- Ang II (angiotensin II) elimination requires >99% suppression of circulating AGT, and under such circumstances, blood pressure decreases substantially.

What Is Relevant?

- Given its stable and sustained efficacy, lasting weeks, RNA interference may offer a unique approach to improving therapy adherence and treating hypertension.

Summary

Maximal blockade of the renin-angiotensin system, achieved by valsartan+siRNA, yielded the greatest reduction in blood pressure and cardiac hypertrophy, whereas AGT lowering alone was as effective as conventional renin-angiotensin system inhibitors. In clinical practice, RNA interference at the AGT level may not only prevent renin-angiotensin system reactivation, but also improve cardiovascular outcome and medication adherence, due to its long-lasting and stable efficacy after a single subcutaneous injection.