Renal Angiotensinogen in Nonhuman Primates is Predominantly Liver-Derived

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Abbreviations:

AGT:	Angiotensinogen
Ang:	Angiotensin
RAS:	Renin angiotensin system
ASO:	Antisense oligonucleotides
NHP:	Nonhuman primates
ACE:	Angiotensin-converting enzyme

Abstract:

AGT (Angiotensinogen) is the unique substrate of the renin-angiotensin system. Liver is the primary source of circulating AGT. The present study determined whether hepatocyte-derived AGT regulates renal AGT accumulation by injecting ASO (antisense oligonucleotides) targeting hepatocyte-derived AGT (GalNAc AGT ASO) into female cynomolgus monkeys. Hepatocyte-specific inhibition of AGT led to profound reductions of plasma AGT concentrations. AGT protein in S1 and S2 of renal proximal tubules was greatly diminished by GalNAc AGT ASO. Given the similarity between nonhuman primates and human, our findings support the notion that renal AGT is predominantly derived from liver, and liver regulates renal angiotensin II production in humans.

AGT (Angiotensinogen) is the unique substrate of the RAS (renin-angiotensin system). While many cells possess the ability to synthesize AGT, studies in mice have demonstrated that plasma AGT is predominantly liver-derived.[1] Indeed, either pharmacological inhibition or genetic deficiency of hepatocyte-derived AGT reduced blood pressure in mice.[2] Yet, particularly in the kidney, it has been argued that locally synthesized AGT may contribute to renal Ang (angiotensin) II generation, possibly in a blood pressure-independent manner, although the literature is inconsistent.[3] Based on this concept, AGT measurement in urine or renal biopsies is often used as an independent marker of renal RAS activation in humans.[4] However, whether renal Ang II generation in humans truly depends on kidney-derived AGT remains uncertain.

AGT is cleaved by renin into two products: Ang I, which consists of 10 amino acids, and des(Ang I)AGT, which has 443 amino acids in mice and 442 amino acids in humans and NHP (nonhuman primates). Although sequences of AGT vary substantially between mouse and human, this protein in humans and NHP is highly conserved.

To distinguish plasma concentrations of total versus intact AGT among species, plasma was collected from male C57BL/6 mice, men, and male cynomolgus monkeys **(Figure A)**. Plasma total AGT concentrations were 3 – 4 μg/mL in mice, 15 – 41 μg/mL in humans, and 11 – 20 μg/mL in cynomolgus monkeys. The percentage of AGT that was intact (Ang I-containing) was 8% in mice, and >60% in both humans and cynomolgus monkeys. Despite these differences in circulating AGT, the distribution of AGT protein accumulation within the kidney was comparable among the 3 species **(Figure B).** AGT protein accumulation was most abundant in the S1 and S2 segments of the renal proximal tubules, modest in the S3 portion of the proximal tubules, and not detectable in glomeruli and other tubules of the kidneys.

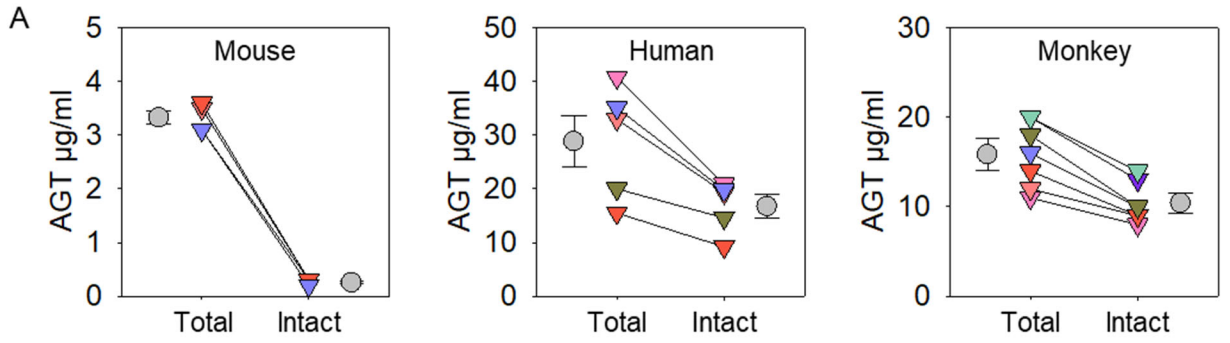

Figure A. Plasma total AGT and intact AGT concentrations in male C57BL/6 mice (N=4), humans (N=5), and cynomolgus monkeys (N=7) were measured by ELISA kits.

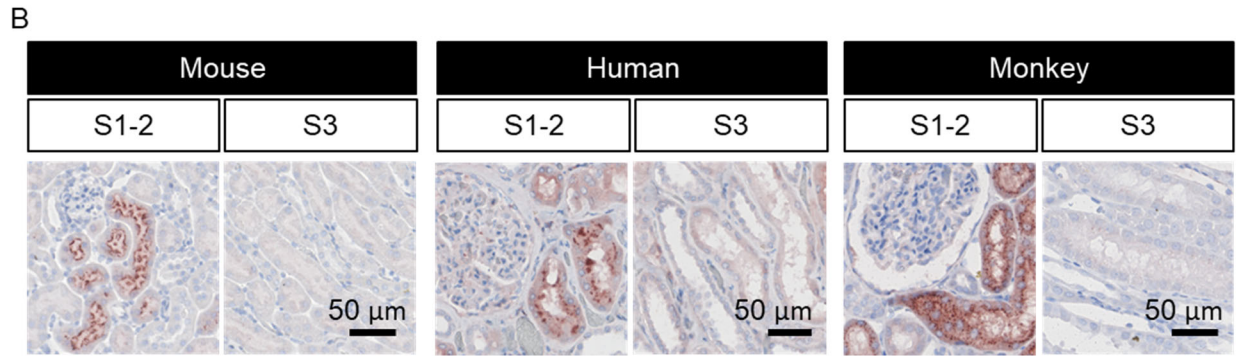

Figure B. Immunostaining of AGT in kidney sections from mice, humans, and cynomolgus monkeys using a rabbit anti-mouse AGT antibody for mouse and a mouse anti-human AGT antibody for both human and monkey.

Given the similarity between humans and cynomolgus monkeys, findings in the latter may have greater translational significance in defining the origin of kidney AGT in humans. To determine whether hepatocyte-derived AGT contributed to AGT protein accumulated in the kidney of NHP, female cynomolgus monkeys (3-4 years of age) were injected subcutaneously with either saline or ASO (antisense oligonucleotides) targeting hepatocyte-derived human AGT (Ionis, GalNAc AGT ASO: conjugated with N-acetyl galactosamine; 2.5 or 10 mg/kg). The human GalNAc AGT ASO had a perfect sequence match to AGT mRNA in cynomolgus monkeys. Saline or ASO was injected on day 1 and day 4, and then once weekly for the subsequent 4 weeks. Neither dose of ASO affected body weight or liver and kidney functions.

Both doses of GalNAc AGT reduced plasma AGT concentrations within 1 week **(Figure C)**. Comparable to mice, AGT mRNA was most abundant in liver, and considerably lower in kidney and adipose tissue **(Figure D)**. Both doses of GalNAc AGT ASO profoundly reduced the hepatic mRNA abundance of AGT **(Figure E)**.

Although the lower dose of GalNAc AGT ASO did not affect renal AGT mRNA abundance, it greatly diminished AGT protein accumulation in the S1 and S2 segments of renal proximal tubules **(Figure F)**. These findings support the notion that hepatocytes supply the bulk of AGT protein to the kidney in NHP, independent of the presence of renal AGT mRNA. **Figure F** also shows the distribution of other RAS components. Renin was observed predominantly in juxtaglomerular cells, and ACE (angiotensin-converting enzyme) and ACE2 were present in all 3 segments of the proximal tubules, being most abundant in the S3 portion. GalNAc AGT ASO did not change the renal distribution of these three enzymes.

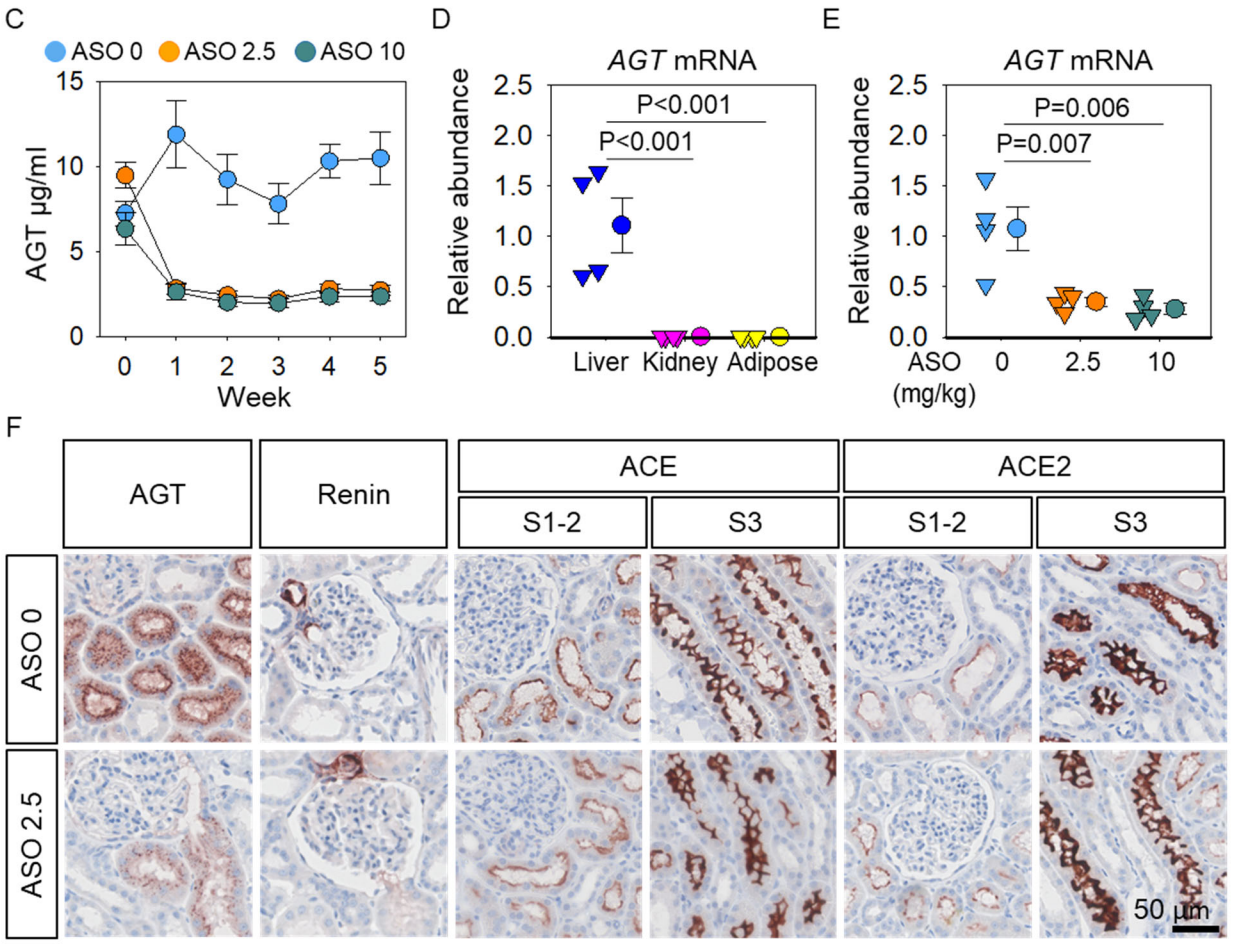

Figure C-F. Female cynomolgus monkeys were injected subcutaneously with either saline (indicated as "ASO 0" in the figure) or GalNAc AGT ASO (2.5 or 10 mg/kg; indicated as "ASO 2.5" and "ASO 10", respectively) for 5 weeks (N = 4/group). **C.** Plasma total AGT concentrations were determined by a Human AGT ELISA kit. (P <0.001 between week 0 and week 1 for both ASO 2.5 and ASO 10)) **D-E.** mRNA abundance of AGT was quantified by qPCR. Data were analyzed using the ΔΔCt method and normalized to the mean of two reference genes: GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and ZFP91 (Zinc finger protein 91). **F.** Immunostaining of AGT, renin, ACE, and ACE2 in kidney sections.

In conclusion, the liver is the major source of AGT in kidneys of cynomolgus monkeys. Hepatic AGT accumulation in the S1 and S2 segments of the proximal tubules coincides with the observation in humans that tubular reabsorption via megalin, an endocytic receptor on the proximal tubules, is the main determinant of urinary AGT.[5] Taken together, the most likely scenario is that renal AGT originates predominantly in the liver. This implies that renal Ang II production in NHP and humans relies on hepatic AGT, and that the concept that AGT in urine or renal biopsies reflects an independent renal RAS needs to be reconsidered.

Statistical Analysis

The data were represented as individual data points and mean ± SEM. For plasma AGT concentrations, a piecewise linear mixed model with a split point at week 1 was performed to compare plasma AGT changes over time among the 3 groups using the "nlme" R package. The estimates of the fixed effect parameters were weighted by the inverse variances at each time point. SigmaPlot version 14.0 (Systat Software Inc., San Jose, CA) was used for statistical analyses of AGT mRNA abundance. Normality and homogeneous variation were tested in all data by Shapiro-Wilk and Brown-Forsythe tests, respectively. The data were log10 transformed to pass normality and equal variance tests, and P-values were calculated by one-way analysis of variance with the Holm-Sidak method. $P < 0.05$ was considered statistically significant.

Reagents

Table 1. Primary antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot #
Mouse AGT	IBL	IBL 28101	0.3 µg/ml	1E-711
Human AGT	IBL	IBL 10417	1 µg/ml	1C-307
Renin	Sigma	HPA005131	1 µg/ml	A47461
ACE	Sigma	HPA029298	2 µg/ml	9722
ACE2	Abcam	ab108252	0.3 µg/ml	GR3344245-2

Table 2. Secondary antibody Kits

Antibody Kit	Vendor or Source	Catalog #	Lot #
ImmPRESS® HRP goat anti-rabbit IgG polymer detection kit	Vector	MP-7451	ZG0629
ImmPRESS® HRP horse anti-mouse IgG, rat adsorbed polymer detection kit	Vector	MP-7422	ZG0520

Table 3. ELISA Kits

ELISA	Vendor or Source	Catalog #	Lot #
Mouse Total AGT Assay Kit	IBL	27413	1B-820
Mouse Intact AGT Assay Kit	IBL	27743	1J-427
Human Total AGT Assay Kit	IBL	27412	2-L011
Human Intact AGT Assay Kit	IBL	27742	2I-018

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Disclosures

Adam Mullick is an employee of Ionis Pharmaceuticals, Inc.

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