# Adipocytes do not significantly contribute to plasma angiotensinogen

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## Abstract

Recently, it has been reported that 25% of plasma angiotensinogen (Agt) is derived from fat. Meanwhile, liver-specific *Agt* knockout (KO) mice have markedly low plasma Agt, which may be due to reduced fat mass. To study the contribution of the fat to plasma Agt, we tested whether increasing fat mass can elevate plasma Agt and blood pressure in liver-*Agt* KO mice. Epididymal fat mass in liver-*Agt* KO mice fed a high-fat diet (HFD) was 4.1-fold larger than that in liver-*Agt* KO mice on a normal-fat diet (NFD). The liver-*Agt* KO mice on NFD were hypotensive with low levels of plasma Agt (on average, 0.11 vs 2.38 μg/ml). HFD slightly increased plasma Agt (0.17 μg/ml) without increase in blood pressure. To further increase fat mass, liver-*Agt* KO mice were fed HFD and simultaneously supplemented with low-dose angiotensin II and compared with control mice. Fat mass was comparable between the two groups. However, liver-*Agt* KO mice had uniformly low plasma Agt (0.09 vs 2.07 μg/ml) and systolic blood pressure (78±12 vs 111±6 mm Hg). In conclusion, adipocyte-derived Agt has essentially no contribution to the plasma concentration and no impact on blood pressure compared to liver-derived Agt.

## Introduction

The renin-angiotensin system (RAS) plays a central role in the regulation of blood pressure, and inappropriate activation of RAS is implicated in hypertension and progression of cardiovascular and renal diseases.[1,2]

The liver is the major source of angiotensinogen (Agt), the precursor of angiotensin II. In addition to the liver, other organs, including the kidney,[3,4] the brain,[5,6] the adrenal gland,[7,8] the testis,[9] the heart,[10,11] the colon,[2] and the adipose tissue,[12–14] also generate *Agt* mRNA and protein. Among them, adipose tissue has been considered as an important source significantly contributing to plasma Agt. Supporting this notion is that the level of *Agt* mRNA normalized by 18S rRNA in adipocytes is 60–80% of that in the liver.[14,15] Although the content of RNA in the adipose tissue is small, the total mass of the adipose tissue is quite large, i.e. 15–30% of total body weight in healthy subjects. Hence, it is thought that adipose Agt may contribute to the hypertension often afflicting obese patients. Indeed, the level of plasma Agt was found to be associated with the degree of obesity in humans[16,17] and mice.[18] Several studies reported that obese rodents showed increased production and secretion of adipocyte Agt, which was accompanied by increase in plasma Agt.[15,18]

Recently, the role of adipocytes in Agt generation was investigated using genetically engineered mice. Massiera et al. reported that transgenic mice overexpressing Agt in adipose tissue showed 20–44% higher plasma Agt levels and hypertension when compared to control mice.[19] More directly, Yiannikouris et al. generated adipocyte-specific *Agt* knockout (KO) mice and demonstrated that plasma Agt level was lower than that in control mice on normal-fat diet (NFD).[20] In addition, these KO mice showed significantly lower blood pressure than control mice on normal- or high-, but not on low-fat diet.[21] These authors concluded that 24–28% of plasma Agt is generated in the adipose tissue in mice fed NFD.[20]

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On the other hand, we recently generated liver-specific *Agt* KO (liver-*Agt* KO) mice.[22] Liver-*Agt* KO mice have markedly low plasma Agt, only 5% of that in control mice. They are severely hypotensive, similar to whole-body *Agt* KO mice. The results appear to indicate that the Agt generated by extrahepatic tissues does not significantly contribute to plasma Agt and is not sufficient to maintain normal blood pressure.

The somewhat controversial results obtained from the two studies may be attributed to reduced fat mass in liver-*Agt* KO mice. Mice knocked out for RAS-related genes are known to have a lipid wasting characteristics, resulting in reduced fat mass.[23,24] Similarly to whole-body *Agt* KO mice, liver-*Agt* KO mice are lean. We hypothesized that increasing fat mass may lead to significant increase in plasma Agt and blood pressure in liver-*Agt* KO mice. Using liver-*Agt* KO mice, which lack the overwhelming liver-derived Agt, the study was conducted to assess the contribution of fat to plasma Agt and blood pressure.

## Materials and methods

### Animal experiments

All animal use was approved by the Animal Experimentation Committee of the Tokai University School of Medicine, Kanagawa, Japan and conformed to the "Guide for the care and use of laboratory animals" developed by National Research Council of the National Academies.

Liver (hepatocyte)-specific *Agt* KO (liver-*Agt* KO) mice, which carry *albumin-Cre* and *Agt^{loxP/loxP}*, were previously reported.[22] Mice carrying only *Agt^{loxP/loxP}* without *albumin-Cre* (control *Agt* mice) were used as control. Both lines were backcrossed with C57BL/6N strain more than 10 times.

*Experiment 1.* Five control *Agt* mice and five liver-*Agt* KO mice were fed a high-fat diet (HFD) (HFD-60, Oriental Yeast Co., Tokyo, Japan), starting at six weeks of age for 20 weeks. Five control *Agt* mice and six liver-*Agt* KO mice were fed NFD. Before starting HFD, and at the end of experiments, body weight and blood pressure were measured, and blood was collected for plasma Agt concentration. After sacrifice, epididymal fat tissues were harvested.

*Experiment 2.* Five liver-*Agt* KO mice were fed HFD and simultaneously supplemented with angiotensin II (100 ng/kg/ min) for eight weeks. For this, Alzet2004 pump containing angiotensin II (0.67 mg/ml in 154 mM NaCl, 100 mM HCl solution) was implanted subcutaneously on the right side of the back under anesthesia with isoflurane. This dose is sufficient to restore normal metabolic phenotypes in renin KO mice,[24] and is known not to raise blood pressure.[25] On the 29th day, the minipump was removed and a new pump was implanted on the left side of the back. On the 56th day, the pump was removed, and five days later the mice were sacrificed and epididymal fat tissues were harvested. These mice were compared with age- and gender-matched control *Agt* mice fed NFD.

For Agt protein and mRNA assays, epididymal fat masses were frozen in liquid nitrogen.

### Food composition

The HFD contains 23.0% of protein by weight (18.2% by calorie), 35.0% (62.2%) of fat, 25.3% (19.6%) of digestible carbohydrate, and 6.6% of fibers. The NFD contains 25.4% of protein by weight (29% by calorie), 4.4% (12.0%) of fat, 50.3% (59%) of digestible carbohydrate, and 4.1% of fibers.

### Blood pressure measurement

Conscious systolic blood pressure was measured by the tail-cuff method using MK-2000 (Muromachi Kikai, Tokyo, Japan) as previously described.[26] In five wild-type (three male, two female) and eight liver-*Agt* KO mice (two male, six female), systolic blood pressure that measured by this method was compared with that measured by the direct catheter method. For the latter, tapered PE50 tubing was inserted into the left common carotid artery under anesthesia with isoflurane (2%). Blood pressure was monitored with a polygraph (RMP 6008M, Nihon Kohden, Japan) 20 min after the surgery for a period of more than 30 min. As shown in Table 1, systolic blood pressure measured by tail cuff method ($x$) and by direct catheter method ($y$) correlate well with regression equation,

$$y = 0.72x + 25.2$$

with values of $R=0.901$ and $p<0.001$.

### Assays for plasma Agt and Agt protein and mRNA in the epididymal fat mass

Western analysis for Agt was performed as previously described.[22] Briefly, under reduced conditions, 5 μg of protein from the adipose tissue or the liver was separated in a Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis gel, and transferred onto Polyvinylidene Difluoride membrane. Agt protein was detected with rabbit anti-Agt antibody (Immuno-Biological Laboratories, Fujioka, Japan) diluted at 1:400 with Can Get Signal immunoreaction enhancer solution (Toyobo, Tokyo, Japan). Actin was detected with rabbit monoclonal anti β-actin antibody (Cell Signaling Technology, Tokyo, Japan) diluted at 1:1000.

Real time RT-PCR was performed for *Agt* and 18S rRNA, with TaqMan primer probe sets (Applied Biosystems, 4331182 and 4319413E) and StepOne Real Time PCR Systems (Applied Biosystems), using the default reaction condition. The relative amount of *Agt* mRNA was determined by the delta-delta Ct method.

**Table 1.** Character of liver-specific angiotensinogen knockout (liver-*Agt* KO) mice.

|  | Wild-type | Liver-*Agt* KO | *p* Value |
|---|---|---|---|
| SBP by tail cuff method (mm Hg) | 115.4±6.9 | 73.1±9.9 | <0.001 |
| SBP by catheter method (mm Hg) | 111.8±3.0 | 76.1±3.9 | <0.001 |
| Relative hepatic *Agt* mRNA/18S rRNA | 100±30.3 | 0.097±0.118 | <0.001 |
| Plasma Agt concentration (ng/ml) | 1390 (1060–1820) | 32.4 (17.1–61.7) | <0.001 |

Agt; angiotensinogen; CI: confidence interval; SBP: systolic blood pressure.
SBP was measured in five wild-type (three male, two female) and eight liver-*Agt* KO mice (two male, six female) by tail cuff method and catheter method. For plasma Agt concentration, geometrical means and 95% CIs are shown, for the others, means and 95% CI are shown. Liver-*Agt* KO mice showed hypotension, very low hepatic *Agt* mRNA and plasma Agt concentration. There is no correlation between residual hepatic *Agt* mRNA/18S rRNA and plasma Agt concentration in liver-*Agt* KO mice.

**Table 2.** High-fat diet causes an increase in abdominal fat.

|  | Normal-fat diet | High-fat diet | *p* Value |
|---|---|---|---|
| Body weight (g) | 26.5±3.0 | 42.4±3.5 | <0.001 |
| Epididymal fat mass (g) | 0.27±0.11 | 2.46±0.23 | <0.001 |
| Total abdominal fat ratio (%) | 10.4±4.1 | 56.4±2.4 | <0.001 |
| Visceral abdominal fat ratio (%) | 4.8±2.1 | 30.8±2.6 | <0.001 |
| Subcutaneous-abdominal fat ratio (%) | 5.6±2.1 | 25.6±0.7 | <0.001 |

Five wild-type male C57BL/6 mice (eight weeks of age) were fed a high-fat diet for 11 weeks and compared with age and gender-matched five wild-type mice fed normal fat diet. Areas of abdominal fat were evaluated by CT scans. Means and 95% confidence intervals are shown.

**Table 3.** *Agt* mRNA in the epididymal fat and liver and plasma angiotensinogen (Agt) in wild-type mice fed normal or high fat diet.

| Diet | Normal-fat diet | | High-fat diet | | *p* Values |
|---|---|---|---|---|---|
| Organ | (1) Liver | (2) Epididymal fat | (3) Liver | (4) Epididymal fat | |
| *Agt* mRNA/18S rRNA (relative amount) | 100.0±25.8 | 171.0±89.0 | 124.8±34.6 | 43.3±6.7 | 0.007 for (3) vs (4), 0.038 for (2) vs (4) |
| RNA content (mg/g tissue) | 5.46±0.92 | 0.16±0.03 | 4.93±1.12 | 0.09±0.02 | <0.001 for (1) vs (2), (3) vs (4), and (2) vs (4) |
| *Agt* mRNA/g tissue (relative amount) | 100.0±37.1 | 4.8±2.4 | 112.8±42.7 | 0.7±0.1 | 0.002 for (1) vs (2),<0.001 for (3) vs (4), 0.011 for (2) vs (4) |
| Plasma Agt (ng/ml) | 1650 (1280–2130) | | 1610 (1380–1880) | | |

*Agt* mRNA/18S rRNA was quantified in the fat and the liver of the mice. The epididymal fat contains a similar level of *Agt* mRNA/18S rRNA to the liver in wild-type mice fed normal fat diet. High-fat diet decreased *Agt* mRNA/18S rRNA in the fat, but not in the liver. Since RNA content was lower in the fat than in the liver, *Agt* mRNA normalized by tissue weight (g) was lower in the fat. High-fat diet further decreased RNA content in the fat. Means and 95% confidence intervals (CIs) are shown.

Plasma and urinary Agt was determined by Enzyme-linked Immunosorbent Assay (ELISA) (IBL, Japan). The sensitivity of the ELISA kit is ≥0.03 ng/ml. The specificity was verified by negative detection of Agt in plasma from whole-body *Agt* KO mice. For the assays, plasma samples of control and liver-*Agt* KO mice were diluted at 1000- and 200-fold, respectively.

### Evaluation of abdominal fat by CT scans

Areas of abdominal fat were calculated from Computed Tomography (CT) scans (15–18 slices) made between the cranial end of the L1 vertebra and the caudal end of the ischium. Average total, visceral, and subcutaneous abdominal fat areas were expressed by percentage of average total non-osseous areas.

### Statistical analysis

Data for plasma Agt concentration were compared after logarithmic transformation. For data shown in Tables 1 and 2, two groups were compared by unpaired *t*-test. For Table 3, data were compared between liver and fat in each diet group by paired *t*-test. Comparisons between the two diet groups were done by unpaired *t*-test. Values of *p* were corrected by Holm's method in order to minimize the inflation of type I errors due to multiple comparisons. For Experiment 1 (Figure 1), data for body weight, epididymal fat mass, adipose tissue mRNA, Agt/β Actin ratio, plasma Agt, and systolic blood pressure were compared by analysis of variance (ANOVA) with the Turley-Kramer method, and they are presented as means ±95% confidence intervals (CIs). For Experiment 2 (Figure 2), data between the two mouse

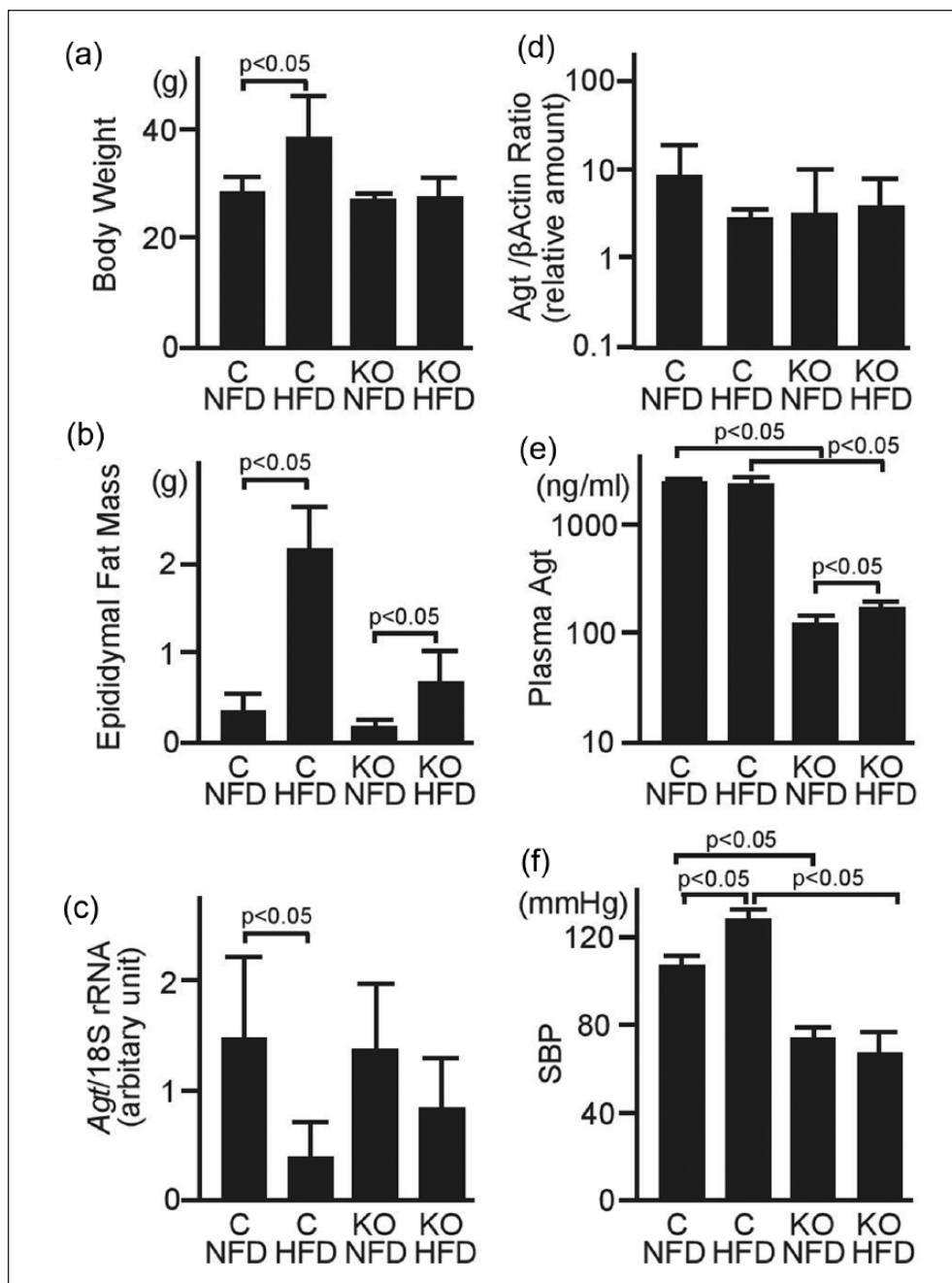**Figure 1.** High-fat diet has only a minor effect on plasma angiotensinogen (Agt). Experiment 1. Control *Agt* mice (C) and liver-specific *Agt* knockout (liver-*Agt* KO) mice were fed normal-fat diet (NFD) or high-fat (HFD) diet for 20 weeks. (a) Body weight at the end of the experiment. HFD increased body weight in control *Agt* mice, but not liver-*Agt* KO mice. (b) Epididymal fat mass. HFD increased epididymal fat mass both in control *Agt* mice and liver-*Agt* KO mice. (c) *Agt* mRNA/18S rRNA ratio in the epididymal fat. HFD decreased adipose *Agt* mRNA in control *Agt* mice, but not liver-*Agt* KO mice. (d) Western analysis for adipose Agt protein. There was no significant difference in Agt/β-actin ratio among four mouse groups. (e) Plasma Agt concentration at the end of the experiment. HFD did not increase plasma Agt in control *Agt* mice. In liver-*Agt* KO mice fed NFD, plasma Agt was only 4.8% of that in control *Agt* mice. Liver-*Agt* KO mice fed HFD showed slight increase in plasma Agt, but the level remained very low compared to control *Agt* mice. (f) Systolic blood pressure (SBP). HFD increased SBP in control mice independently of plasma Agt. This increase in SBP by HFD is greater than those reported in other studies[21,27,28] using radiotelemetry. Severe hypotension in liver-*Agt* KO mice was not restored by HFD. Geometrical means (for (e)) or means (for the others) are shown. Error bars represent 95% confidence intervals.

groups were compared by unpaired *t*-test. Body weight and systolic blood pressure were compared with the generalized

estimating equation. Plasma Agt was logarithmically transformed and analyzed with the generalized estimating
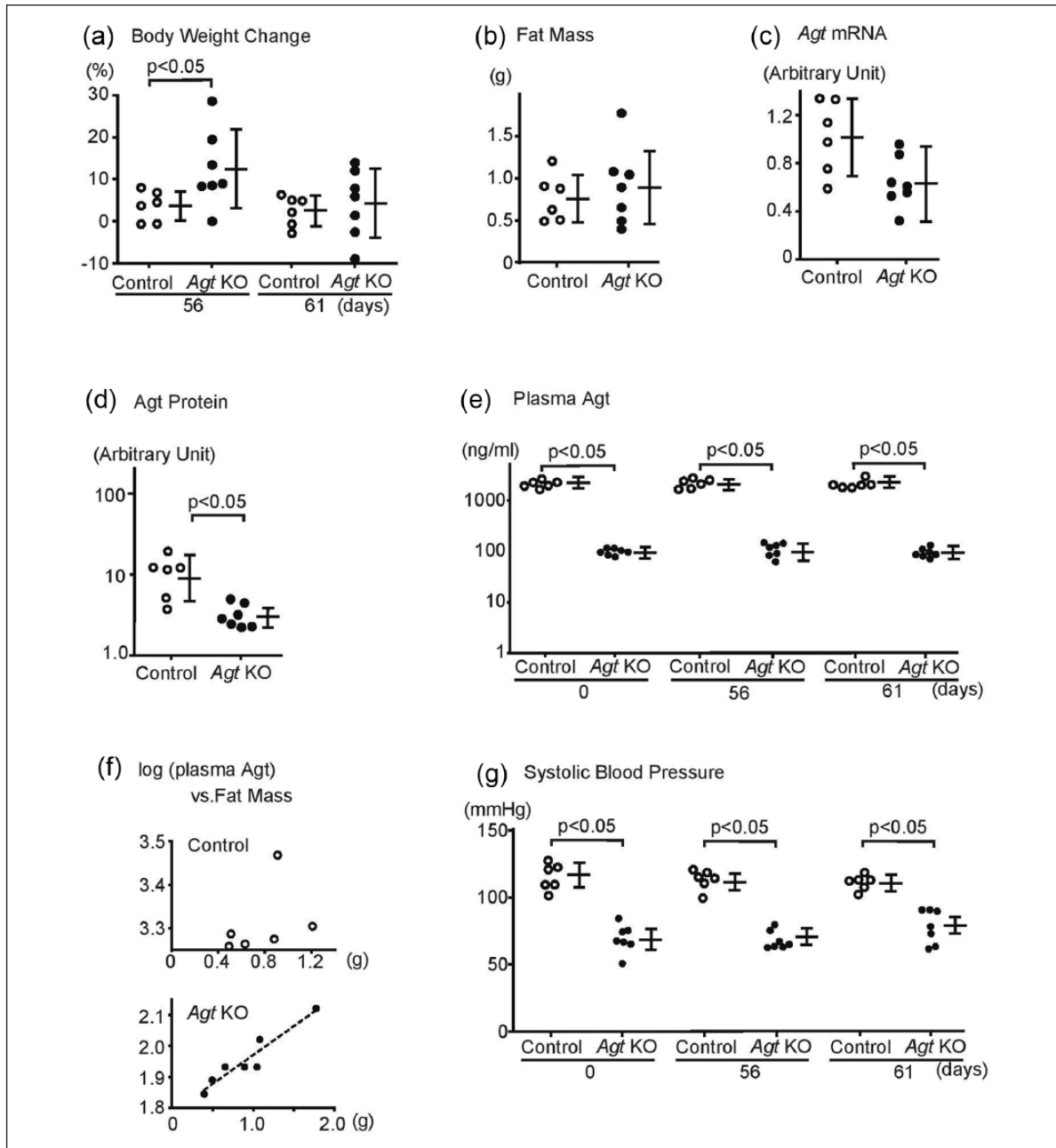
**Figure 2.** High-fat diet (HFD) with angiotensin II supplementation did not increase plasma angiotensinogen (Agt) and blood pressure. Experiment 2. Liver-specific *Agt* knockout (liver-*Agt* KO) mice were fed HFD and simultaneously supplemented with non-pressor dose of angiotensin II (100 ng/kg/min) from day 0–day 56. On the 56th day, the angiotensin II infusion was stopped and the mice were kept on HFD until the 61st day. These mice were compared with age- and gender- matched control *Agt* mice fed normal-fat diet (NFD). (a) Body weight change. Liver-*Agt* KO mice showed more body weight gain at day 56 (12.5±6.3 vs 3.6±2.7%, *p*<0.05), but this difference was not seen at day 61 (4.2±5.6 vs 1.7±2.6%). (b) Epididymal fat mass at the end of the experiment. The epididymal fat mass in liver-*Agt* KO mice+HFD+angiotensin II group was increased to a comparable level to that of control *Agt* mice. (c) *Agt* mRNA/18S rRNA in the epididymal fat tissue. Liver-*Agt* KO mice showed a slight but significantly lesser amount of *Agt* mRNA in the fat. (d) Western analysis for adipose Agt protein. Liver-*Agt* KO mice showed significantly less Agt/β-actin ratio than control *Agt* mice. (e) Plasma Agt concentration. Liver-*Agt* KO mice uniformly and continuously showed much lower plasma Agt concentration than control *Agt* mice throughout the experimental period. (f) Relationship between plasma Agt concentration (log-transformed) and epididymal fat mass. Plasma Agt concentration was significantly associated with fat mass in liver-*Agt* KO mice, but not in control *Agt* mice. (g) Systolic blood pressure (SBP). Liver-*Agt* KO mice uniformly and continuously showed much lower blood pressure than control *Agt* mice throughout the experimental period. In (a)–(e) and (g), individual data are shown by scattered plot and means (a,b,c,g) or geographical means (d,e) and 95% CI are shown by horizontal bar with error bars.

equation. The analyses were performed using KyPlot (Kyens Lab) or SPSS (IBM) software. Values were regarded as significant at two-sided $p<0.05$.

## Results

As previously reported[22] and shown in Table 1, liver-*Agt* KO mice are severely hypotensive. Their plasma Agt concentration is uniformly low, but not undetectable. In liver-*Agt* KO mice, hepatic *Agt* mRNA was barely detectable by the real-time RT-PCR method with Ct value >35. By delta-delta Ct method, hepatic *Agt* mRNA in liver-*Agt* KO mice was calculated as about 0.1% of that in wild-type mice. However, this value was not correlated with the plasma Agt concentration, indicating that low plasma Agt is generated in non-hepatic tissues in liver-*Agt* KO mice.

The adipose tissue is a candidate of the extrahepatic source of plasma Agt. We determined *Agt* mRNA in wild-type mice fed NFD or HFD (Tables 2 and 3). As reported previously,[14,15] the epididymal fat showed a comparable level of *Agt* mRNA normalized by 18S rRNA to the liver of wild-type mice fed NFD. Feeding HFD for 11 weeks markedly increased abdominal fat. Average epididymal fat weight increased 9.1-fold, and average total, visceral, and subcutaneous abdominal fat areas increased 5.4-, 6.4-, and 4.6-fold, respectively. HFD decreased *Agt* mRNA/18S rRNA in the fat tissue, but not in the liver. Since RNA content was lower in the fat than in the liver, *Agt* mRNA normalized by tissue weight was lower in the fat. HFD further decreased RNA content, hence *Agt* mRNA amount was normalized by tissue weight in the fat, as well, which may nullify the effect of the increase in fat amount. Plasma Agt concentration was not different between the two groups. Since liver-derived Agt may mask potential change in the adipose contribution to plasma Agt, we performed the following two experiments using liver-*Agt* KO mice.

### Experiment 1

We fed control and liver-*Agt* KO mice with HFD and compared with those fed NFD. At baseline, the mean body weight of liver-*Agt* KO and control *Agt* mice was not statistically different (17.7±2.3 and 19.4±1.3 g). After the feeding period of 20 weeks, the bodyweight of control *Agt* mice fed HFD was, on average, 38.3±7.1 g, which was greater than that of control *Agt* mice fed NFD, 28.0±2.7 g ($p=0.01$). In contrast, the bodyweight of liver-*Agt* KO mice fed HFD was no different from that of liver-*Agt* KO mice fed NFD (26.3±1.9 vs 27.1±3.6 g) (Figure 1(a)).

Mean epididymal fat mass was 7.3-fold greater in control *Agt* mice fed HFD than those fed NFD (2.2±0.5 vs 0.3±0.2 g, $p<0.001$) (Figure 1(b)). The increase in epididymal fat mass with HFD was 4.1-fold in liver-*Agt* KO (0.7±0.4 vs 0.2±0.1 g, $p<0.01$).

HFD significantly reduced *Agt*/18S rRNA in the adipose tissue (Figure 1(c)). With NFD, liver-*Agt* KO mice showed a level of adipose *Agt*/18S rRNA similar to that of control *Agt* mice. In contrast to control *Agt* mice, the decrease in adipose *Agt*/18S rRNA seen with HFD was modest and not significant in liver-*Agt* KO mice.

Western analysis showed no significant difference in the amount of Agt protein among the four mouse groups (Figure 1(d)). HFD did not significantly change plasma Agt in control *Agt* mice (2.38 (2.24–2.53) µg/ml on NFD vs 2.21 (1.86–2.63) µg/ml on HFD) (Figure 1(e)). As previously reported, plasma Agt concentration in liver-*Agt* KO mice fed NFD was extremely low, on average, 0.11 (0.09–0.14) µg/ml, which was only 4.8% of that in control *Agt*+NFD group. Liver-*Agt* KO mice fed HFD showed slightly higher plasma Agt concentration, 0.17 (0.16–0.20) µg/ml than the liver-*Agt* KO+NFD group. This difference is significant only when the data are analyzed after logarithmic transformation. Nonetheless, this value remained low, only 7.6% of that in the control *Agt*+HFD group.

At baseline, systolic blood pressure measured by the tail-cuff method in liver-*Agt* KO mice was, on average, 76±5 mm Hg, remarkably lower than that in control *Agt* mice, averaging 103±5 mm Hg ($p<0.001$) (data not shown). After the feeding periods, control *Agt* mice fed HFD showed higher systolic blood pressure than those fed NFD (127±6 vs 106±5 mm Hg, $p<0.001$) (Figure 1(f)), although plasma Agt concentration was not different. On the other hand, both liver-*Agt* KO+NFD and liver-*Agt* KO+HFD groups were similarly hypotensive, with systolic blood pressure averaging 73±6 and 67±9 mm Hg, respectively, although the latter showed a slightly higher plasma Agt concentration. Thus, HFD increased blood pressure in control *Agt* mice independently of plasma Agt, and the slight increase in plasma Agt seen in liver-*Agt* KO mice was not accompanied by a detectable increase in blood pressure.

### Experiment 2

In the above experiment, HFD only modestly increased fat mass and did not affect body weight in liver-*Agt* KO mice. We, therefore, aimed to more effectively increase body-weight in liver-*Agt* KO mice. Since angiotensin II deficiency is reported to cause lipid wasting,[24] we fed liver-*Agt* KO mice with HFD and simultaneously supplemented with a non-pressor dose of angiotensin II (100 ng/kg/min) for eight weeks, and compared them with age- and gender-matched control *Agt* mice fed NFD. On the 56th day, liver-*Agt* KO mice showed more bodyweight (34.5±4.2 vs 31.8±2.2 g, $p<0.05$) and more bodyweight gain ($p<0.05$) (Figure 2(a)), We, therefore, stopped angiotensin II infusion on that day. To avoid a possible direct effect of angiotensin II infusion on fat *Agt* mRNA, we set a five-day interval and completed the experiment on the 61st day.

During that five-day period, the liver-*Agt* KO mice slightly lost bodyweight. The epididymal fat mass of liver-*Agt* KO mice was comparable to that of control *Agt* mice (0.91±0.42 vs 0.77±0.29 g) (Figure 2(b)).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis showed that liver-*Agt* KO mice with HFD and angiotensin II supplementation had slightly less amount of *Agt* mRNA in the fat tissue than control *Agt* mice (*p*=0.03) (Figure 2(c)).

Western analysis revealed that *Agt* protein was reduced in liver-*Agt* KO mice given HFD and angiotensin II (Figure 2(d)).

Plasma Agt concentration in the liver-*Agt* KO mice was uniformly and markedly lower than that in control *Agt* mice (0.09±0.02 vs 2.07±0.45μg/ml, *p*<0.001) despite the bodyweight gain (Figure 2(e)). Although the mice lost weight during the last five-day period, plasma Agt concentration in the liver-*Agt* KO mice was low throughout the experimental period. In liver-*Agt* KO mice, plasma Agt concentration was significantly associated with epididymal fat mass (*R*=0.95, *p*<0.001), while there was no such association in control *Agt* mice (Figure 2(f)). Systolic blood pressure in the liver-*Agt* KO mice was also uniformly and continuously lower than that in control *Agt* mice (78±12 vs 111±6 mm Hg, *p*<0.001) (Figure 2(g)).

## Discussion

In the present study, we assessed the contribution of adipocytes to plasma Agt in the absence of the overwhelmingly dominant hepatocyte-derived Agt. To increase fat mass, we used HFD with or without angiotensin II supplementation.

In Experiment 1, plasma Agt concentration and epididymal fat weight of individual liver-*Agt* KO mice were not correlated. A possible contribution of the fat can be observed only when liver-*Agt* KO mice on HFD and liver-*Agt* KO mice on NFD groups were compared. HFD slightly increased plasma Agt, on average, by 53 ng/ml in liver-*Agt* KO mice, while it increased epididymal fat weight, on average, by 0.52 g. Assuming that the increased plasma Agt was ascribed to the increase in fat mass and that the relationship between plasma Agt concentration and epididymal fat mass is linear, every 1 g increase in epididymal fat is expected to accompany 102 ng/ml increase in plasma Agt. In Experiment 2, plasma Agt and epididymal fat weight of the individual liver-*Agt* KO mice were positively correlated (Figure 2(f)), indicating that every 1 g increase in the epididymal fat is expected to accompany 42.6 ng/ml increase in plasma Agt. On the other hand, the average plasma Agt concentration in control *Agt* mice on NFD was 2073 ng/ml. In this regard, in an earlier study, Yiannikouris et al. estimated that 25% of plasma Agt is derived from the adipose tissue.[20] If this is the case, about 500 ng/ml of plasma Agt is generated by the adipose tissue in control *Agt* mice on NFD. In conjunction with our

observed relationship between fat weight and plasma Agt, the weight of their epididymal fat would be estimated to be approximately 5–12 g, i.e. 15–36 times the average epididymal fat mass in normal mice fed HFD.

Of note, the above estimation is based on the data obtained from liver-*Agt* KO mice and may overrate the contribution of the fat to plasma Agt. In control *Agt* mice, Agt synthesis in the adipose tissue is suppressed as fat mass increases,[18] which was also observed in the present study (Table 3 and Figure 1(c)). Our assessment of the contribution of the fat is indirect while precise assessment can be made by direct comparison of liver-adipose dual *Agt* KO mice and liver-*Agt* KO mice. Nevertheless, HFD did not affect liver *Agt* mRNA, and the efficiency of liver *Agt* gene deletion is uniformly almost complete in liver-*Agt* KO mice, validating our indirect assessment.

Overall, the current study indicates that adipocytes have very little contribution to plasma Agt in the presence of hepatocyte-derived Agt. Importantly, in both experiments, HFD did not increase systolic blood pressure in liver-*Agt* KO mice, indicating that adipocyte-derived Agt has no appreciable impact on blood pressure in the presence of hepatocyte-derived Agt. As observed in the control *Agt* mice of Experiment 1, HFD increases systolic blood pressure, but this occurs independently of plasma Agt. In this matter, mechanisms thus far proposed include hyperinsulinemia, high leptin level, hyperactivity of sympathetic nervous system, and increase in aldosterone and increase in sodium retention.[29]

The present study confirmed that adipocyte expresses a large amount of *Agt* mRNA. Since plasma contains a high concentration of Agt protein, the adipose tissue in wild-type mice is expected to contain a considerable amount of liver-derived Agt in the interstitium. Our study demonstrated an intense Agt band in the adipose tissue of liver-*Agt* KO mice, indicating that the adipose tissue generates a substantial amount of Agt protein. This is in contrast to the kidney. Previously, we found that renal proximal tubular cells in the S3 segment synthesized a large amount of *Agt* mRNA, but the Agt protein was immediately secreted into the urine and not retained in the kidney. This renal *Agt* mRNA does not contribute to plasma Agt or renal angiotensin II.[22] In the adipose tissue, synthesized Agt protein appears to be mostly confined within the cell and not secreted into the plasma. In contrast to our present findings, it was previously reported that Agt was secreted constitutively and was not stored in secretory vesicles in a pituitary cell line.[30] The underlying mechanism is interesting but remains to be determined. Alternatively, although the fat has high *Agt* mRNA/18S RNA and high Agt protein/g levels, the content of RNA and protein is not comparably high. In addition, adipose RNA content further decreases when fat mass increases. This may be, at least, one reason why the fat has little contribution to plasma Agt.

Previously, Massiera et al. reported that transgenic overexpression of Agt in the adipose tissue increased plasma Agt

and blood pressure in wild-type mice, and partially restored plasma Agt and blood pressure in adipocyte-*Agt* KO mice.[19] Based on these experiments, these authors speculated that adipocyte-derived Agt is an important source of plasma Agt and has a role in blood pressure regulation. In their study, however, the transgenic adipose tissue secreted a four-fold amount of Agt compared to wild-type adipose tissue.

In the present study, we analyzed only the epididymal fat, and not subcutaneous fat. However, visceral fat tissues such as the epididymal fat generates a greater amount of Agt than the latter[31,32] and thus can be regarded as representative Agt-generating white adipose tissue.

We infused angiotensin II because this is the only way to increase fat mass in liver-*Agt* KO mice. A previous study reported that the infusion of angiotensin II increased *Agt* mRNA in the adipose tissue.[14] Although we may overestimate the contribution of the fat on plasma Agt, we can draw the same conclusion that the contribution of the fat to plasma Agt is small. In addition, in the present study, no change was observed in plasma Agt during the following five days after the discontinuation of angiotensin II, suggesting that angiotensin II infusion has no impact on plasma Agt in this experimental setting.

Recently, Yiannikouris et al. reported that adipocyte-specific *Agt* KO mice either on a low-fat diet or HFD showed similar plasma Agt levels to control mice on the same diet.[21] This finding is in concert with our finding, but not with their earlier observation in adipocyte-specific *Agt* KO mice on NFD.[20] Interestingly, they showed that adipocyte-specific *Agt* KO significantly decreased angiotensin II levels both in the adipose tissue and in the plasma on HFD, independently of plasma Agt. This decrease in angiotensin II was accompanied by a decrease in blood pressure. These results suggest that angiotensin II is generated from Agt within the adipose tissue, released into the systemic circulation, and has a significant impact on blood pressure. In our study, HFD failed to increase blood pressure despite the increase of the fat mass, suggesting that the angiotensin II locally generated within the adipose tissue has only a minor effect on blood pressure in the presence of hepatocyte-derived Agt. Since plasma renin activity in liver-*Agt* KO mice is extremely high, the failure of blood pressure increase suggests that angiotensin II is generated within the adipocyte independently of the plasma renin.

Although our study showed that the adipose tissue has an inappreciable contribution to the plasma Agt when compared to the liver, locally generated Agt and angiotensin II within the adipocyte may have direct effects on the adipose tissue in an autocrine and/or paracrine manner. These include promoting lipogenesis[33,34] and lipid accumulation,[33,35] induction of proinflammatory cytokines,[36] recruitment of macrophages,[37] increase in reactive oxygen species,[38] and decrease in adiponectin.[39] These may contribute to insulin resistance in the diabetes and the metabolic syndrome.[40,41]

In conclusion, although adipocytes generate a substantial amount of Agt, they have essentially very little contribution to the plasma concentration and an inappreciable impact on blood pressure in the presence of hepatocyte-derived Agt. This indicates that obesity-induced hypertension occurs independently of plasma Agt. The mechanism for the confinement of adipose Agt and its functional role await further investigation.

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