ONLINE SUPPLEMENT

Adipocyte Deficiency of Angiotensinogen Prevents Obesity-Induced Hypertension in Male Mice

Frederique Yiannikouris[1], Manisha Gupte[1], Kelly Putnam[1], Sean Thatcher[1], Richard Charnigo[2], Debra L. Rateri[3], Alan Daugherty[1,3], Lisa A. Cassis[1]
Graduate Center for Nutritional Sciences[1], Department of Statistics[2],
Saha Cardiovascular Research Center[3]
University of Kentucky, Lexington, KY

Supplemental Materials and Methods.

Experimental protocol. Male mice (2 months of age) of each genotype were fed a LF (10% kcal as fat; D12450B; Research Diets Inc, New Brunswick, NJ) or HF diet (60% kcal as fat; D12492, Research Diets Inc, New Brunswick, NJ) for 16 weeks. Diets were matched in protein content (20% kcal) and provided energy at 3.85 or 5.25 kcal/gm (LF and HF, respectively). Diets were provided to mice *ad libitum*. Fat and lean mass were measured on conscious mice at study endpoint using NMR spectroscopy (Echo MRI®).
Measurement of plasma and tissue AngII. Plasma AngII concentrations were quantified in male mice fed a LF or HF diet as described previously.[1] For tissue quantification of AngII, adipose tissue or liver (1:10 wt/vol) were homogenized on ice in buffer (0.1 N HCl, 80% ethanol, 50 mM EDTA, 0.5 mM o-phenanthroline, 0.1 mM pepstatin), centrifuged (20,000 g, 4^{o}C for 20 min), and the supernatants stored at -20^{o}C overnight. The next day, centrifugation was repeated, and supernatants were diluted with 0.1% orthophosphoric acid (0.1% wt/vol) and stored at 4^{o}C for 6 hours. This process was repeated, and then samples were processed over C_{18} mini-columns. Eluted peptides were vacuum concentrated, and angiotensin peptides quantified by radioimmunoassay using an anti-rat AngII antibody as described previously.[1] To quantify renin-like activity, explants of adipose tissue (10-15 mg) were incubated in buffer containing captopril (1 μM) and EDTA (0.05 M) for 4 hours. Angiotensin I released into the media was quantified by radioimmunoassay as described previously.[1.2]

Quantification of plasma renin, total renin, and AGT. Plasma renin activity (PRA) was quantified indirectly by incubating mouse plasma (8 μl) at 37^{o}C (30 min) in phosphate buffer containing EDTA (0.05 M) and enalapril (10 μM), followed by quantification of angiotensin I by radioimmunoassay as described previously.[1. 2] Plasma renin concentration (PRC) was quantified by incubating mouse plasma (8 μl) with exogenous AGT (25 nM) prepared from nephrectomized rats at 37EC (30 min) using the method described above. Plasma (10 μl) concentrations of AGT were quantified using a mouse total angiotensinogen assay kit (Immuno-Biological Laboratories Co, Gunma, Japan) as described previously.[3] This kit does not distinguish intact AGT from des-asp AGT. Plasma total renin (reinin+prorenin) was quantified using the rat Prorenin/Renin Total Antigen Assay (Molecular Innovation, MI, USA)

Measurement of blood pressure*.* Blood pressure was measured by radiotelemetry during week 16 according to methods described previously.[4] Briefly, anesthetized (isoflurane) mice were implanted with carotid artery catheters and telemetry devices during week 15 of LF or HF feeding, allowed 1 week to recover, and then pressure was recorded continuously (5 minute sampling) for 3-5 days. Inclusion criteria were (1) pulse pressures $\geq$ 20 mmHg, (2) standard deviation of the pulse pressure $\leq$ 9 mmHg and (3) the presence of diurnal differences in SBP between the night and light cycle.

Measurement of glucose tolerance. Mice were fasted for 6 hours before quantification of glucose tolerance. Blood glucose was quantified at 0 min before injection of glucose solution (2 mg/kg body weight, ip) and at 15, 30, 60 and 120 min

after injection as described previously.[24]

Tissue RNA extraction and quantitative RT-PCR. Tissue RNA was quantified as described previously.[24] Estimation of amplified gene products was normalized to 18S RNA for liver and kidney and analyzed using the $2^{-\Delta\Delta Ct}$ method. For adipose tissue (HF versus LF diet) , amplified gene products were normalized to the average of IPO8 and FBXL10 mRNA abundance. The primer sequences are illustrated in Table S1.

Field Code Changed

Statistical analysis. Data are expressed as mean ± SEM. All data were analyzed using SigmaPlot. Ordinary and repeated measures ANOVAs were employed to compare diet and genotype groups on quantitative outcome measures, followed by post-hoc tests with Holm-Sidak adjustments for multiple comparisons. When the assumptions underlying the ANOVA's were not otherwise met, we nonlinearly transformed the observations before running the ANOVA's to obtain valid p-values; however, for ease of interpretation, the Figures are based on the untransformed observations. Figure 6 data were analyzed by three-way ANOVA on log-transformed responses, with the modification that mouse-specific random effects were included. Statistical significance was declared at $P<0.05$.

References

1. Daugherty A, Rateri DL, Lu H, Inagami T, Cassis LA. Hypercholesterolemia stimulates angiotensin peptide synthesis and contributes to atherosclerosis through the AT1A receptor. *Circulation.* 2004;110:3849-3857.

2. Cassis LA, Rateri DL, Lu H, Daugherty A. Bone marrow transplantation reveals that recipient AT1a receptors are required to initiate angiotensin II-induced atherosclerosis and aneurysms. *Arterioscler Thromb Vasc Biol.* 2007;27:380-386.

3. Yiannikouris F, Karounos M, Charnigo R, English VL, Rateri DL, Daugherty A, Cassis LA. Adipocyte-specific deficiency of angiotensinogen decreases plasma angiotensinogen concentration and systolic blood pressure in mice. *Am J Physiol Regul Integr Comp Physiol*. 2012;302:R244-251.

4 . Gupte MN, Boustany-Kari CM, Bharadwaj K, Police S, Thatcher S, Gong MC, English VL, Cassis LA. Ace2 is expressed in mouse adipocytes and regulated by a high fat diet. *Am J Physiol Regul Integr Comp Physiol.* 2008;295:R781-788, 2008.

Table S1

Gene	Forward 5'-3'	Reverse 5'-3'
(pro)renin receptor	AGCCCGTGGCCGGTGGAATA	TGGTGGCGGGTGCTTTAGGGA
ACE	GGAGTACTTCCAACCGGTCA	GCCTTGGCTTCATCAGTCTC
AGT	GTACAGACAGCACCCTACTT	CACGTCACGGAGAAGTTGTT
Tonin	CTGCTCCGCCTCAGCAAGCC	TGCTGCCCCAGCCTGACACT
Cathepsin D	CGGCGACCTCTGGCTTTAAG	GGACCCAGCAGGCTATGTCAAG
18s	AGTCGGCATCGTTTATGGTC	CGAAAGCATTTGCCAAG
IPO8	ACCAGGACCCGTCACGTCG	ATCCACGGCAGGAGGTCGGT
FBXL10	AAGTATGCCTCCAACCTGCC	TTTTTGGGGTGCTCGTCTGA

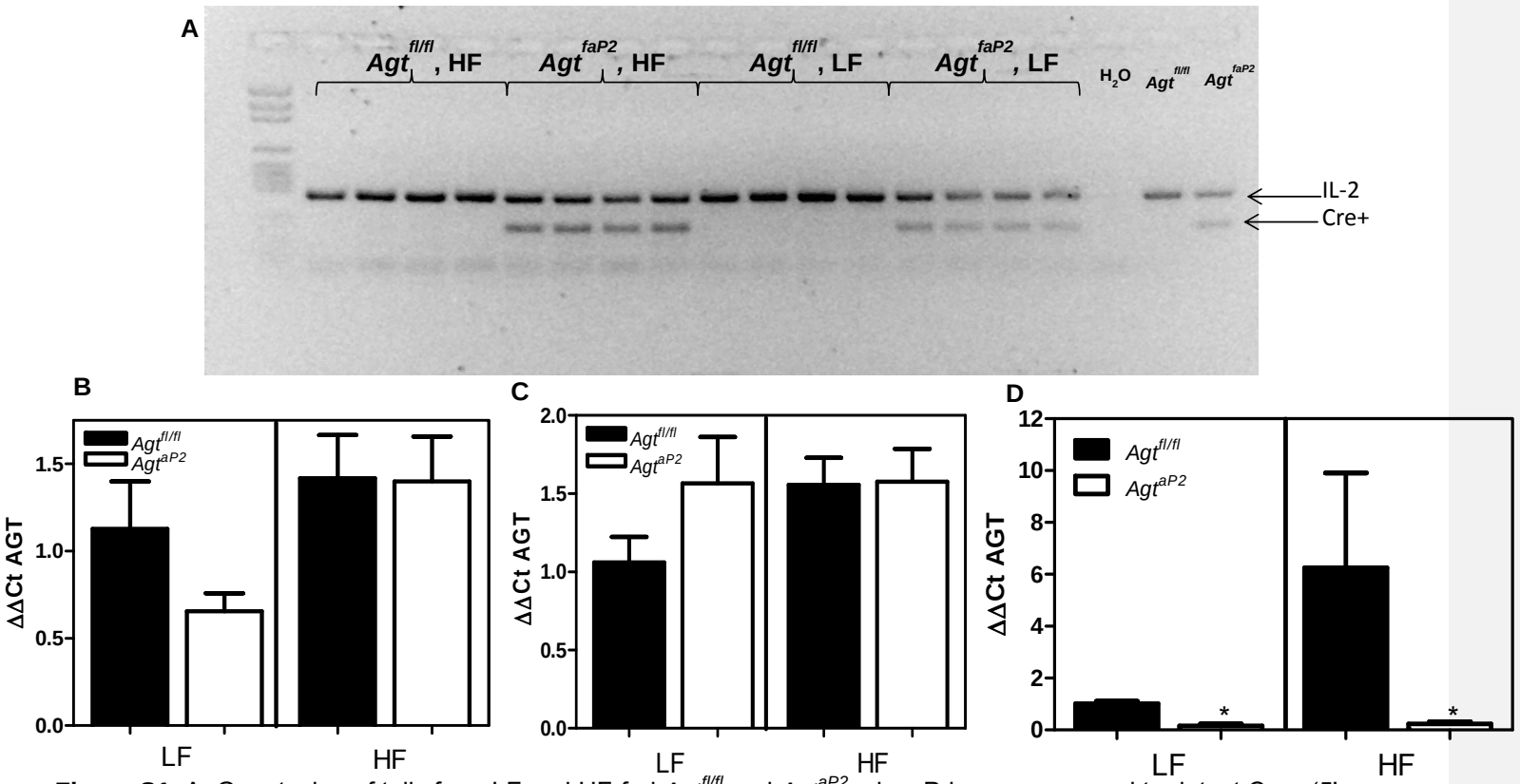
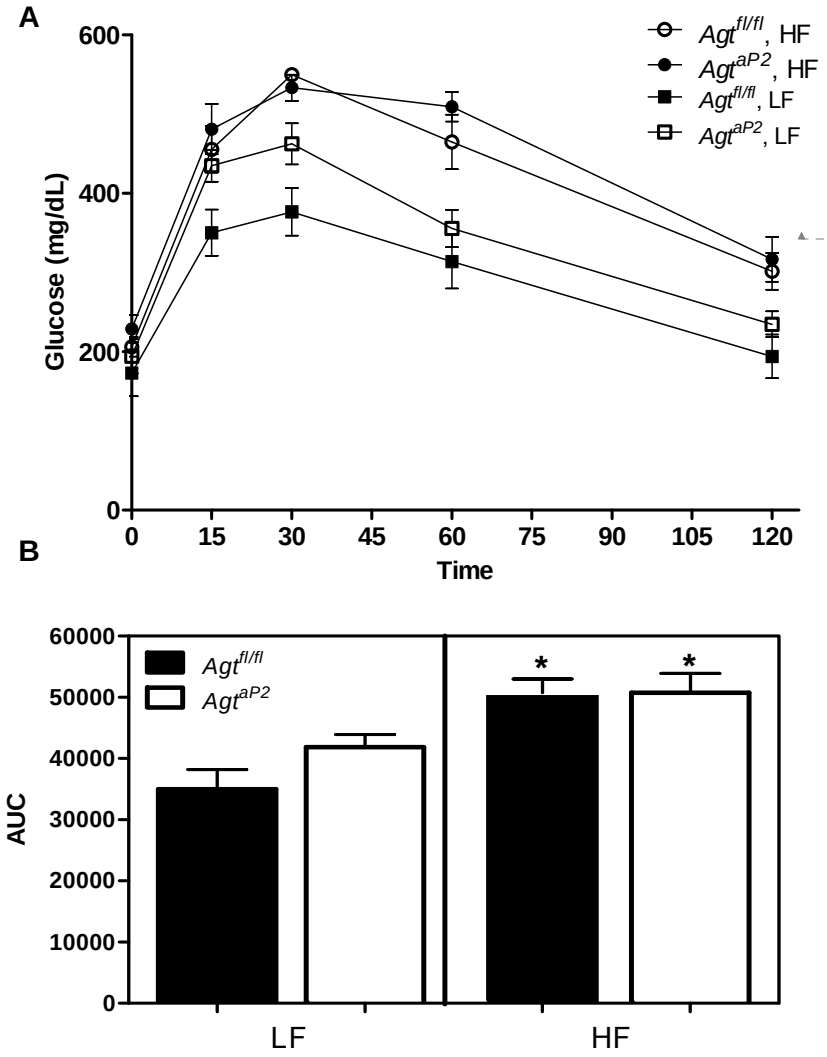


Figure S1. A. Genotyping of tails from LF and HF-fed *Agt*$^{fl/fl}$ and *Agt*^{aP2} mice. Primers were used to detect Cre+ (5'-ACCTGAAGATGTTCGCGATT and 5'-CGGCATCAACGTTTTCTTTT), IL-2 gene (5'-CTAGGCCACAGAATTGAAAGATCT and 5'-GTAGGTGGAAATTCTAGCATCATCC). Cre+ mice were identified by a product of 182bp. B. AGT mRNA abundance in liver; C. AGT mRNA abundance in kidney; D. AGT mRNA abundance in white adipose tissue. Data are mean ± SEM from n = 6-10 mice/group. *, P<0.05 compared to *Agt*$^{fl/fl}$ within diet group.



Field Code Changed

Figure S2. Glucose tolerance tests in LF and HF-fed *Agt*$^{fl/fl}$ and *Agt*^{aP2} mice. A, Blood glucose concentrations at several time points after administration of glucose (2 mg/kg body weight). B, Area under the curve (AUC) for data in A, above. Data are mean ± SEM from n = 7-10 mice/group. *, P<0.05 compared to LF within genotype.

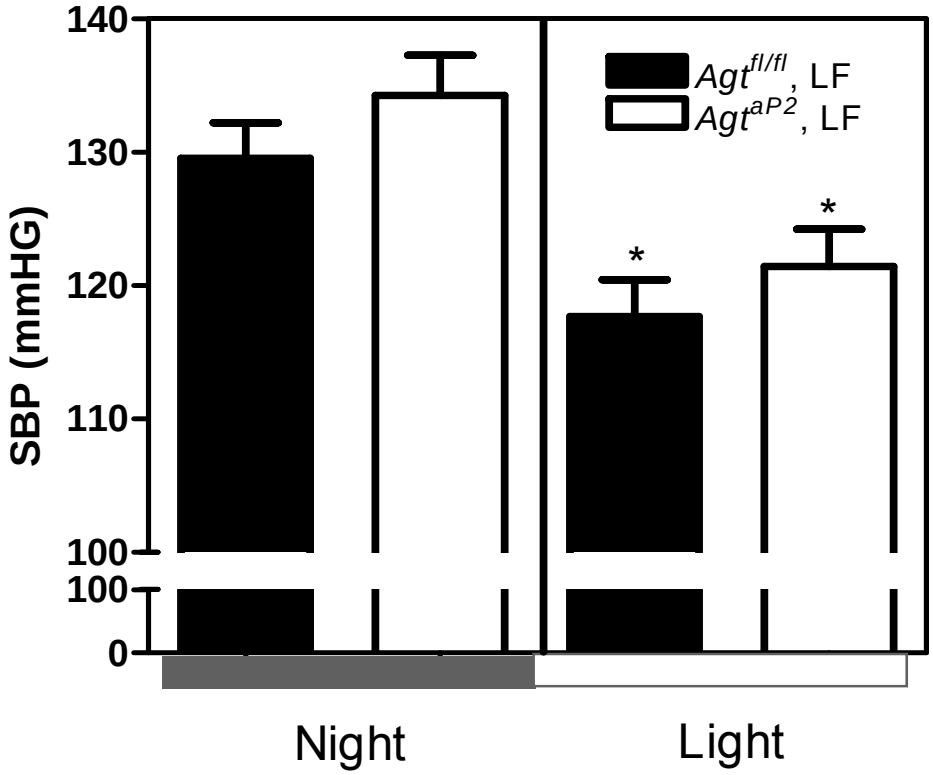


Figure S3. SBP (12 hour averages for each mouse) of LF-fed *Agt*$^{fl/fl}$ and *Agt*^{aP2} mice during the night and light cycle. Carotid artery catheters and radiotelemeters were implanted at week 15 of LF, mice were allowed one week to recover, and SBP was recorded for 3 - 5 days. Data are mean ± SEM from n = 6-7 mice/group (LF) over the 3 - 5 days of recording. *, P<0.05 compared to night cycle within genotype.

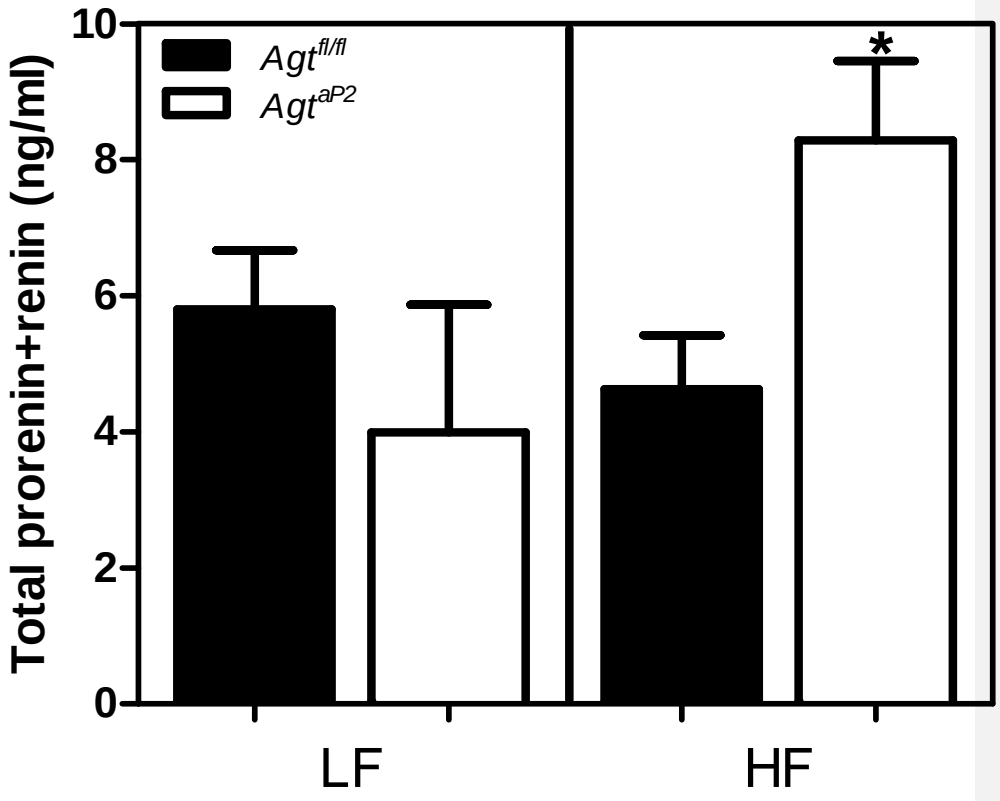


Figure S4. Total renin (renin+prorenin) in plasma of LF and HF-fed *Agt*$^{fl/fl}$ and *Agt*^{aP2} mice. Data are mean ± SEM from n = 5-6 mice/group. *, P<0.05 compared to LF within genotype.

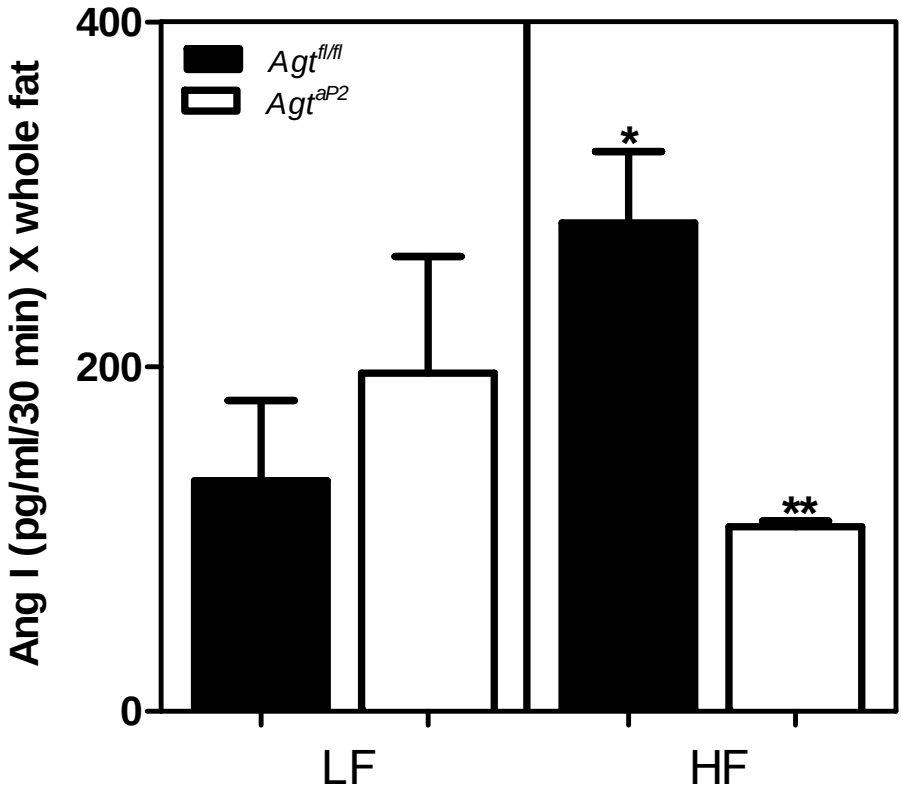
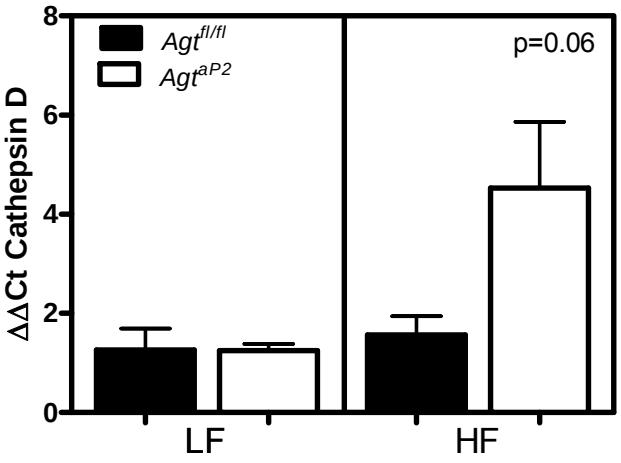


Figure S5. Renin-like activity, as evidenced by generation of angiotensin I, in explants of adipose tissue from LF and HF-fed *Agt*$^{fl/fl}$ and *Agt*^{aP2} mice. Data are mean ± SEM from n = 3-4 mice/group. *, P<0.05 compared to LF within genotype. **, P<0.05 compared to *Agt*$^{fl/fl}$ within diet group.

A



B

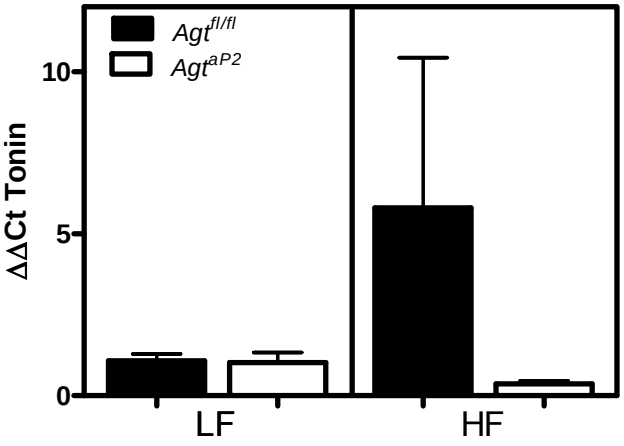


Figure S6. Cathepsin D (A) and tonin (B) mRNA abundance in adipose tissue from LF and HF-fed *Agt*$^{fl/fl}$ and *Agt*^{aP2} mice. Data are mean ± SEM from n = 5-7 mice/group. P = 0.06 genotype by diet interaction.