ONLINE SUPPLEMENTAL MATERIAL

Gene Trapping Uncovers Gender-Specific Mechanisms for Upstream Stimulatory Factors 1 and 2 in Angiotensinogen Expression

Sungmi Park, Xuebo Liu, Deborah R. Davis, and Curt D. Sigmund
Departments of Pharmacology, Roy J. and Lucille A. Carver College of Medicine,
University of Iowa, Iowa City, IA 52242

Supplemental Methods:

Animals. Due to an inability to obtain USF1 null mice we employed a USF1 gene trap ($USF1^{GT}$) allele ($USF1^{Gt\ (XG830)\ Byg}$) obtained from BayGenomics (now the Mutant Mouse Regional Resource Center, MMRRC, affiliated with the KnockOut Mouse Project, KOMP). XG830 ES cells were injected into the blastocysts in the University of Iowa Gene Targeting Facility and the chimeras were bred to C57BL/6 to confirm germline transmission. Mice carrying a hAGT transgene[1] were bred with $USF1^{GT}$ mice. Double transgenic mice carrying hAGT and $USF1^{GT}$ were confirmed by PCR based genotyping in the transgenic facility at the University of Iowa.

Gene expression. Animals were sacrificed by CO_2 asphyxiation between 9-12 am. Tissues were collected by blunt dissection, weighed, snap frozen at -80°C immediately, and total RNA was extracted using TRIzol Reagent according to the protocol provided by the manufacturer (Invitrogen) and QIAGEN RNeasy Mini kit's manual. The expression levels of Usf1, Usf2, mAgt and hAGT genes were assessed by real-time RT-PCR using TaqMan primer/probe sets from Applied Biosystems (Usf1, Mm01290210_g1; Usf2, Mm00495959_m1; Agt, Mm00599662_m1; hAGT, Hs01586213_m1). The quantitive expression of p53, Fasn, and PPARγ were performed by SYBR green mix using iCycler (Biorad). Primer sequences follows: 1) p53 5'-AAAGGATGCCCATGCTACAGAGGA-3', and 5'-AGTAGACTGGCCCTTCTTGGTCTT-3'; 2) Fasn 5'-TGCTCCCAGCTGCAGGC-3', and 5'-GCCCGGTAGCTCTGGGTGTA-3'; 3) PPARγ 5'-GATGGAAGACCACTCGCATT-3', and 5'-AACCATTGGGTCAGCTCTTG -3'. All data were normalized by beta actin probe and quantified by the $2^{-\Delta\Delta CT}$ method.[2,3]

Western blot. Antibodies against USF1 (1:10000 sc-229X, Santa Cruz Biotechnology), USF2 (1:10000 sc-861X, Santa Cruz Biotechnology), hAGT (1:10000, gift from Duane Tewksbury, Marshfield Medical Research Foundation, Marshfield, Wis), β-actin (1:5000 ab8227, Abcam) and albumin primary antibody (1:10000 sc46293, Santa Cruz Biotechnology) were used for western blotting. Quantitative Western analysis was performed using imaging software (ImageJ, NIH) after normalization by β-actin for tissues or albumin for plasma.

Gel Shift and Super shift Assays. Nuclear extracts were prepared from liver samples and 7ug per lane were used for electrophoretic mobility shift assay (EMSA). Double-stranded DNA oligos were labeled by ^{32}P ATP. The sequences were as follows; 5'-GAT CTAAATAGGGC**X**TCGTGACCCGGCCAGG-3' where X=A or C for the -20 SNP in hAGT. Gel shifts were carried out using 5% polyacrylamide gel and USF1 (sc-229X), USF2 (sc-861X) (1 µg/reaction) were added for super shift under the same condition. A 2-50 excess of unlabelled double stranded DNA (C or mutant G at -20 region of hAGT promoter sequence) were used as competitors to confirm specific DNA-protein interactions.

Chromatin Immunoprecipitation (ChIP). Mouse liver tissues were harvested and frozen. Tissues were minced on ice and incubated in PBS containing 1% (v/v) formaldehyde at

room temperature (15 min). The reaction was stopped by addition of glycine (0.125M, 5 min at room temperature), homogenized on ice, and then rinsed with ice-cold PBS including proteinase inhibitors three times. The pellet was resuspended in lysis buffer with proteinase inhibitors (EZ-CHIP, Millipore) and sonicated on ice (Amplitude 30%, time 12 seconds, cooling 30 seconds, 10-15 times using a Sonic Dismembrator Model 500, Fisher Scientific). The size of the sonicated chromatin was verified as between 200-1000 bp by electrophoresis. Immunoprecipitation was performed with the EZ-CHIP kit using 5ug antibody against USF1 (sc-229X), USF2 (sc-861X), or non specific IgG as a negative control, RNA polymerase II provided by the kit as a positive control as described by us.[4] Real-time PCR reactions utilized SYBR-Green mix (Applied Biosystems). The sequence for amplification of the proximal region of the hAGT promoter (-129-+76) was 5'- AGTGTCGCTTCTGGCATCTGT -3' and 5'-AGAGACAA GACCGAGAAGGAG-3'.

Intravenous Adenoviral Delivery of shRNA against USF2 (AdshUSF2) in vivo. AdshUSF2.3 expressing the 21-nt core sequence (5'-GGATCGTCCAGCTTTCGAAAA-3' for acute Usf2 inhibition *in vivo* was prepared as described.[4] Intravenous jugular cannulation, adenoviral delivery, and extraction of proteins from tissues were performed as described.[4] We injected $2X10^{10}$ pfu of AdshGFP as a control or $1X10^{10}$ pfu of AdshUSF2.3 and tissues were harvested after 5 days.

Plasma Aldosterone. Plasma aldosterone was determined using an ELISA kit from Cayman Chemical (Cat # 1004377) after 1:2 dilution of plasma.

Metabolic rate: Individual Mice were placed into a water-jacketed 2 L beaker (Ace Glass, Vineland, NJ) maintained at 30°C, and room air was drawn through the chamber at 300 mL/min (R2 flow control, AEI) described as in [5] .

Food and fluid Intake: Mice were acclimated to Nalgene (Rochester, NY) single-mouse-sized metabolism cages for at least 2 days before the measurement for water intake, food intake, and urine volume averaged over 24 hour collections for a 3 day period.

References

1. Yang G, Merrill DC, Thompson MW, Robillard JE, Sigmund CD. Functional expression of the human angiotensinogen gene in transgenic mice. *J Biol Chem*. 1994;269:32497-32502.

2. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402-408.

3. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008;3:1101-1108.

4. Dickson ME, Tian X, Liu XB, Davis DR, Sigmund CD. Upstream Stimulatory Factor Is Required for Human Angiotensinogen Expression and Differential Regulation by the A-20C Polymorphism. *Circulation Research*. 2008;103:940-U83.

5. Grobe JL, Grobe CL, Beltz TG, Westphal SG, Morgan DA, Xu D, de Lange WJ, Li H, Sakai K, Thedens DR, Cassis LA, Rahmouni K, Mark AL, Johnson AK, Sigmund CD. The brain Renin-angiotensin system controls divergent efferent mechanisms to regulate fluid and energy balance. *Cell Metabolism*. 2010; 12:431-442.

Supplemental Table S1. Physiologic and Metabolic Parameters

		10% Fat Diet		60% Fat Diet	
		$USF1^{WT}$	$USF1^{GT}$	$USF1^{WT}$	$USF1^{GT}$
Female		(n≥6)	(n≥6)	(n≥6)	(n≥6)
Plasma Aldo	pg/mL	1158 ± 48	1314 ± 11*	1214 ± 43	1252 ± 22
Body Weight	g	20.9 ± 0.7	21.3 ± 0.6	29.5 ± 2.2†	28.2 ± 1.6†
Food Intake	Kcal/day	13.6 ± 0.3	13.7 ± 0.4	7.0 ± 0.4†	7.3 ± 1.2†
Glucose	mg/dL	146 ± 26	160 ± 32	142 ± 8	129 ± 12
Water Intake	mL/day	2.8 ± 0.1	3.4 ± 0.6	1.8 ± 0.2†	1.9 ± 0.4†
Urine Output	mL/day	1.0 ± 0.1	1.4 ± 0.2	0.5 ± 0.1†	0.4 ± 0.1†
VO_2	mL/100g/min	3.18 ± 0.02	3.59 ± 0.10*	3.03 ± 0.09	3.50 ± 0.08*
RQ		0.92 ± 0.02	0.87 ± 0.01	0.84 ± 0.03†	0.83 ± 0.03
Heat	Kcal/hr	0.20 ± 0.01	0.22 ± 0.01	0.26 ± 0.03	0.25 ± 0.01

		10% Fat Diet		60% Fat Diet	
		$USF1^{WT}$	$USF1^{GT}$	$USF1^{WT}$	$USF1^{GT}$
Male		(n≥6)	(n≥6)	(n≥6)	(n≥6)
Plasma Aldo	pg/mL	N.D	N.D	N.D	N.D
Body Weight	g	26.7 ± 1.1	27.2 ± 1.6	41.7 ± 2.2†	37.0 ± 2.0†
Food Intake	Kcal/day	13.2 ± 0.6	15.4 ± 0.3*	8.0 ± 0.8†	8.2 ± 0.4†
Glucose	mg/dL	140 ± 13	136 ± 19	166 ± 23	170 ± 7
Water Intake	mL/day	2.7 ± 0.3	3.1 ± 0.5	1.8 ± 0.5†	1.8 ± 0.2†
Urine Output	mL/day	0.7 ± 0.2	1.7 ± 0.3*	0.7 ± 0.1	0.8 ± 0.1†
VO_2	mL/100g/min	2.83 ± 0.05	2.91 ± 0.09	2.39 ± 0.09†	2.61 ± 0.13
RQ		0.89 ± 0.01	0.95 ± 0.02*	0.84 ± 0.01†	0.83 ± 0.02†
Heat	Kcal/hr	0.23 ± 0.01	0.23 ± 0.02	0.28 ± 0.02†	0.30 ± 0.01†

N.D., not determined
*, P<0.05 vs. $USF1^{WT}$
†, P<0.05 vs. 10% fat diet

Supplemental Figures

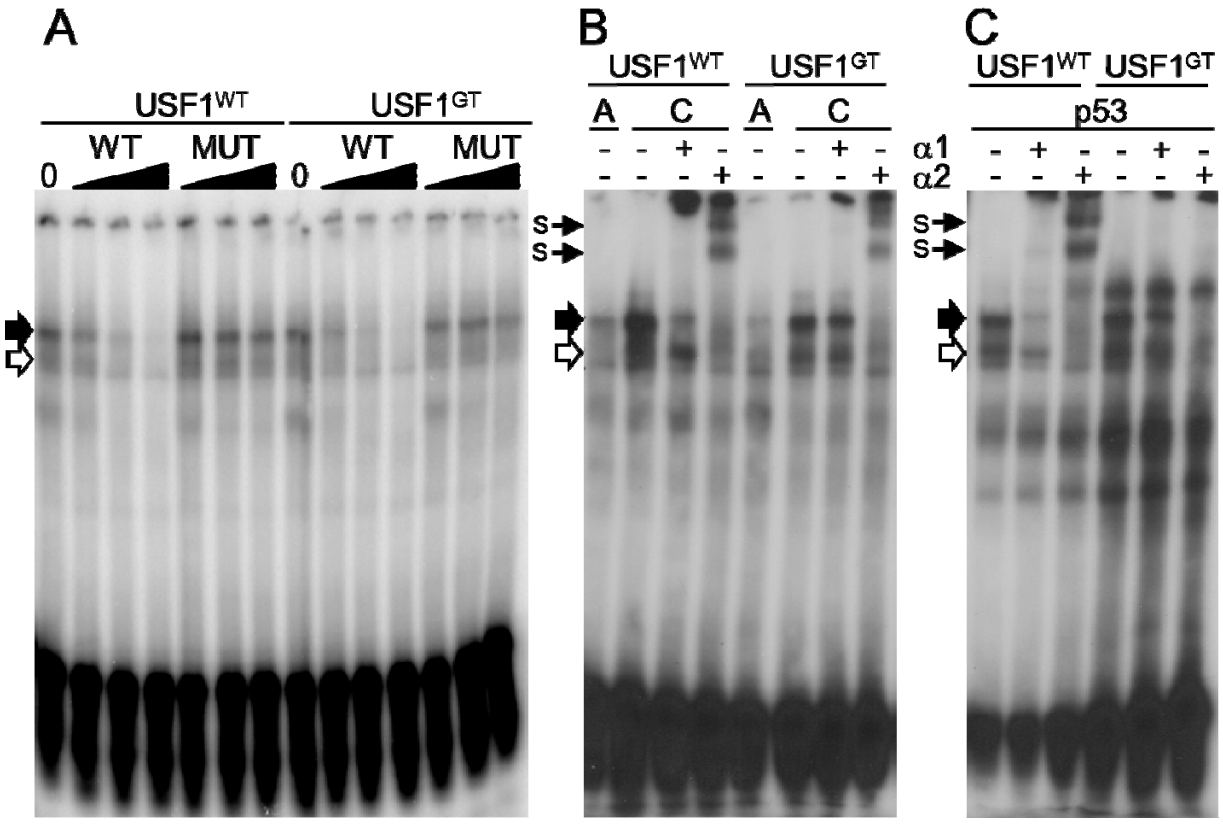

Figure S1. ***EMSA of liver nuclear extract from USF1^{WT} and USF1^{GT} male mice*.**

A. Competition EMSA using labeled -20C probe. Ramped increases in cold wildtype (WT) and mutant (MUT) probes are indicated by the closed triangles. The source of the extracts (USF1^{WT} and USF1^{GT}) is indicated. The mutant probe substitutes -20G for -20C. B. EMSA and supershift using -20A (A) or -20C (C) probes and antisera directed against USF1 (α1) and USF2 (α2). C. EMSA and supershift using p53 probes and antisera directed against USF1 (α1) and USF2 (α2). Closed arrowhead, major shift product; open arrowhead, minor shit product; S-arrows, supershift products.