Journal of the American Heart Association

ORIGINAL RESEARCH

Persistent Renin-Angiotensin System Sensitization Months After Body Weight Recovery From Severe Food Restriction in Female Fischer Rats

Aline M. A. de Souza, PhD; Hong Ji, MD; Xie Wu, BS; Kathryn Sandberg, PhD; Crystal A. West, PhD

BACKGROUND: Prior exposure to periods of severe food restriction (sFR) is associated with increased risk of developing hypertension and cardiovascular disease later in life.

METHODS AND RESULTS: To investigate the mechanism of these long-term adverse effects of sFR, 4-month-old female Fischer rats were divided in 2 groups and maintained on a normal diet ad libitum (control) or on an sFR diet with 60% reduction in daily food intake for 2 weeks that resulted in a 15% reduction in body weight. After the 2-week sFR period ended, both groups received normal chow ad libitum for 3 months. Within 2 weeks after refeeding was initiated in the sFR group, body weight was restored to control levels; however, plasma angiotensinogen (1.3-fold; $P<0.05$), Ang-[1-8] (2.0-fold; $P<0.05$), and angiotensin-converting enzyme activity (1.1-fold; $P<0.01$) were all elevated 3 months after refeeding. Angiotensin type 1 receptor activity was also increased as evidenced by augmented pressor responses to angiotensin-[1-8] ($P<0.01$) and depressor responses to the angiotensin type 1 receptor antagonist, losartan ($P<0.01$) in the sFR group.

CONCLUSIONS: These results indicate that sensitization of the renin-angiotensin system persisted months after the sFR period ended. These findings may have implications for women who voluntarily or involuntarily experience an extended period of sFR and thus may be at increased risk of developing cardiovascular disease through sensitization of the renin-angiotensin system even though their body weight, mean arterial pressure, and heart rate appear normal.

Key Words: body weight recovery ■ calorie restriction ■ sensitization

Severe food restriction (sFR) can be the result of psychological conditions such as anorexia nervosa, environmental stressors, or economic forces (eg, very low food security).[1,2] Irrespective of the cause, sFR can result in adverse cardiovascular health-related consequences. Acutely, sFR can cause hypotension, bradycardia, long QT intervals, arrhythmias, and myocardial infarction.[3,4] However, the long-term cardiovascular effects of sFR after body weight (BW) recovery has not been extensively investigated.

We have shown previously that 14 days of sFR (60% reduction in daily food intake) in female Fisher 344 rats caused hypotension, bradycardia, and plasma volume contraction, which resulted in activation of the renin-angiotensin system (RAS).[5] Specifically, we found an increase in plasma levels of angiotensinogen, angiotensin-converting enzyme (ACE) activity and the octapeptide angiotensin-[1-8] (also known as angiotensin II) as well as mRNA expression of the angiotensin type 1 receptor (AT_1R) in mesenteric arteries. However, the pressor responses to angiotensin-[1-8] were diminished even though the RAS was activated and AT_1Rs remained responsive to the depressor effects of a specific AT_1R antagonist. Whether this reprogramming of

Correspondence to: Crystal A. West, PhD, Department of Medicine, Georgetown University, Building D, Room 396, 4000 Reservoir Road, NW, Washington, DC 20057. E-mail: caw240@georgetown.edu

For Sources of Funding and Disclosures, see page 12.

JAHA is available at: www.ahajournals.org/journal/jaha

CLINICAL PERSPECTIVE

What Is New?

- We found that a low-calorie diet caused sensitization to the renin-angiotensin system even after the body weight was recovered and the food intake normalized.

What Are the Clinical Implications?

- Women who experienced short periods of low calorie intake and body weight loss may be at higher risk of developing hypertension and other cardiovascular diseases.

Nonstandard Abbreviations and Acronyms

ACE	angiotensin-converting enzyme
AT_1R	angiotensin type 1 receptor
BP	blood pressure
BW	body weight
HR	heart rate
MAP	mean arterial pressure
RAS	renin-angiotensin system
sFR	severe food restriction

the RAS persists after BW has recovered attributable to refeeding is currently unknown.

The purpose of this study was to investigate the long-term impact of sFR on the regulation of the RAS after refeeding. Therefore, we investigated key components of the angiotensin-[1-8] synthetic pathway and the expression and activity of the AT_1R in sFR female Fischer (F344) rats 3 months after refeeding. We chose the female model of sFR because more women than men voluntarily engage in sFR diets and experience eating disorders that result in rapid weight loss.[6] Furthermore, conducting this investigation enabled us to compare our findings at 3 months after refeeding with our previous studies on the acute effects of sFR on the RAS in female rats. We also measured the vasoconstrictor responses to exogenous angiotensin-[1-8] in mesenteric arteries and pressor and depressor responses to AT_1R activation and blockade, by angiotensin-[1-8] and losartan, respectively.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

Animal maintenance and experimental protocols were all performed with prior approval from the Georgetown University Animal Care and Use Committee (Protocol no. 2016.1182), and all procedures were in accordance with institutional guidelines and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. These approvals are also in accordance with this journal's policies and regulations on animal experimentation. Unnecessary distress to the animals was avoided, and efforts were made to minimize the number of animals used in the study.

Animals, Diet, and Housing

Female Fischer (F344) rats weighing 170 to 180 g at 3 to 4 months of age were purchased from Envigo Corporation (Frederick, MD). At 5:00 PM each day, food intake and BW were determined for each animal before the food was replenished, as we described.[7] Following this 2-week period, the rats were randomly divided into a control (n=28) and sFR (n=28) group. During the sFR period, both control and sFR animals were housed in individual cages on a 12-hour light-dark cycle at room temperature (24°C). The amount of food provided to individual rats in the sFR group was determined by averaging the food consumed during the prior 2 weeks for that specific animal. This normal level of food intake per individual rat was then used as the basis for calculating a 60% reduction in daily food intake for that animal. After the 2-week period of sFR ended, the sFR rats were given food ad libitum. All rats remained in single housing for an additional 2 weeks after the sFR period ended to enable accurate assessment of daily food and water intake. After this point, the rats were transitioned to paired housing to avoid the stress of single housing.[8] Note: within 1 week of refeeding, food intake in the sFR rats became indistinguishable from the control group. All animals received a standard rat diet with calories provided by 24.6% protein, 13.1% carbohydrate and 62.3% fat (Rodent diet 20, #5053; LabDiet, Marlborough, MA) and had ad libitum access to water. The only difference between the 2 groups was the amount of food offered during the sFR period.

Chronic Measurement of Mean Arterial Pressure and Heart Rate

Rats at 3 months of age were implanted with radio transmitters (#HD-S10; Data Sciences International; St. Paul, MN) in the left femoral artery after anesthesia (2.5% isoflurane at 1 L/min oxygen) and the battery pack was placed in the left flank subcutaneously, as described.[9] The analgesic carprofen (5 mg/kg; RIMADYL #10000319; Zoetis, Parsippany, NJ) was

administered subcutaneously for up to 3 to 4 days after surgery, and the health of the rats was closely followed for at least a week after surgery. After recovery from surgery (20 days), mean arterial pressure (MAP) and heart rate (HR) recordings were taken every 5 minutes for 10 seconds. Night (6 PM to 6 AM) and day (6 AM to 6 PM) averages were calculated using a Data Acquisition and Analysis System (Dataquest ART v4.36; Data Sciences International, St. Paul, MN). Baseline MAP and HR were recorded 2 days before the sFR protocol was initiated. The transmitter was kept on during the sFR protocol and also during the refeeding period for up to 2 months. However, at that point, the transmitters began to fail. Therefore, in a parallel set of experiments, 7-month-old rats were implanted with radio transmitters (#PA-C10, Data Sciences International) during their second month of refeeding, as described above. The blood pressure (BP) dipping response was calculated by averaging both the night and day MAP measurements over the last 3 days of the 2-week sFR period or the last 3 days of the 3-month refeeding period, as described.[10]

Acute Measurement of MAP and HR

Three months after refeeding, polyethylene catheters were implanted into the femoral artery of anaesthetized rats (2.5% isoflurane at 1 L/min oxygen), as described previously.[11] The arterial catheter was connected to a pressure transducer (MLT0699; ADI Instruments, Bella Vista, Australia) and a signal amplifier (ETH-400; CB Sciences Inc., Milford, MA). The analog signal from the amplifier was digitized using a 12-bit analog-to-digital converter (PowerLab/400; ADI Instruments). The pulsatile arterial pressure was recorded at 1000 Hz using Windows software (Chart v. 7.0, ADI Instruments). Pulse-to-pulse analysis was used to calculate MAP and HR from the pulsatile arterial pressure measurements.[12] After baseline values for MAP and HR were obtained by averaging the values over a 5-minute period, drugs were infused into the femoral vein.

Drug Infusions

Angiotensin-[1-8]

Thirty minutes after vehicle (0.9% NaCl) infusion, angiotensin-[1-8] was infused in bolus (0.1 mL/100 g of BW) followed by 30-minute intervals before the next-higher dose was administered: (50, 100, 200, 400 ng/kg). The time course of MAP and HR following angiotensin-[1-8] infusion was calculated from continual 5-second averages immediately following injection. The dose response of the change in MAP and HR from baseline was calculated from the 10-second average 40 seconds after agonist infusion.

Losartan

Thirty minutes after vehicle (0.9% NaCl) infusion, losartan was infused in bolus (0.05 mL/100 g of BW) followed by a 15-minute interval before the next-higher dose was administered (0.037, 0.15, 0.63, 2.5, 10 mg/kg). The time course of MAP and HR following losartan infusion was calculated from continual 30-second averages immediately following injection. The dose response of the change in MAP and HR from baseline was calculated from the 30-second average 180 seconds after antagonist infusion.

RAS Fingerprint

After 3 months of refeeding, whole blood was collected by cardiac puncture from anesthetized rats in the presence of heparin and a protease inhibitor cocktail that completely blocked angiotensin-[1-8] metabolism. This proprietary cocktail contained inhibitors against numerous proteases including: aspartic proteases (pepstatin A), cysteine proteases (p-hydroxymercuribenzoic acid), metalloproteases (ethylenediaminetetraacetic acid, 1,10-phenanthroline), serine proteases, and renin and aminopeptidases A and N specific inhibitors. The final concentration was 5% v/v (Attoquant Diagnostics, Vienna, Austria). The samples were centrifuged (2000*g*) for 10 minutes at 4°C and stored at −80°C until mass spectrometry was used to analyze angiotensin peptide concentrations.

Stable isotope-labeled internal standards (200 pg/mL) for each angiotensin-[1-8] metabolite were added to each sample including: angiotensin-[1-10], angiotensin-[1-9], angiotensin-[1-8], angiotensin-[1-7], angiotensin-[1-5], angiotensin-[2-10], angiotensin-[2-8], angiotensin-[2-7], angiotensin-[3-8], and angiotensin-[3-7]. Samples were subjected to liquid chromatography–tandem mass spectrometry analysis using a reversed-phase analytical column (Acquity UPLC C18, Waters, Milford, MA) after C18-based solid-phase extraction. Samples were next subjected to a XEVO TQ-S triple quadrupole mass spectrometer (Waters) in multiple reaction monitoring mode. Peptide recovery in each sample was determined using internal standards for each angiotensin peptide. Angiotensin peptide concentrations were calculated based on the corresponding response factors determined from calibration curves conducted on the original sample matrix. Only integrated signals exceeding a signal-to-noise ratio of 10 were considered.

Plasma Angiotensinogen

Plasma angiotensinogen was measured by ELISA (Cat no. LS-F27853, LSBio LifeSpan BioSciences,

Seattle, WA). Plasma samples (100 µL/well, diluted 1:5 in ELISA buffer) were incubated at 37°C for 1 hour with horseradish peroxidase–labeled C-terminal antibody (50 µL). After rinsing the wells 5 times with Washing Buffer, substrate (100 µL) was added to each well followed by a 20 minutes incubation at 37°C. Sulfuric acid (50 µL at 0.5 mol/L) was added to each well to stop the reaction. The absorbance values of the plasma samples were measured at 450 nm and compared with the standard curve using rodent angiotensinogen (250–5000 pg/mL).

Plasma ACE Activity

Plasma ACE activity was measured in 96-well microtiter plates using the fluorogenic substrate Abz-Phe-Arg-Lys(Dnp)-Pro-OH.[13] Each well contained 80 µL of reaction buffer (1 mol/L NaCl, 0.5 mmol/L $ZnCl_2$, 75 mmol/L Tris, pH 7.5) in the presence of vehicle or the ACE inhibitor, captopril (20 µmol/L). After adding 10 µL of fluorogenic substrate to each well, 10 µL of plasma (diluted 1:10) was immediately added to achieve a final substrate concentration of 60 µmol/L. Product formation was determined at 37°C by following the fluorescence as a function of time at an excitation wavelength of 320 nm and an emission wavelength of 410 nm using a fluorescence plate reader (FLUOstar Omega). Initial velocities were determined from the rate of fluorescence increase over the 10- to 60-minute time course corresponding to the linear range of the assay. Enzyme kinetics were analyzed using Prism (version 8.0, GraphPad Software Inc, La Jolla, CA). Non-ACE activity was defined as enzyme activity measured in the presence of captopril. Specific ACE activity was defined as total peptidase activity minus non-ACE activity.

AT_1R mRNA

The mesenteric arteries were dissected from the intestinal wall and placed in phosphate buffered saline at 4°C. Under an Olympus dissecting microscope, the fat and veins were removed from the vessels, and the tissue was snap frozen in a dry ice–methanol bath and transferred to a −80°C freezer until further use. Ceramic microbeads were used to homogenize the vessels. Total RNA was extracted according to the RNeasy protocol (Bio-Rad, Hercules, CA). High-capacity cDNA reverse transcription (Applied Biosystems, Foster City, CA) was used to reverse transcribe the purified RNA (1 µg). Real-time polymerase chain reaction was used to quantitate AT_1R cDNA with an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The polymerase chain reaction reaction mixture consisted of RNase-free water, SYBR green supermix and 300 nmol/L specific primers as previously described[14]: angiotensin type 1 receptor (AT_1R)—F: 5′-CTC AAG CCT GTC TAC GAA AAT GAG-3′; R: 5′-TAG ATC CTG AGG CAG GGT GAA T-3′; and, beta-actin—F: 5′-CCCATCTATGAGGGTTACGC-3′, R: 5′TTTAATGTCACGCACGATTTC-3′. Standard curves were used to calculate the AT_1R cDNA in mesenteric arteries.

$AType_1R$ Vascular Activity

Mesenteric arteries were mounted onto wires in a myography apparatus (Multi Myograph System 620M, DMT-USA, Inc., Ann Arbor, MI) and then preconstricted with 10^{-5} mol/L phenylephrine to determine a maximum response and to ensure vessel function. If a vessel did not constrict, the vessel was not used for further experiments. Concentration-response curves to angiotensin-[1-8] were then conducted as we described.[15]

Plasma Aldosterone

Plasma aldosterone was measured by ELISA (Cat no. ADI-900-173, Enzo Life Sciences, NY). Plasma samples (100 µL/well, diluted 1:5) were incubated overnight at 4°C after addition of the antibody (50 µL). After washing the wells 3 times, substrate (200 µL) was added to each well followed by a 1-hour incubation at room temperature. Trisodium phosphate solution was added to each well to stop the reaction. Absorbance values were measured at 405 nm.

Reagents

Angiotensin-[1-8] and captopril were purchased from Sigma (St. Louis, MO). Isoflurane was purchased from Petterson Veterinary (Greeley, CO). Abz-Phe-Arg-Lys(Dnp)-Pro-OH was purchased from GenScript (Piscataway, NJ). The ELISA kits for angiotensinogen and aldosterone were purchased from Enzo (Hamburg, Germany) and LifeSpan BioSciences (Seattle, WA), respectively.

Statistical Analysis

The data were analyzed by Prism software (v. 8.0, GraphPad, La Jolla, CA). Body weight, food intake, enzyme activity, and peptide concentrations were analyzed initially using the Shapiro-Wilk normality test and, following normality, were analyzed using the Student nonpaired *t* test to assess differences between groups; data that did not follow the normality distribution would be analyzed by the Mann–Whitney test. Time courses and dose responses were analyzed by 2-way ANOVA for repeated measurement followed by the Bonferroni post hoc test to analyze differences between groups. Significance was defined by $P<0.05$.

RESULTS

Body and Tissue Weights

Before initiation of the sFR protocol, both groups of rats had similar BW (control, 185±3.1 g, n=6 versus sFR, 185±1.9 g, n=6) and age (13 weeks old). After 14 days of 60% sFR, the BW in the sFR group was decreased by 15%, while BW in the control rats increased by 4.7% (control, 194±1.1 g, n=6 versus sFR, 158±1.2, n=6; $P<0.001$). Within 1 week of the refeeding protocol, BW (Figure 1A) and food intake (Figure 1B) in the sFR-refed rats reached levels observed in the control rats.

Not only there were no long-term differences in BW between the control and sFR-refed groups at 7 months of age, no long-term changes were observed in the growth rate as indexed by tibia size and most organ wet weights including the heart and kidneys (Table 1). The uterus wet weight was used as a bioassay for estrogen. No differences in uterine wet weights were found suggesting there were no differences in estrogen levels between the control and sFR-refed groups 3 months after refeeding (Table 1). The only difference observed was in the adrenal, which was twice as large in the sFR-refed rats compared with the control group.

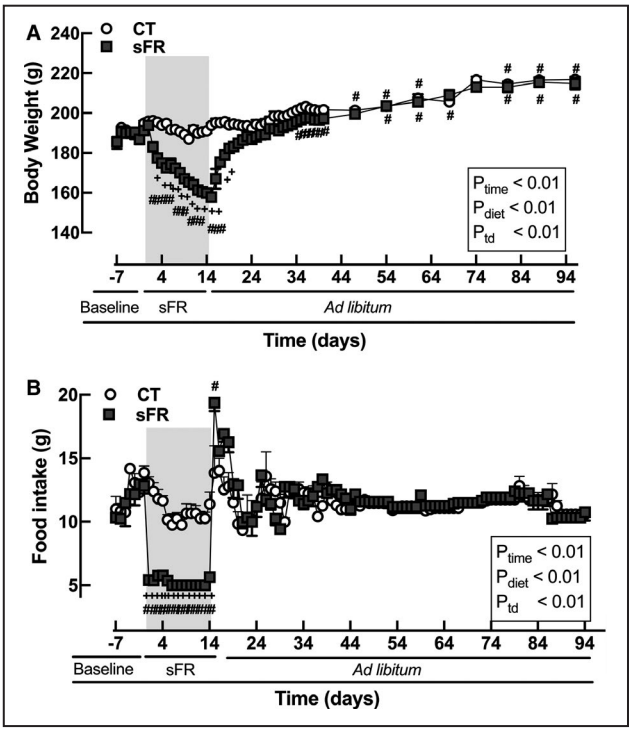

Figure 1. Effects of severe food restriction (sFR) followed by refeeding on body weight (BW) and food intake in control and sFR rats.

Shown is (**A**) BW and (**B**) food intake in control (white circle; n=6) and sFR (gray square; n=6) animals at baseline, during the sFR period (light gray bar) and after refeeding (sFR-refed). Data were analyzed using 2-way ANOVA for repeated measurement with the factors of time and its probability of effect (P_{time}), diet and its probability of effect (P_{diet}), and the probability of an interaction between time and diet (P_{td}); $^{+}P < 0.05$ vs control, same time point, by Bonferroni post hoc test; $^{\#}P<0.05$ vs baseline, same diet, by Bonferroni post hoc test. Error bars are represented as SEM.

Table 1. Effect of Severe Food Restriction and Refeeding on Tissue Weight

Tissue	Control Mean±SEM (n)	sFR Mean±SEM (n)	*P* Value
Tibia, cm	3.83±0.07 (6)	3.93±0.07 (6)	0.36
Heart, g	0.563±0.009 (6)	0.565±0.008 (5)	0.85
Left kidney, g	0.73±0.02 (6)	0.77±0.01 (6)	0.10
Right kidney, g	0.73±0.02 (6)	0.75±0.02 (6)	0.45
Uterus, g	0.59±0.05 (6)	0.69±0.07 (6)	0.31
Adrenal, g	0.040±0.003 (6)	0.079±0.01 (6)	0.0059*

Tissue weight normalized to the tibia size in rats on a control or severe food restricted (sFR) followed by refeeding diet. Values are expressed as the mean.

*$P<0.05$ vs control, same tissue, by unpaired Student *t* test.

MAP and HR

Similar to our previous findings,[5] the MAP of the sFR group was decreased by 20 mm Hg compared with the control group after 14 days on the sFR diet (Figure 2A). However, the MAP recovered to normal levels within 2 weeks of refeeding ad libitum (Figure 2A).

While the control rats showed a typical pattern of BP dipping between the active (night) and inactive (day) periods,[16] no dipping was observed during the sFR period (Figure 2A and 2C). Three months after refeeding, however, BP dipping was restored, and no BP dipping differences were observed between the control and sFR-refed group (Figure 2A and 2C).

As we found previously,[5] the sFR diet lowered HR (Figure 2B). By 8 days, the sFR rats had an 85-bpm reduction in HR (control, 385±4 bpm, n=6 versus sFR, 300±5 bpm, n=4; $P<0.001$) and the lowered HR continued for the duration of the sFR period; however, HR in the sFR rats returned to normal levels within 2 weeks of refeeding (Figure 2B).

Angiotensin-[1-8] and Losartan MAP and HR Responses

Injection of exogenous angiotensin-[1-8] (200 ng/kg) into the circulation produced a higher peak pressor response in the sFR compared with the control group (peak MAP: control, 96±2.6 mm Hg, n=6 versus sFR, 107±2.0 mm Hg, n=6; $P=0.008$) (Figure 3A). There was also a trend to produce a higher peak pressor response at half that dose (peak MAP: control, 89±1 mm Hg, n=6 versus sFR, 93±1 mm Hg, n=6; $P=0.061$) (Figure 3B). The angiotensin-[1-8] dose response showed the sFR-refed animals reached the maximum

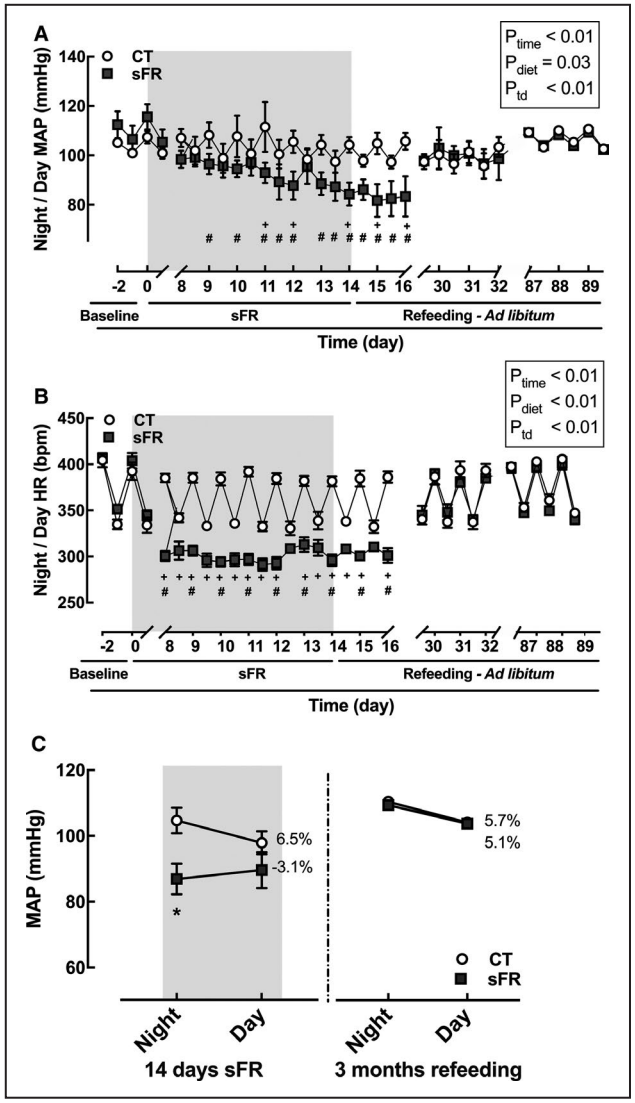

Figure 2. Effects of severe food restriction (sFR) followed by refeeding on mean arterial pressure (MAP) and heart rate (HR) in control and sFR rats determined by telemetry.
Shown is night and day (**A**) MAP and (**B**) HR in control (white circle; n=6) and sFR (gray square; n=6) rats at baseline, during the sFR period (light gray bar) and after refeeding (sFR-refed). Data were analyzed using 2-way ANOVA for repeated measurement with the factors of time and its probability of effect (P_{time}), diet and its probability of effect (P_{diet}), and the probability of an interaction between time and diet (P_{td}); $^{+}P<0.05$ vs control, same time point, by Bonferroni post-hoc test; $^{\#}P<0.05$ vs baseline, same diet, by Bonferroni post hoc test. Shown is (**C**) the MAP dipping response in control (white circle; n=6) and sFR (gray square; n=6) rats averaged over the last 3 days of the (left panel) sFR period or (right panel) refeeding period. $^{*}P<0.05$ vs CT, same time point, by Student *t* test. Error bars are represented as SEM.

pressor response at lower doses than the control group, indicating increased sensitivity to the AT_1R agonist (Figure 3B). Injection of vehicle (0 ng/kg) had no effect on MAP in both groups. Differences were also detected in angiotensin-[1-8]–mediated effects on HR; the HR responses to angiotensin-[1-8] were lower in the sFR-refed rats (Figure 3C), and this effect was dose dependent (Figure 3D).

Infusion of AT_1R antagonist, losartan, caused a lower BP response in sFR-refed rats when compared with the control group. The largest difference in the depressor response to 0.62 mg/kg losartan occurred at 180 seconds (Figure 4A). The losartan dose response showed the sFR-refed rats reached a maximum depressor response at lower doses than the control group, indicating increased sensitivity to the AT_1R antagonist (Figure 4B). Injection of vehicle (0 mg/kg) had no effect on MAP in both groups. Differences were also detected in losartan-mediated effects on HR; the HR responses to losartan were higher in the sFR-refed rats (Figure 4C), and this effect was magnified at 2.50 mg/kg (Figure 4D).

Plasma Levels of Angiotensin Metabolites

To address the increased pressor response to angiotensin-[1-8], angiotensin peptides involved in angiotensin-[1-8] synthesis and catabolism were measured by the RAS-Fingerprint assay in control and sFR-refed rats. Plasma levels of angiotensinogen and angiotensin-[1-8] in the sFR-refed rats were 29% and 99% higher, respectively, compared with the control rats (Figure 5). No differences were observed in plasma levels of other detectable angiotensin metabolites including angiotensin-[1-10], angiotensin-[2-10], angiotensin-[2-8], and angiotensin-[3-8]. Note: angiotensin-[1-7] was below the limits of assay detection.

Plasma ACE Activity

ACE is the major enzyme responsible for synthesis of angiotensin-[1-8]. Thus, plasma ACE activity was compared in control and sFR-refed rats using a fluorogenic assay. A time course of ACE activity showed there was more substrate formed by plasma ACE and higher enzyme activity in the sFR-refed compared with the control rats (Figure 6).

AT_1R Expression and Function

To assess if the increased pressor response to angiotensin-[1-8] in the sFR-refed group was attributable to increased expression of the AT_1R in small resistance vessels, AT_1R mRNA was determined in mesenteric arteries by quantitative polymerase chain reaction. No differences were observed between control and sFR-refed rats in AT_1R mRNA expression in mesenteric vessels (Figure 7A).

To assess if the increased pressor response to angiotensin-[1-8] in the sFR group was due to increased AT_1R vasoconstrictor activity, AT_1R vasoconstrictor responses to angiotensin-[1-8] were determined in

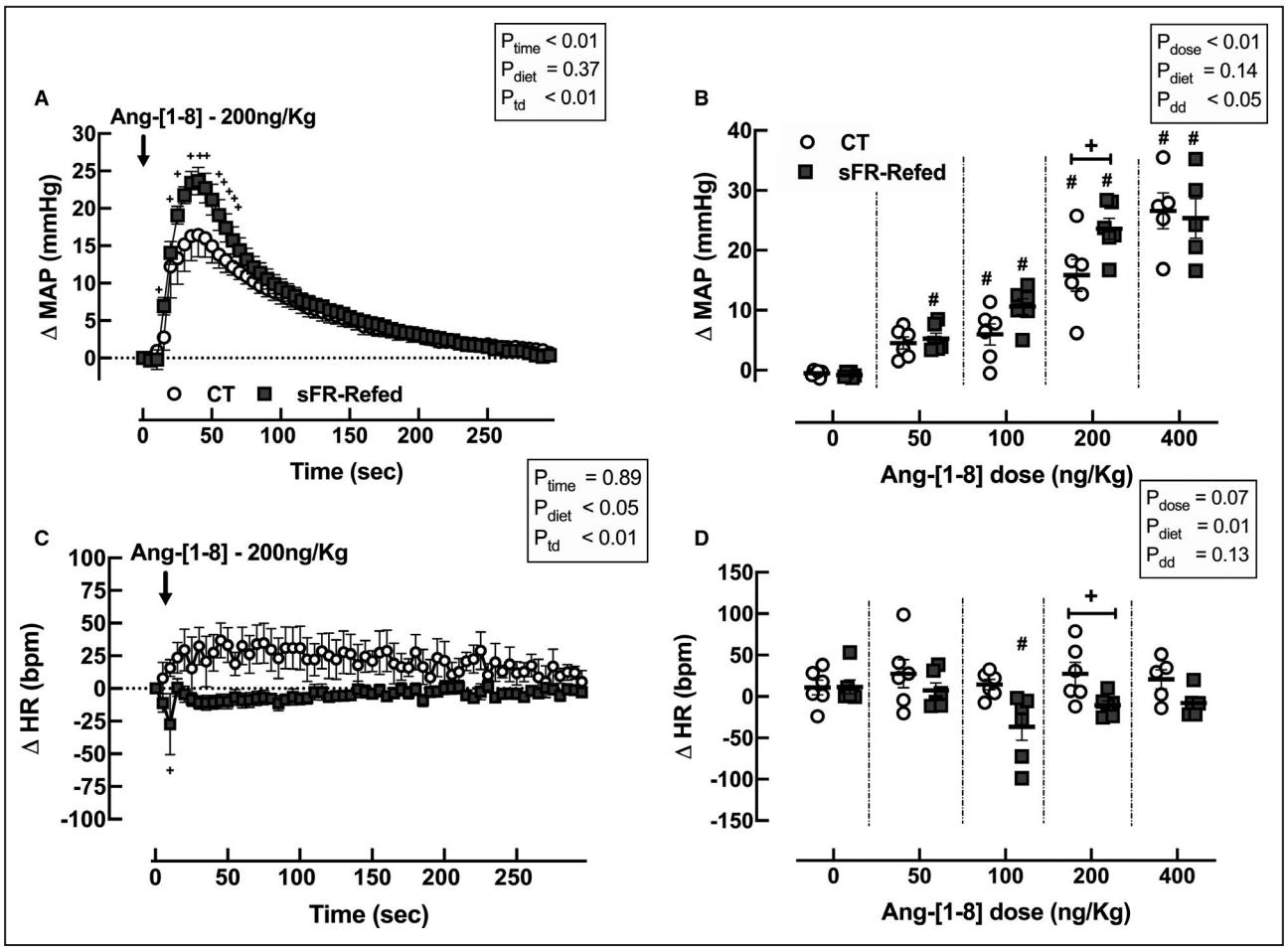

Figure 3. Effects of severe food restriction (sFR) followed by refeeding on angiotensin-[1-8] mean arterial pressure (MAP) and heart rate (HR) responses in control and sFR-refed rats.
Shown are the differences in (**A** and **B**) MAP and (**C** and **D**) HR responses to angiotensin-[1-8] as a function of (**A** and **C**) time (after a bolus i.v. infusion of angiotensin-[1-8] at 200 ng/kg) and (**B** and **D**) angiotensin-[1-8] dose (40 seconds after infusion and repeated with increasing bolus doses of angiotensin-[1-8] every 30 minutes) in control (white circle; n=6) and sFR (gray square; n=6) rats compared with baseline. **A** and **C**, Data were analyzed using 2-way ANOVA for repeated measurement with the factors of time and its probability of effect (P_{time}), diet and its probability of effect (P_{diet}), and the probability of an interaction between time and diet (P_{td}); $^{+}P<0.05$ vs control, same time point, by Bonferroni post hoc test. **B** and **D**, Data were analyzed using 2-way ANOVA for repeated measurement with the factors dose and its probability of effect (P_{dose}), diet and its probability of effect (P_{diet}), and the probability of an interaction between dose and diet (P_{dd}); $^{+}P<0.05$ vs control, same time point, by Bonferroni post hoc test; $^{\#}P<0.05$ vs baseline, same diet, by Bonferroni post hoc test. Error bars are represented as SEM.

mesenteric arteries by wire myography. Vasoconstrictor responses to angiotensin-[1-8] were small and peaked at 10 nmol/L before tachyphylaxis downregulated the vasoconstrictor response; however, no differences were observed between control and sFR-refed rats (Figure 7B).

Plasma Levels of Aldosterone Before and After Angiotensin-[1-8] Infusion

No differences in basal plasma aldosterone were observed between control and sFR-refed rats (Figure 8, left panel). Following angiotensin-[1-8] infusion, plasma aldosterone was reduced by 65% in the control rats, whereas there was no significant reduction in plasma aldosterone in the sFR rats (Figure 8, right panel).

DISCUSSION

This is the first report demonstrating a long-lasting dysregulation of the RAS following a 2-week exposure to sFR. Specifically, the major finding of this study was that 3 months after the sFR period ended, the in vivo pressor response to angiotensin-[1-8] (Figure 3B) and the depressor response to losartan (Figure 4B) were shifted to the left. Thus, the angiotensin-[1-8] pressor and losartan depressor responses remained sensitized months after BW, BP, and HR returned to normal.

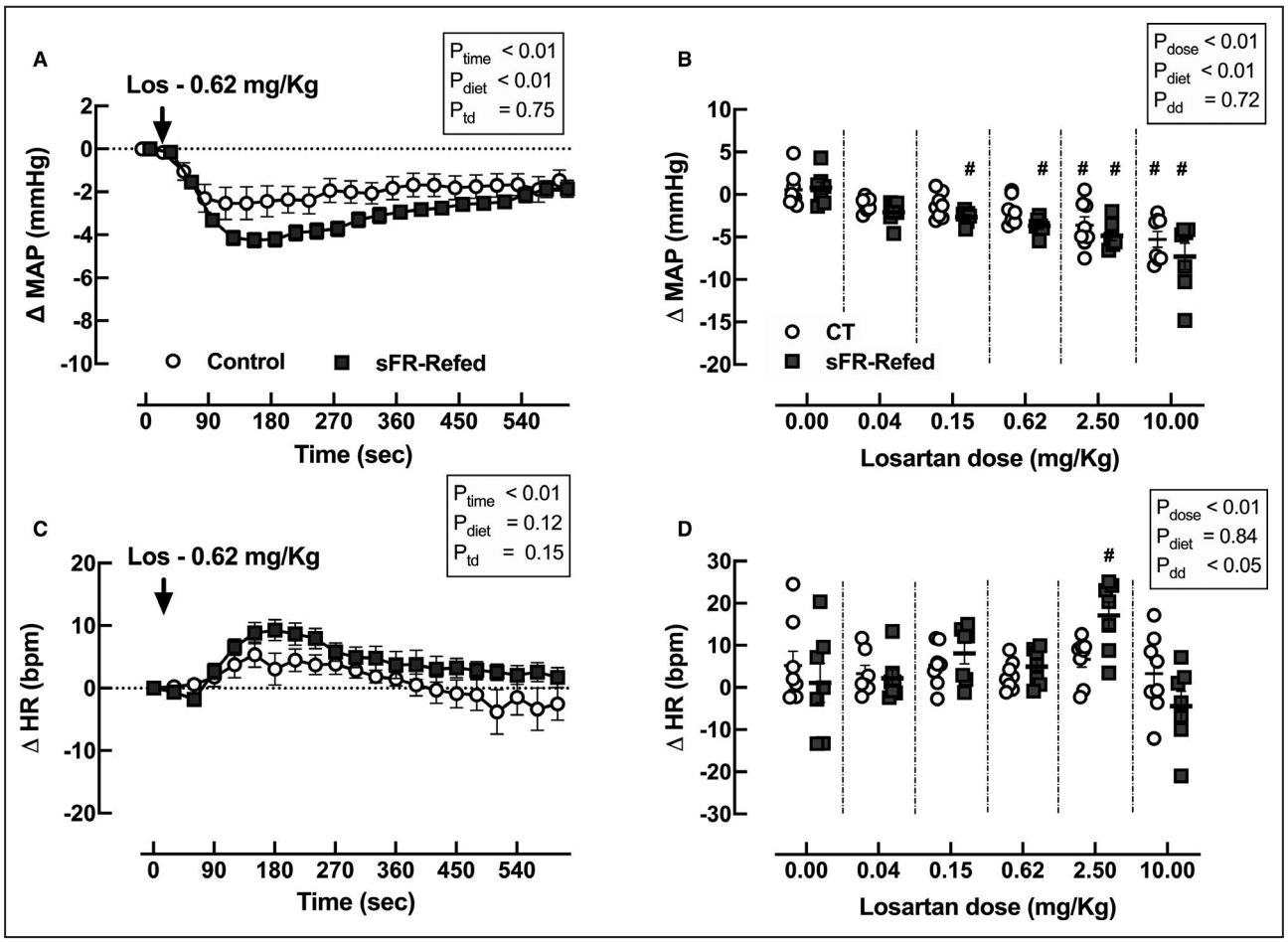

Figure 4. Effects of severe food restriction (sFR) followed by refeeding on losartan mean arterial pressure (MAP) and heart rate (HR) responses in control and sFR-refed rats.
Shown are the differences in (**A** and **B**) MAP and (**C** and **D**) HR responses to losartan as a function of (**A** and **C**) time (after a bolus intravenous infusion of losartan at 0.62 mg/kg) and (**B** and **D**) losartan dose (180 seconds after infusion and repeated with increasing bolus doses of losartan every 30 minutes) in control (white circle; n=8) and sFR-refed (gray square; n=7) rats compared with baseline. **A** and **C**, Data were analyzed using 2-way ANOVA for repeated measurement with the factors time and its probability of effect (P_{time}), diet and its probability of effect (P_{diet}), and the probability of an interaction between time and diet (P_{td}). **B** and **D**, Data were analyzed using 2-way ANOVA for repeated measurement with the factors dose and its probability of effect (P_{dose}), diet and its probability of effect (P_{diet}), and the probability of an interaction between dose and diet (P_{dd}); $^{\#}P<0.05$ vs baseline, same diet, by Bonferroni post hoc test. Error bars are represented as SEM.

The RAS is in a constant state of flux. This major volume regulatory system rapidly changes to maintain water and electrolyte homeostasis. A reduction in plasma volume is a well-known stimulus for angiotensin-[1-8] production.[17] When plasma volume is reduced by hemorrhage, angiotensin-[1-8] rises in the plasma causing increased vasoconstriction, aldosterone release, and renal sodium/water retention.[18] This activation of the RAS is a survival response designed to counteract the drop in BP. Severe FR also activates the RAS by this same survival mechanism because the acute effects of sFR include a marked drop in plasma volume and a reduction in BP.[5]

Dietary sodium is another regulator of RAS activity. Changes in plasma angiotensin-[1-8] occur rapidly in response to fluctuations in dietary sodium; low sodium increases plasma angiotensin-[1-8], while high sodium reduces plasma levels.[19] Thus, the RAS maintains water and electrolyte homeostasis by rapidly increasing or reducing its metabolic activity. In the case of a 2-week sFR diet, the RAS, however, did not return to homeostasis. Plasma angiotensin-[1-8] remained elevated 3 months after the sFR period ended (Figure 5). Furthermore, the higher pressor responsiveness to angiotensin-[1-8] and the greater depressor response to losartan in the sFR rats was dose dependent, suggesting that the AT_1R in sFR-refed rats had increased sensitivity to its agonist, angiotensin-[1-8] (Figure 3B) and the AT_1R antagonist, losartan (Figure 4B).

These findings support other studies that have demonstrated sensitization of the RAS under various

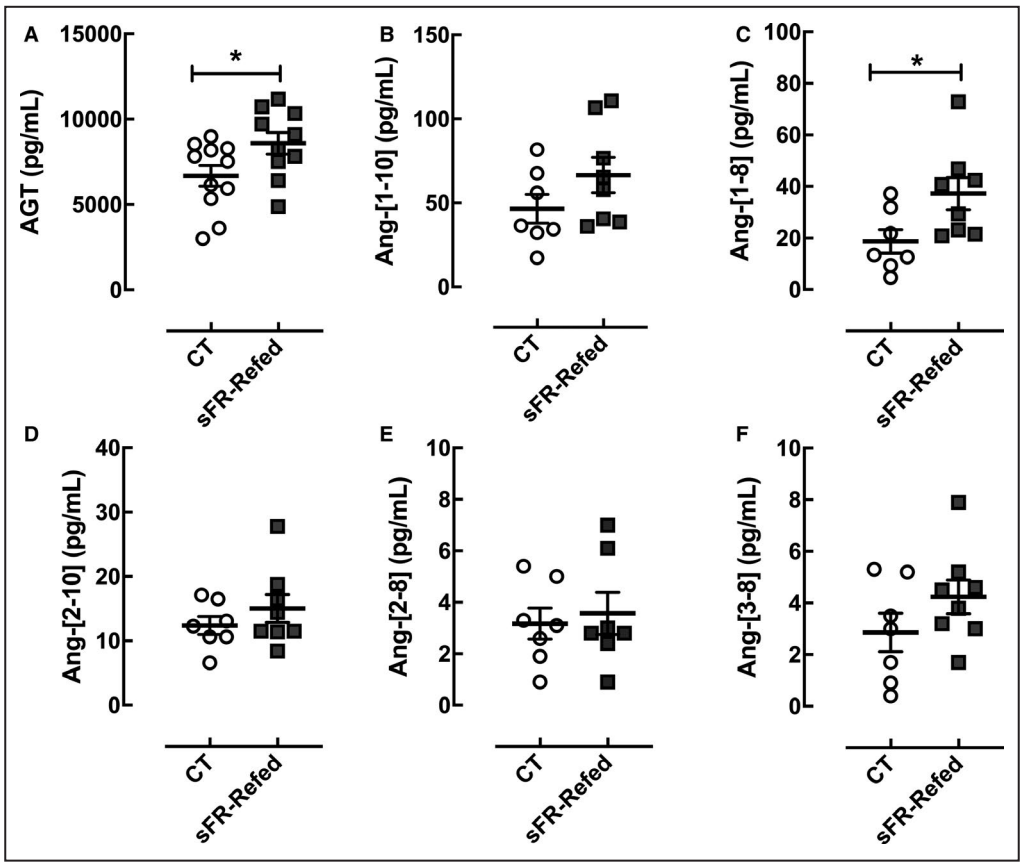

Figure 5. Effects of severe food restriction (sFR) followed by refeeding on angiotensin peptides in control and sFR-refed rats.
Shown are the plasma levels of (**A**) angiotensinogen (control, n=11; sFR, n=10) (**B**) angiotensin-[1-10] (control, n=7; sFR, n=8), (**C**) angiotensin-[1-8] (control, n=7; sFR, n=8), (**D**) angiotensin-[2-10] (control, n=7; sFR, n=8), (**E**) angiotensin-[2-8] (control, n=7; sFR, n=7), and (**F**) angiotensin-[3-8] (control, n=7; sFR, n=7) in control (white circle) and sFR-refed (gray square) rats. Angiotensin-[1-7] levels were below the level of assay detection. *P<0.05 vs control by Student t test. Error bars are represented as SEM.

conditions. Prior exposure to a low nonpressor dose of angiotensin-[1-8] was shown to sensitize male rats to the pressor effects of angiotensin-[1-8] 1 week later.[20] Maternal malnutrition also elevated plasma angiotensin-[1-8], and this increased RAS activity induced adverse metabolic programming in offspring including increased susceptibility to developing salt-sensitive hypertension later in life.[21]

Response sensitization occurs when a stimulus is injurious or threatening and activates defensive physiological and behavioral responses. This sensitized capacity to increase sympathetic nervous system activity in response to physiological and psychosocial challenges provides a working hypothesis to explain how prior exposure to stressors can lead to increased systemic vascular resistance and high BP.[22] Thus, 2 weeks on a sFR diet is sufficient to cause a long-lasting response sensitization of the RAS.

The sFR-induced sensitization of the RAS could be due to an increase in ligand availability, AT_1R expression, or AT_1R function. We previously showed that sFR increases ligand availability acutely by increasing circulating angiotensinogen, angiotensin-[1-8], and ACE activity.[5] This study found that ligand availability is chronically increased by sFR. The angiotensin-[1-8] synthetic pathway remained upregulated months after the sFR period ended. Plasma levels of angiotensinogen, angiotensin-[1-8], and ACE activity were all elevated in sFR-refed rats when compared with control animals (Figures 5 and 6). Increased availability of angiotensin-[1-8] is also observed in a myriad of diseases including hypertension, congestive heart failure, chronic kidney disease, and sepsis.[23,24] Furthermore, the excessive circulating angiotensin-[1-8] contributes to the progression of these diseases as evidenced by the effectiveness of ACE inhibitors and AT_1R blockers in treating these conditions.

Increased AT_1R expression can also contribute to RAS sensitization. We found the sFR-refed rats were more sensitive to the pressor effects of angiotensin-[1-8] (Figure 3) and the depressor effects of losartan (Figure 4), suggesting increased activity of AT_1Rs.

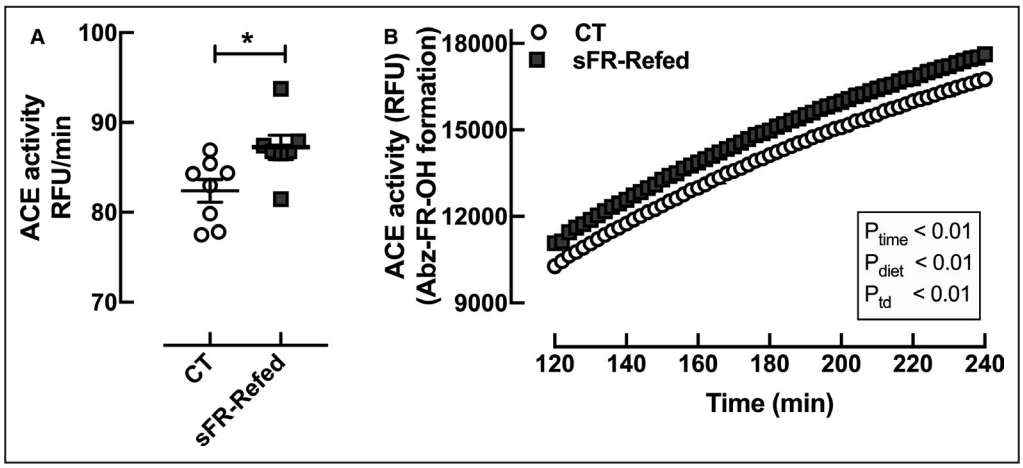

Figure 6. Effects of severe food restriction (sFR) followed by refeeding on angiotensin-converting enzyme (ACE) in control control and sFR-refed rats.
Shown is ACE activity expressed as the amount of product formed (RFU) as a function of (**A**) time and (**B**) relative fluorescent units per min (RFU/min) in control (white circle; n=8) and sFR (gray square; n=8) rats. Data were analyzed by (**A**) Student *t* test, **P*<0.05 vs control and (**B**) 2-way ANOVA for repeated measurement with the factors time and its probability of effect (P_{time}), diet and its probability of effect (P_{diet}), and the probability of an interaction between time and diet (P_{td}). Error bars are represented as SEM.

However, when AT_1R expression was analyzed in mesenteric vessels, no differences between control and sFR rats were observed 3 months after refeeding (Figure 7A). Not only were there no detectable differences in AT_1R mRNA levels, there were no functional differences in AT_1R-mediated vasoconstriction; vessel contraction to angiotensin-[1-8] was indistinguishable from control (Figure 7B) even though plasma angiotensinogen and angiotensin-[1-8] were elevated. While increased AT_1R activity was not observed in mesenteric arteries ex vivo, increased AT_1R activity in sFR rats could occur via increased levels of angiotensin-[1-8] in vivo. These findings, however, cannot rule out additional contributions to increased AT_1R activity from other vascular beds or sympathetic neuron activation.

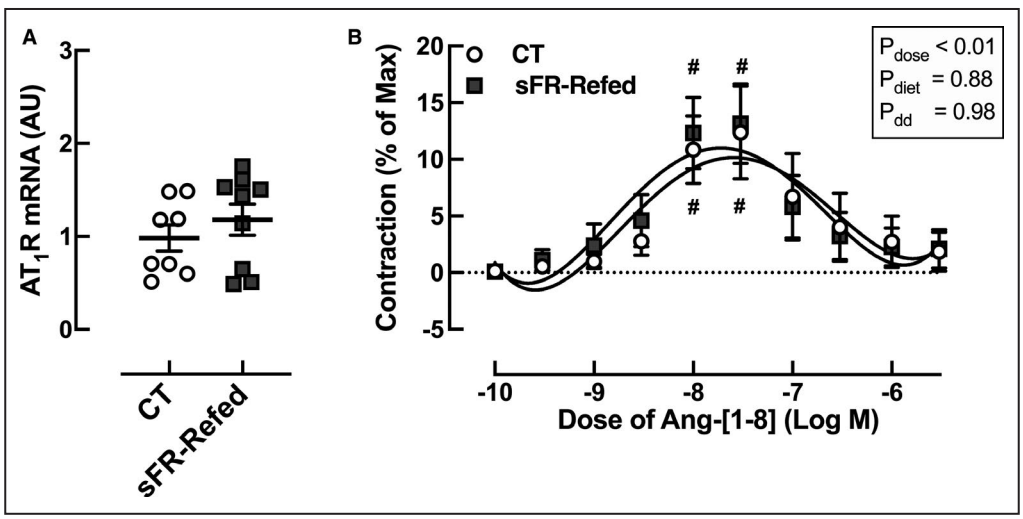

Figure 7. Effects of severe food restriction (sFR) followed by refeeding on AT_1R mRNA expression and vasoconstrictor responses in mesenteric vessels from control and sFR-refed rats.
Shown is AT_1R (**A**) mRNA expression and (**B**) percent maximum vasoconstrictor responses to angiotensin-[1-8] in mesenteric vessels from control (white circle; n=8) and sFR-refed (gray square; n=9) rats. Data were analyzed by (**A**) Student *t* test, *P*=0.39 vs control and (**B**) 2-way ANOVA for repeated measurement with the factors dose and its probability of effect (P_{dose}), diet and its probability of effect (P_{diet}), and the probability of an interaction between dose and diet (P_{dd}); $^{\#}P$<0.05 vs baseline, same diet, by Bonferroni post hoc test. Error bars are represented as SEM.

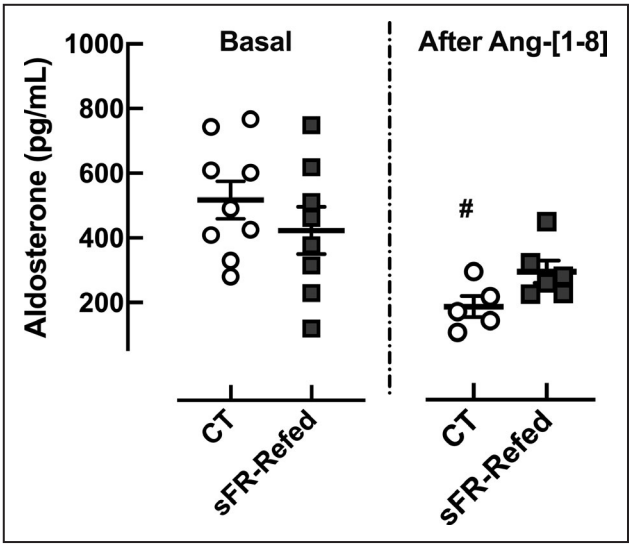

Figure 8. Effects of severe food restriction (sFR) followed by refeeding on plasma aldosterone responses in control and sFR-refed rats.
Shown is (**A**) basal aldosterone (control, n=9; sFR-refed, n=8) and (**B**) aldosterone 30 minutes after 400 ng/kg angiotensin-[1-8] infusion (control, n=5; sFR-refed, n=6) in the plasma of control (white circle) and sFR-Refed (gray square) rats. $^{\#}P<0.05$ vs basal, same diet, by Student *t* test. Error bars are represented as SEM.

As we found previously,[5] after the 8th day of sFR, HR was lower than control rats and after the 12th day, MAP also fell (Figure 2). After refeeding ensued, the MAP returned to baseline by day 5, whereas it took 12 days for the HR to return to basal levels. These findings indicate that HR regulation is more sensitive to the restrictive diet than the BP. We also found the sFR diet impaired the circadian rhythm for both MAP and HR (Figure 2). Nocturnal dipping of arterial BP is a feature of normal circadian rhythm, and its absence, which is called nondipping, is associated with more severe end-organ damage and increased risk of cardiovascular events, especially in hypertensive patients.[25] Nondippers show impairment in autonomic system functions that include abnormal parasympathetic and sympathetic activities.[26] Studies suggest that restricted feeding influences not only the phase but also the amplitude of clock gene oscillations in a tissue-specific manner. Such observations suggest differential responsiveness of organs to feeding-derived entrainment factors exists, relative to other signals (eg, direct innervation from the suprachiasmatic nucleus).[27]

While the sFR diet had no effect on heart and kidney weights, the diet did increase the weight of the adrenal gland. Adrenal function may also have been impaired. angiotensin-[1-8] stimulates the synthesis of aldosterone in the zona glomerulosa of the adrenal cortex. While basal plasma levels of aldosterone were indistinguishable between the control and sFR rats, the aldosterone negative feedback response to angiotensin-[1-8] infusion was impaired in sFR rats (Figure 8). These findings suggest sFR alters adrenal function by disrupting the angiotensin-[1-8]- aldosterone feedback loop.

In conclusion, despite BW, BP, and HR recovery 3 months after the sFR period had ended, the RAS remained sensitized in female Fischer rats due to increased activity of the angiotensin-[1-8] synthetic pathway; the precursor and major synthetic enzyme remained upregulated. This study also suggests the contribution of mesenteric resistance vessels to the increased pressor responses to angiotensin-[1-8] was attributable to increased levels of angiotensin-[1-8] rather than to increased expression of mesenteric AT_1Rs. Moreover, increased expression of AT_1Rs in other vascular beds or within the central nervous system also likely contributes to RAS sensitization in sFR rats.

Clinical Implications

Few studies have examined the long-term effects of prior exposure to periods of inadequate food intake on the cardiovascular system. One study of men who experienced the Leningrad siege during 1941–1944 showed that starvation around puberty (ages 9–15) was more strongly associated with high systolic BP and stroke in adult life with no effect on the body weight after 30 years.[28] The etiology of primary hypertension is largely unknown; however, this early and sustained reprogramming of the RAS in the female rat warrants further study not only in females regarding this potential mechanism for cardiovascular disease development but also in males to determine if sFR has similar long-term effects on the RAS after refeeding.

Perspective

These findings warrant further study of individuals who voluntarily (eg, anorexia) or involuntarily (eg, natural disaster) experience an extended period of sFR and thus may be at increased risk of developing cardiovascular disease through sensitization of the RAS even though their BW, MAP, and HR appear normal. These individuals may be especially vulnerable if subjected to a second stressor later in life that targets the RAS (eg, hypertension).

ARTICLE INFORMATION

Received April 27, 2020; accepted June 15, 2020.

Affiliations

From the Department of Medicine, Georgetown University, Washington, DC.

Acknowledgments

The authors are thankful to Dr Marko Poglitsch (Fingerprint) for his contribution to the angiotensin peptide measurements.

Author contributions: Conception and design of the experiments: Drs de Souza, West, and Sandberg; collection, analysis, and interpretation of data: Drs de Souza, West, Ji, Sandberg, and Wu; drafting and revising the article critically for important intellectual content: de Souza, West, Ji, and Sandberg. All authors approved the final version of the manuscript. All the individuals cited qualify for authorship, and all the authors approved the final version of this manuscript.

Sources of Funding

This work was supported by the following grants: American Heart Association: 19POST34380744 (de Souza), American Society of Nephrology: Carl W. Gottschalk Research Scholar Grant (West), NIH: UL1-TR001409 (Sandberg) and R01-HL119380 (Sandberg, Ji).

Disclosures

None.

REFERENCES

1. Victora CG, Vaughan JP, Kirkwood BR, Martines JC, Barcelos LB. Risk factors for malnutrition in Brazilian children: the role of social and environmental variables. *Bull World Health Organ*. 1986;64:299–309.
2. Muller O, Krawinkel M. Malnutrition and health in developing countries. *CMAJ*. 2005;173:279–286.
3. Casiero D, Frishman WH. Cardiovascular complications of eating disorders. *Cardiol Rev*. 2006;14:227–231.
4. Koschke M, Boettger MK, Macholdt C, Schulz S, Yeragani VK, Voss A, Bar KJ. Increased QT variability in patients with anorexia nervosa—an indicator for increased cardiac mortality? *Int J Eat Disord*. 2010;43:743–750.
5. de Souza AMA, West CA, de Abreu ARR, Pai AV, Mesquita LBT, Ji H, Chianca D Jr, de Menezes RCA, Sandberg K. Role of the renin angiotensin system in blood pressure allostasis-induced by severe food restriction in female Fischer rats. *Sci Rep*. 2018;8:10327.
6. Montani JP, Schutz Y, Dulloo AG. Dieting and weight cycling as risk factors for cardiometabolic diseases: who is really at risk? *Obes Rev*. 2015;16(suppl 1):7–18.
7. de Souza AA, de Menezes RC, Abreu AR, Araujo GR, Costa DC, Chianca DA Jr. Increased alpha1-adrenoreceptor activity is required to sustain blood pressure in female rats under food restriction. *Life Sci*. 2015;128:55–63.
8. Mumtaz F, Khan MI, Zubair M, Dehpour AR. Neurobiology and consequences of social isolation stress in animal model-a comprehensive review. *Biomed Pharmacother*. 2018;105:1205–1222.
9. Fekete A, Sasser JM, Baylis C. Chronic vasodilation produces plasma volume expansion and hemodilution in rats: consequences of decreased effective arterial blood volume. *Am J Physiol Renal Physiol*. 2011;300:F113–F118.
10. Douma LG, Solocinski K, Holzworth MR, Crislip GR, Masten SH, Miller AH, Cheng KY, Lynch IJ, Cain BD, et al. Female C57BL/6J mice lacking the circadian clock protein PER1 are protected from nondipping hypertension. *Am J Physiol Regul Integr Comp Physiol*. 2019;316:R50–R58.
11. Loss Ide O, Fernandes LG, Martins CD, Cardoso LM, Silva ME, Dias-da-Silva VJ, Moraes MF, Chianca DA Jr. Baroreflex dysfunction in rats submitted to protein restriction. *Life Sci*. 2007;81:944–950.
12. Gomide JM, de Menezes RC, Fernandes LG, Silva FC, Cardoso LM, Miranda PH, da Silva LG Jr, Lima MP, Pesquero JL, Foureaux G, et al. Increased activity of the renin-angiotensin and sympathetic nervous systems is required for regulation of the blood pressure in rats fed a low-protein diet. *Exp Physiol*. 2013;98:57–66.
13. Carmona AK, Schwager SL, Juliano MA, Juliano L, Sturrock ED. A continuous fluorescence resonance energy transfer angiotensin I-converting enzyme assay. *Nat Protoc*. 2006;1:1971–1976.
14. Sullivan JC, Pollock DM, Pollock JS. Altered nitric oxide synthase 3 distribution in mesenteric arteries of hypertensive rats. *Hypertension*. 2002;39:597–602.
15. Wang D, Wang C, Wu X, Zheng W, Sandberg K, Ji H, Welch WJ, Wilcox CS. Endothelial dysfunction and enhanced contractility in microvessels from ovariectomized rats: roles of oxidative stress and perivascular adipose tissue. *Hypertension*. 2014;63:1063–1069.
16. Sufiun A, Rafiq K, Fujisawa Y, Rahman A, Mori H, Nakano D, Kobori H, Ohmori K, Masaki T, Kohno M, et al. Effect of dipeptidyl peptidase-4 inhibition on circadian blood pressure during the development of salt-dependent hypertension in rats. *Hypertens Res*. 2015;38:237–243.
17. McKinley MJ, Walker LL, Alexiou T, Allen AM, Campbell DJ, Di Nicolantonio R, Oldfield BJ, Denton DA. Osmoregulatory fluid intake but not hypovolemic thirst is intact in mice lacking angiotensin. *Am J Physiol Regul Integr Comp Physiol*. 2008;294:R1533–R1543.
18. Xiang L, Clemmer JS, Lu S, Mittwede PN. Impaired blood pressure compensation following hemorrhage in conscious obese Zucker rats. *Life Sci*. 2013;93:214–219.
19. Aguilera G, Menard RH, Catt KJ. Regulatory actions of angiotensin II on receptors and steroidogenic enzymes in adrenal glomerulosa cells. *Endocrinology*. 1980;107:55–60.
20. Xue B, Zhang Z, Johnson RF, Johnson AK. Sensitization of slow pressor angiotensin II (Ang II)-initiated hypertension: induction of sensitization by prior Ang II treatment. *Hypertension*. 2012;59:459–466.
21. Kawakami-Mori F, Nishimoto M, Reheman L, Kawarazaki W, Ayuzawa N, Ueda K, Hirohama D, Kohno D, Oba S, Shimosawa T, et al. Aberrant DNA methylation of hypothalamic angiotensin receptor in prenatal programmed hypertension. *JCI Insight*. 2018;3:e95625.
22. Johnson AK, Xue B. Central nervous system neuroplasticity and the sensitization of hypertension. *Nat Rev Nephrol*. 2018;14:750–766.
23. Ames MK, Atkins CE, Pitt B. The renin-angiotensin-aldosterone system and its suppression. *J Vet Intern Med*. 2019;33:363–382.
24. Hsieh MS, How CK, Hsieh VC, Chen PC. Preadmission antihypertensive drug use and sepsis outcome: impact of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs). *Shock*. 2020;53:407–415.
25. Dai S, Huang B, Zou Y, Liu Y. Associations of dipping and non-dipping hypertension with cardiovascular diseases in patients with dyslipidemia. *Arch Med Sci*. 2019;15:337–342.
26. Okutucu S, Karakulak UN, Kabakci G. Circadian blood pressure pattern and cardiac autonomic functions: different aspects of same pathophysiology. *Anadolu Kardiyol Derg*. 2011;11:168–173.
27. McGinnis GR, Young ME. Circadian regulation of metabolic homeostasis: causes and consequences. *Nat Sci Sleep*. 2016;8:163–180.
28. Sparen P, Vagero D, Shestov DB, Plavinskaja S, Parfenova N, Hoptiar V, Paturot D, Galanti MR. Long term mortality after severe starvation during the siege of Leningrad: prospective cohort study. *BMJ*. 2004;328:11.