Hypotension and bradycardia during caloric restriction in mice are independent of salt balance and do not require ANP receptor

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Hunt, Lisa M., Emily W. Hogeland, Maria K. Henry, and Steven J. Swoap. Hypotension and bradycardia during caloric restriction in mice are independent of salt balance and do not require ANP receptor. Am J Physiol Heart Circ Physiol 287: H1446–H1451, 2004.—We hypothesized that caloric restriction (CR)-induced hypotension would correlate with increased sodium excretion through an atrial natriuretic peptide (ANP)-dependent mechanism. To test this hypothesis, the cardiovascular parameters of c57/Bi mice were measured with radiotelemetry while urine was collected. The 23-h mean blood pressure (BP) dropped from 108.6 ± 1.8 to 92.7 ± 2.4 mmHg, and 23-h heart rate dropped from 624 ± 5 to 426 ± 13 beats/min over 7 days of CR at 29°C. Contrary to our hypothesis, urine sodium excretion decreased by 55% by day 7 of CR. Consistent with decreased sodium excretion was the drop in plasma ANP (from 82.4 ± 4.3 to 68.0 ± 5.8 pg/ml). To explore the possibility that CR lowers BP through an ANP receptor-dependent mechanism that is independent of its effect on sodium retention, we measured the cardiovascular parameters of mice deficient in the ANP receptor (NPR1−/−) or the ANP clearance receptor (NPR3−/−). Mean BP fell from 117.1 ± 3.9 to 108.0 ± 4.7 mmHg in the NPR1−/− mice and from 87.0 ± 2.4 to 78.4 ± 1.7 mmHg in the NPR3−/− mice during CR. These data indicate that the hypotension induced by CR does not depend on increased sodium excretion. Rather, it appears that the mouse responds to the low BP induced by CR with an increase in sodium reabsorption. Furthermore, circulating ANP levels and data from NPR1−/− and NPR3−/− mice suggest that the ANP pathway may not be involved in the cardiovascular response to CR.

blood pressure; heart rate; standard deviation of the interbeat interval; radiotelemetry; atrial natriuretic peptide

During chronic periods of negative energy balance, arterial blood pressure (BP) falls significantly although the mechanism(s) causing this hypotension remain elusive (9, 15, 17, 37, 40, 41, 43, 44). The initial stages of negative energy balance are associated with natriuresis and diuresis (4, 7, 18, 23, 36, 38), which has been proposed to play a part in the hypotension of caloric restriction (CR). One of the principle hormones involved with both natriuresis and BP is atrial natriuretic peptide (ANP; reviewed in Refs. 3 and 20). Numerous lines of evidence have demonstrated the importance of ANP in the acute regulation of BP. Acute infusions of ANP established its role in natriuresis, inhibition of the renin-angiotensin-aldosterone axis, and ultimately systemic hypotension (3, 20). ANP also profoundly impacts the autonomic nervous system (ANS) both centrally and peripherally (10, 16, 21, 34, 35), resulting in bradycardia, a drop in total peripheral resistance, and BP. Importantly, CR resembles ANP infusions in both bradycardia and a drop in BP, with the ANS as the prime mediator of these cardiovascular effects (9, 17, 18, 37, 41, 43).

It was not until the generation of mice with alterations in ANP signaling that its role in the chronic regulation of BP was demonstrated. First, overexpression of ANP from the liver results in hypotension (2, 39), whereas mice missing the ANP gene are hypertensive (14). Second, mice missing the receptor for ANP (NPR1−/−) are hypertensive and exhibit enlarged cardiac mass (28). Furthermore, increasing expression of the ANP receptor results in a dose-dependent drop in BP (29). Finally, mice missing the clearance receptor for ANP (NPR3−/−) have an elevated half-life of ANP with subsequent low BP (24).

In the current study, we hypothesized that the hypotension associated with CR is dependent on an increase in ANP-dependent signaling. Strong circumstantial evidence led us to the hypothesis that ANP mediates the hypotension and bradycardia associated with CR. Natriuresis is a common response among different animals in response to fasting (4). Second, ANP levels have been shown to increase during negative energy balance (4, 23), although this is not observed universally (7, 26, 33). Finally, the hypotension induced by CR requires an intact sympathetic nervous system (SNS) signaling (41) and ANP is known to dampen SNS activity (19). We tested this hypothesis using radiotelemetry for cardiovascular measurement in conscious mice that were either wild type (c57/Bi), NPR1−/−, or NPR3−/−.

MATERIALS AND METHODS

Animals. All animals were obtained from Jackson Laboratories. Approximately 6-mo-old female c57/Bi mice (n = 8), NPR1−/− mice (n = 10), or NPR3−/− mice (n = 3) were used in the cardiovascular studies. Animals were maintained at 29°C on a 12:12-h light-dark cycle, dark from 11 AM to 11 PM. The Williams College Institutional Animal Care and Use Committee approved all animal studies.

Implantation of BP telemeters. Mice were anesthetized initially with 5% isoflurane in an oxygen stream and maintained on 1–2% isoflurane. Mice were kept on a heating pad (38°C) throughout implantation of the BP telemeter (model PAC20; Data Sciences International) in the left common carotid artery (40). Mice were maintained on a heating pad for 48 h after the surgery and housed individually at 29°C for 1 wk to allow time for recovery from the surgery.

Cardiovascular data and urine collection. After recovery from surgery, c57/Bi mice were placed in urine collection cages, which were placed on receivers (model RPC-1, Data Sciences International) for the telemeters. Experiments performed on NPR1−/− and NPR3−/− mice were conducted in standard housing, with no urine collection. Animals were fed ad libitum a high-carbohydrate, low-fat diet.
liquid diet (AIN76A; Dyets) supplemented with a vitamin mix (300050; Dyets) and a mineral mix (201200; Dyets). We opted for a liquid diet because preliminary studies demonstrated that on powdered food or biscuits, some food was dropped into the urine collection tube, artificially raising the mineral content of the urine. The feeding bottle for the liquid diet was placed so that contamination of the urine from the liquid diet was not possible. After placement of mice into the urine collection cages, the cardiovascular parameters from the mice took 3–4 days to stabilize. At this point, animals were fed ad libitum for 2 more days for baseline urine collection and cardiovascular data collection. Over the ad libitum feeding time, caloric intake was measured to be 18 kcal/day. At the onset and through the duration of CR, the mice received 50% of normal food intake, which was 9 kcal/day. When the CR food was made, twice the amount of vitamins and minerals were added so that there were no intake deficiencies. Animals were calorically restricted for 7 days. At all times, drinking water was available ad libitum. Data from the BP telemeters was recorded at 500 Hz. Two-minute data streams were obtained every 10 min. From the pressure waveform, the following cardiovascular parameters were obtained: heart rate, systolic BP, diastolic BP, mean BP, pulse pressure, and the standard deviation of the interbeat interval (SDIBI). Between 10 AM and 11 AM, cardiovascular data were not obtained while the animals were cared for (urine collection, cage cleaning, body weight measurement, watering, and feeding). The dark cycle cardiovascular parameters were averaged from data collected between 11 AM and 10 PM and the light cycle parameters were averaged from data collected between 11 PM and 10 AM; 23-h averages were generated from data collected between 11 AM and 10 AM the next day. Ambient temperature was set at 29°C to prevent the mice from entering torpor during the CR period.

Urine content measurements. The following were measured from the collected urine: creatinine (Sigma), sodium (flame photometry), osmolarity (Precision Systems osmometer S), and protein content (Bio-Rad). Glomerular filtration rate (GFR) was estimated from urinary excretion of creatinine. Urine excretion of sodium, milliosmoles, and protein are expressed relative to GFR.

ANP measurement. Plasma ANP levels were measured in female C57/Bl mice euthanized at the end of the light cycle. A sample size (n) of 6 was used at each time point (ad libitum, and 50% CR for each of 1–4 and 5 days). Plasma ANP was measured with the use of an RIA kit (Phoenix Pharmaceuticals).

Statistics. Data are reported as means ± SE. One-way ANOVA, within the circadian cycle and across days, was performed to determine the effects of CR on the dependent variables. The P < 0.05 level of confidence was accepted for statistical significance.

RESULTS

C57/Bl mice and CR. Both cardiovascular parameters and urine contents were measured simultaneously in c57/Bl mice during ad libitum feeding and throughout a 7-day period of 50% CR. Mean BP (Fig. 1) as well as systolic and diastolic pressures (data not shown) fell significantly throughout the 7-day CR period. Twenty-three-hour mean BP dropped from 108.6 ± 1.8 during ad libitum feeding to 92.7 ± 2.4 mmHg during the last day of 50% CR. Similarly, heart rate fell during the CR period (Fig. 1). The 23-h heart rate dropped significantly from 624 ± 5 beats/min during ad libitum feeding to 426 ± 13 beats/min during the last day of 50% CR. BP and heart rate dropped in both the light cycle and dark cycle (data not shown). Urinary creatinine, sodium, osmolarity, and protein content were assessed from these same animals over the same time frame to determine whether the drop in BP and heart rate during CR was associated with a change in renal excretion. GFR, as estimated from creatinine clearance, was ~0.08 ml/min and did not change throughout the period of CR (from 0.080 ± 0.010 during ad libitum feeding to 0.081 ± 0.003 ml/min during the last day of 50% CR). In contrast to our hypothesis, 23-h sodium excretion increased significantly on the first day of 50% CR, from 4.2 ± 0.6 to 7.7 ± 1.5 mg sodium, followed by a drop to 1.1 ± 0.4 mg sodium by the last day of CR (Fig. 2). This was also true when expressed relative to GFR (ad libitum = 54 mg sodium·ml⁻¹·min⁻¹; first day of 50% CR = 78 mg sodium·ml⁻¹·min⁻¹; last day of 50% CR = 24 mg·ml⁻¹·min⁻¹). Similarly, excretion of the total number of millimoles fell from 2.3 ± 0.5 to 1.1 ± 0.1 millimoles (Fig. 2), which remained true when expressed relative to GFR (27.0 ± 2.5 to 17.4 ± 1.7 mosmol·ml⁻¹·min⁻¹). Total protein excretion significantly decreased from 0.91 mg protein during ad libitum feeding to 0.66 mg during the last day of 50% CR.

ANP and CR. Although sodium excretion decreased throughout the period of CR, and not increased as we had hypothesized, we tested the hypothesis that ANP plays an important role in cardiovascular regulation during CR in a manner that is independent of its actions on sodium excretion. Two approaches were taken to test the hypothesis. First, a correlative approach was taken to measure ANP plasma levels during CR. As Fig. 3 shows, ANP levels fell throughout a 5-day period of CR, consistent with the fall in sodium excretion seen in Fig. 2. The second approach was one that utilized genetic models. Cardiovascular parameters during ad libitum feeding and during a 7-day period of CR were measured in NPR1-/- or NPR3-/- mice. NPR1-/- mice displayed hypertension, as has been seen previously (28). Contrary to our hypothesis, 23-h mean BP dropped from 117.1 ± 3.9 to 108.0 ± 4.7 mmHg during the last day of 50% CR (Fig. 4). Similarly, 23-h heart rate dropped significantly from 500 ± 22 beats/min during ad libitum feeding to 424 ± 16 beats/min.
during the last day of 50% CR (Fig. 4). BP and heart rate fell in both the light and dark cycles (data not shown). NPR3−/− mice, missing the clearance receptor for ANP with subsequent elevated levels of circulating ANP, were hypotensive (Fig. 5) as has been reported previously (22). Similar to the c57/Bl and NPR1−/− mice, CR caused a drop in both mean BP (from 87.0 ± 2.4 to 78.4 ± 1.7 mmHg during the last day of 50% CR) and heart rate (from 437 ± 22 to 344 ± 22 beats/min during the last day of 50% CR) of NPR3−/− mice, although these mice have relatively low BP and heart rates during ad libitum feeding (Fig. 5). This was true in both the dark and light cycle (data not shown).

**SDIBI.** SDIBI was calculated for the mice of all three genotypes. CR elevated SDIBI for all mice throughout the 7-day CR period (average of days 6 and 7 is shown in Fig. 6). An increase in SDIBI has been seen in other studies with wild-type mice (40, 41, 44) and is interpreted as a relative increase in parasympathetic input or decrease in sympathetic input into the heart (30).

![Fig. 2. A 23-h sodium excretion and 23-h total osmole excretion in the urine during a 7-day bout of CR in c57/Bl mice. While cardiovascular parameters were measured during CR in mice shown in Fig. 1, urine was collected on a daily basis. Urine sodium content was measured using flame photometry and osmolarity was measured with an osmometer. Sodium excretion increased significantly during the first day of CR, but fell below pre-CR values by the fourth day of CR. Similar to sodium excretion during CR, the total number of osmoles excreted in the urine decreased throughout the bout of CR. Data are shown as means ± SE. *P < 0.05 vs. pre-CR.](image1)

![Fig. 3. Circulating atrial natriuretic peptide (ANP) throughout a 5-day period of 50% caloric restriction in c57/Bl mice. Mice (n = 6 at each time point) were euthanized at the end of the light cycle and blood was drawn. Plasma ANP levels dropped significantly by the third day of 50% CR. Data are shown as means ± SE. *P < 0.05 vs. pre-CR.](image2)

![Fig. 4. Effect of CR on mean BP and heart rate during a 7-day period of 50% CR in mice deficient in ANP receptor (NPR1−/−). NPR1−/− mice are missing the functional receptor for ANP. Mean BP dropped significantly by the sixth day of CR, whereas heart rate fell significantly by the third day of CR in these mice. Data are shown as means ± SE. *P < 0.05 vs. pre-CR.](image3)

![Fig. 5. Effect of CR on mean BP and heart rate during a 7-day period of 50% CR in mice deficient in ANP clearance receptor (NPR3−/−). NPR3−/− mice are missing the clearance receptor for ANP and have low BP due to elevated ANP levels (24). Both mean BP and heart rate fell significantly over the 7 days of CR in these mice. Data are shown as means ± SE. *P < 0.05 vs. pre-CR.](image4)
**DISCUSSION**

*Salt balance, ANP, and BP.* The relationship between salt intake and BP has been well known for many decades (recently reviewed in Ref. 5). In the current study, we held sodium intake constant while restricting the caloric intake of mice to specifically examine the impact of CR on sodium excretion and BP. One of the central findings of this study was that the hypotension and bradycardia that occur during a bout of CR were independent of natriuresis. Increased sodium excretion was observed only during the first day of CR (Fig. 2), before a change in mean BP (Fig. 1), heart rate (Fig. 1), or circulating ANP levels (Fig. 3). In the absence of changes in circulating ANP, the early onset natriuresis during CR has been attributed to withdrawal of renal sympathetic activity (18) and hypoinsulinemia (38). With longer-term CR (>3 days), the drop in sodium excretion (Fig. 2) coincides with the fall in circulating ANP (Fig. 3) and BP (Fig. 1). This is contrary to our prediction that BP falls during CR in response to rising ANP levels.

*ANP, NPR-A, and BP.* ANPs actions are mediated through its receptor, NPR-A (20). This receptor not only responds to ANP peripherally, it also responds to 1) ANP centrally, 2) brain natriuretic peptide peripherally and centrally, and 3) to a lesser extent, C-natriuretic peptide (3, 20). Therefore, it seemed plausible that this receptor could potentially mediate the hypotensive effect of CR independent of both circulating ANP levels and natriuresis. Therefore, we used mice deficient in NPR-A (NPR1−/−) to test whether this receptor plays a role in the cardiovascular effects of CR. NPR1−/− mice displayed bradycardia and only a moderate drop in mean BP during CR (Fig. 4). The time frame of the cardiovascular depression of the NPR1−/− as well as the absolute magnitude of the changes were different from the c57/Bi mice (NPR1−/− responses were smaller and took longer to occur). Certainly, the different genetic backgrounds of the NPR1−/− mice and the c57/Bi mice may play an important role with the differential cardiovascular response to CR. Therefore, care should be taken when making comparisons of the change in cardiovascular parameters between these two strains of mice. However, it does remain clear that some degree of bradycardia and hypotension can occur in the absence of the ANP receptor.

_NPR-C and BP._ The natriuretic peptides also interact with NPR-C, first referred to as the “biologically silent” ANP receptor (22). NPR-C lacks the intracellular signaling domain of NPR-A (30) and acts as the ANP clearance receptor. Fasting has been shown to decrease the expression of NPR-C in adipose tissue in rats (33). In fact, NPR-C expression in adipose tissue has been proposed to play a major role in sodium retention and obesity-associated hypertension (6). Because different studies have reported that negative energy balance either increases circulating ANP (4, 23) or decreases circulating ANP (Fig. 3; Refs. 7, 26, and 33), we performed a pilot study on NPR3−/− mice (n = 3). As shown in Fig. 5, the NPR3−/− mice respond to CR with depressed cardiovascular parameters. While interpretation of the data should be conservative due to the low sample size as well as the different genetic background from the c57/Bi mice, these data suggest that the ANP clearance receptor is not required for some of the bradycardia and hypotension associated with CR.

*Heart rate and the ANS.* ANP also profoundly impacts the ANS at many levels (3, 19, 20), which is relevant here due to the important role of the ANS in CR-induced hypotension and bradycardia (9, 17, 18, 37, 41, 43). The interaction between ANP and the ANS was first seen when infusions of ANP lowered BP that was accompanied by a drop in heart rate and not the expected baroreflex-mediated tachycardia (1). Interestingly, this combination of bradycardia and hypotension is also observed in animals undergoing CR (40, 41, 44). ANP appears to influence the ANS both centrally and peripherally (10, 16, 21, 34, 35) and includes suppression of SNS outflow from the central nervous system, suppression of catecholamine release from nerve endings, and a lowering of the activation threshold of vagal afferents. The CR-induced bradycardia observed in all three strains of mice in the present study could be the result of 1) decreased SNS input, 2) increased parasympathetic nervous system (PNS) input, or 3) a drop in the intrinsic heart rate. While it is formally possible that the bradycardia could be mediated through a lowering of the intrinsic heart rate, a response that has been seen in fasted rats (15), the heart rate responses observed in this study are likely mediated by alterations in SNS and/or PNS input to the heart. The increase in SDIBI observed during CR (Fig. 6) indicates a decreased SNS, increased PNS input to the heart (42), or both. In fact, a recent report (41) suggests that both the SNS and PNS are involved with the bradycardia. Because the mice used here were of different genetic backgrounds, interpretations and comparisons among the mice should be made with caution. However, an increase in SDIBI can occur in the absence of either the NPR-A or NPR-C receptor (Fig. 6), suggesting that alterations in the ANS can occur in the absence of either receptor.

Numerous other hormones have been linked to modulation of the autonomic nervous system, cardiovascular parameters, and reflect ingestive behavior. These include those derived from the gut (8, 25, 27), such as ghrelin and PYY3–36, as well as those derived from fat (31, 32, 40), including leptin and adiponectin. Each of these hormones can influence the cardiovascular system, and perhaps it is the altered signaling of these hormones, and not ANP, that is responsible for the sympatholytic nature of CR. One further possibility linking ingesting behavior and BP regulation is water intake. Williams et al. (44) have shown that mice calorically restricted at 30°C drink ~40% less water than ad libitum fed mice at the same tem-

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**Fig. 6. Standard deviation of the interbeat interval (SDIBI), a measure of relative autonomic input into the heart, was calculated before the onset of CR (ad libitum), and during CR (averaged over the last 2 days). SDIBI significantly increased during CR in all three types of mice. Data are shown as means ± SE. *P < 0.05 vs. ad libitum feeding.**

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**ANP AND CARDIOVASCULAR PARAMETERS**

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perature. Although water balance was not assessed here, it may be that a negative water balance over the 7-day CR period occurred, and this was sufficient to lower BP.

Mice were examined in this study at 29°C, an ambient temperature (Ta) that calls for little SNS activity due to the reduced requirement for SNS-mediated thermogenesis at temperatures near the thermoneutral zone for mice (12). Evidence supporting this idea is the relatively long SDIBIs seen during ad libitum feeding in the animals tested (10–12 ms; Fig. 6). It may be that at a Ta that engages the SNS for thermogenesis (i.e., Ta below the thermoneutral zone), ANP and its receptor would play a greater role in modulating the ANS. However, we chose a Ta of 29°C, a temperature that prevents mice from entering torpor during the CR phase (11, 40), to avoid the complication of torpor-related cardiovascular changes.

In summary, the early onset of natriuresis seen in mice (Fig. 2) and other organisms, including humans (4, 7, 18, 23, 36, 38), during negative energy balance appears to be independent of both circulating ANP and BP. Furthermore, the long-term effect of CR in mice was enhanced renal reabsorption of sodium, likely reflecting the drop in ANP levels and drop in mean BP. Hence, whereas sodium intake plays a critical role in regulation of blood volume and pressure (5, 13), sodium excretion and ANP signaling appear to play little role in the hypertensive effect of CR.

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