Prenatal high-salt diet in the Sprague Dawley rat programs blood pressure and heart rate hyperresponsiveness to stress in adult female offspring

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Abstract

Several animal models have been developed to study fetal programming of hypertension. One model involves feeding high-salt (HS) diet to rats before and during pregnancy, during lactation, and after weaning for 10 days. In the present investigation, we limited HS diet to the prenatal period in an attempt to find a narrower critical window for fetal programming. The HS diet did not result in low birth-weight offspring. In the adult offspring, radiotelemetry was used to assess blood pressure and heart rate in the conscious unstressed state. As adults, the HS offspring were not hypertensive compared to normal-salt (NS) control animals. However, the pressor and tachycardic responses to 1-hr of restraint were significantly enhanced in HS female offspring and recovery after restraint was delayed. This was accompanied by an increase in relative expression of corticotropin-releasing hormone (CRH) mRNA in the paraventricular nucleus of the hypothalamus during basal and stressed conditions. There was no augmented stress response or relative increase in CRH mRNA in adult HS male offspring. When challenged with one week of 8% NaCl diet as adults, neither HS male nor female offspring exhibited salt sensitivity compared to NS groups. These data show that a high-salt diet limited to the prenatal period is not sufficient to program hypertension in adult offspring. However, this narrower critical period is sufficient to imprint a lasting hyperresponsiveness to stress, at least in adult female offspring. These data indicate that excessive maternal salt intake during pregnancy can adversely affect the cardiovascular health of adult offspring.
Key words: CRH, paraventricular nucleus, hypertension, radiotelemetry, sodium chloride, pregnancy, prenatal nutrition physiology
In recent years, several animal models have been developed to study developmental origins (also known as fetal programming) of adult diseases, including hypertension. Most of these have been models of low birth weight in an attempt to better understand clinical data in humans that suggest low birth weight is associated with hypertension in later life. Of these, the protein undernutrition model has received the most attention. Limiting maternal protein intake during pregnancy produces small offspring in rats. These offspring are hypertensive as adults (24,31,45). Another model has used ligation of the uterine arteries to produce underperfusion of the placenta. Offspring are small and develop hypertension as adults (1,36). A third model involves injection of dexamethasone into pregnant animals near parturition. Once again, offspring are small and develop hypertension later in life (11,44). All of these models provide evidence that the fetal environment can have lasting effects on blood pressure.

Contreras et al. have shown that perinatal high salt can also produce a lasting hypertension in Sprague Dawley rats (8). This model involved giving a high-salt diet before and during pregnancy, during lactation, and to weaned offspring for 10 days before switching to a normal-salt chow. We have used this model to show that hypertension is present in the offspring as early as four weeks and may involve increased sympathetic nervous activity secondary to increased activation of central angiotensin II AT1 receptors (37). While this model can produce permanent hypertension, it is not strictly a model of fetal programming since the high-salt diet is also given during lactation and after weaning. In the present study, we limited the high-salt diet exposure to pregnancy (and one-week preconception). We hypothesized that a prenatal high-salt diet would be sufficient to produce lasting hypertension in adult offspring.
There are also several reports that the fetal environment can permanently affect the stress axis in adults (6,20,42). Of interest to us are the reports that the blood pressure response to different stressors may be enhanced in adult offspring of rats subjected to adverse environments including hypoxia, malnutrition, or restraint/heat stress during pregnancy (17,30,38). This hyperactive pressor response occurred even in cases where the prenatal insult did not produce lasting hypertension in the offspring. Even without frank hypertension, a programmed pressor hyperresponsiveness during stress could ultimately lead to permanent elevation in arterial pressure (14). The second hypothesis addressed was that a prenatal high-salt diet would be sufficient to impart an enhanced cardiovascular response to acute stress in adult offspring. If present, such a hyperresponsiveness could be due to an augmentation of descending stress-related sympathetic pathways mediated by increased hypothalamic corticotropin releasing hormone (CRH) or enhanced activation of the hypothalamic-pituitary-adrenal (HPA) axis. To this end, the expression of mRNA for CRH in the paraventricular nucleus of the hypothalamus (PVN) and plasma levels of corticosterone were examined during basal and stressed conditions.

Sprague Dawley rats are not genetically salt sensitive, that is, a high-salt diet produces only a modest increase in blood pressure (15,28). However, there is some indication that perinatal high salt and other models of fetal programming may impart a significant permanent salt sensitivity (8,34,45). The final hypothesis addressed was that a maternal high-salt diet limited to the period of pregnancy would produce offspring that were salt sensitive as adults.
There is considerable evidence that the effects of fetal programming are sex specific (6,9,21,43). However, there is no clear consensus about whether the effects predominate in males or females. This depends, in part, on the model of fetal programming and on the parameters measured in the adult offspring. Nevertheless, it seems appropriate to consider the possibility that high-salt during pregnancy could have different effects in male and female offspring. To this end, the sex of the offspring was included as a factor in all analyses. Portions of this work have been reported in abstract form (16,32).
Materials and Methods

Prenatal HS and NS protocol

All protocols were approved by the IACUC of Brigham Young University. Female Sprague Dawley (Harlan Sprague Dawley) rats received 8% NaCl Harlan Teklad diet (product ID# 92012) as the HS treatment or 0.7% NaCl (product ID# 96329) as the NS control treatment. Proven breeder male Sprague Dawley rats were given standard lab chow (Harlan Teklad Rodent Diet (0.29% Na⁺, 0.49% Cl⁻)) during this time. Rats were allowed to adjust to the experimental diet (HS or NS) while caged separately for one week prior to breeding, after which they were placed in hanging wire cages in breeding pairs. Once vaginal mucus plugs were found (usually 1-5 days), the males were removed and the females were placed in polycarbonate cages and allowed to eat experimental diet and drink water ad libitum. Corncob bedding was changed every 2-3 days. Water and food intake were measured during gestational days 17-20 for some rats (HS, n=4; NS, n=4), and body weight at day 20 of gestation was measured in other rats (HS, n=5; NS, n=4). Daily food intake was determined by subtracting the weight of pellets remaining each morning and the weight of crumbs collected from below each cage from the weight of pellets given on the prior day. Daily water intake was determined by measuring the water remaining each morning in bottles that were filled on the prior day. Pregnant dams were allowed to progress to delivery. Litter size, birth weights, and sex were determined within 24 hours of delivery and litters were culled to 10, keeping an equal ratio of males and females except for 1 NS litter that had 4 males and 4 females and 1 HS litter that had 5 males and 4 females. After parturition, experimental diet was discontinued and dams were fed standard lab chow while lactating. Pups were weaned on PD 21 and given
standard lab chow to eat. Hence, the only exposure of offspring to the high-salt environment was during the in utero period.

**Implantation of radiotelemetry probe**

Adult offspring were anesthetized with ketamine/acepromazine (140 mg/kg/1.4 mg/kg, *im*) and the abdominal aorta was exposed through a ventral midline incision. Blood pressure probes (TA11PA-C40, DSI, Arden Hills, MN) were inserted into the aorta distal to the renal arteries and cemented in place with Vetbond (3M, St. Paul, MN) and secured with a cellulose (0.5 cm²) patch. After the incision was closed, rats were injected with Rimadyl (5 mg/kg, *im*, Pfizer, Exton, PA) and allowed one week to recover in plastic cages placed on top of radio receiving units (RPC-1, DSI, 1 per rat). During this recovery week children’s acetaminophen syrup was added to the drinking water to provide analgesia. Only animals without surgical complications were included in the study (2 NS female offspring were excluded). Blood pressure and HR were monitored using a scheduled sampling protocol which included 30 seconds of recording (500Hz) every 20 minutes throughout the light and dark cycles. Data were digitized and stored on a hard drive using Dataquest ART software (DSI).

**Expression of CRH mRNA**

Animals were killed by decapitation and, in some rats, trunk blood was collected into chilled tubes containing 5 µl of heparin (2,000 U/ml). Brains were quickly removed and frozen in plastic molds containing O.C.T. compound. The blocks of tissue were stored at -93°C until processing for in situ hybridization.
In situ hybridization.

Coronal sections (20 µm) through the PVN were cut (1 in 3 series) with a cryostat and thaw-mounted onto slides (Superfrost Plus, Fisher Scientific). One series of sections was subsequently fixed (4% buffered paraformaldehyde) and acetylated. Hybridization, in situ, was carried out overnight at 57°C using a 33P-UTP-labeled (3 X 10^6 cpm per slide) antisense riboprobe to CRH mRNA (Dr. Kelly Mayo, Northwestern University, Evanston, IL). Unincorporated probe was removed by incubating the slides in RNAse (14 µg/ml, Sigma) for 30 min followed by washes in buffer without RNAse, 1X SSC (Saline Sodium Citrate, room temperature) and 0.5X SSC (60°C). Visualization of the hybridized sections, together with 14C-standards (American Radiolabeled Chemicals, Inc., St. Louis, MO) utilized 48-hr exposure to autoradiographic film (Hyperfilm MP, Amersham Biosciences). Autoradiographic films were then developed using a standard x-ray developer.

For data analysis, sections through the whole extent of the PVN were scanned from each brain. The region bounded by both wings of the PVN was outlined as the region of interest and mean optical density (O.D., calibrated to µCi/g using the 14C standards) and area (in square pixels) were determined (Scion Image, Scion Corporation, Frederick, MD). An identical area over the region just dorsal to the PVN was used to determine the background O.D. For each section, the O.D. (total minus background) was multiplied by the area and all values from a given brain were averaged. Since not all sections were processed in the same assay, the data required normalization. This was done by dividing the value for each individual rat by the average of the NS offspring for
given assay. Within each sex, the normalized data for HS and NS groups during basal and stressed conditions were compared using two-way ANOVA.

*Corticosterone assay*

Blood was centrifuged for 15 min at 4°C and plasma was stored at -20°C until the time of assay. Radioimmunoassay for corticosterone was performed using a commercially available kit (MP Biomedicals, Orangeburg, NY).

*Experimental protocols*

*Basal recordings*

One week after surgery, resting MAP and HR was measured as outlined above for 7 days. During this time, the animals were left undisturbed except for periodic changing of cages and routine feeding by the animal care facility workers. For males, adult offspring ranged in age from 2 to 6 months at the time of initial surgery and came from 10 different litters (5 HS and 5 NS). For females, adult offspring ranged in age from 2 to 4.5 months and came from 12 different litters (6 HS and 6 NS).

For each rat, the 36 data points recorded for each light or dark period for each day were averaged separately to give 7 light-period values and 7 dark-period values. Within male or female groups, 2-way ANOVA for repeated measures was used to compare resting MAP and HR between HS and NS groups over the 7 days (SigmaStat, SSI, Richmond, CA). Light-period values were compared separately from dark-period values.

*Acute restraint stress*

Two weeks after surgery, scheduled sampling was changed from a 30-second sample once every 20 minutes to a 30-second sample once every 5 minutes. The experimental procedure room was kept quiet the morning before the stress. Beginning at
13:00, several samples were taken to establish initial MAP and HR. At the end of the final five-minute baseline period, rats were placed into Plexiglas restraining tubes (5 cm x 7 cm x 14 cm for rats under 300 g and 5 cm x 8 cm x 15 cm for rats greater than 300 g) which were placed back into the home cages sitting on top of the radio receivers in time to record the next five-minute sample. After one hour, rats were removed from the restraining cages and allowed to recover in their home cages for an additional hour. After recovery, scheduled sampling was changed back to one 30-second sample every 20 minutes. For each rat, pre-stress values for MAP and HR were averaged (2-10 values) to give a single baseline number. The change in MAP and HR from the initial was then determined for each 5-min sample during the 60 minutes of stress and the 60 minutes of recovery. Two-way ANOVA with repeated measures (time) was used to compare the effects in NS and HS offspring across the 120 minutes. Where appropriate post hoc analysis was performed using the Student-Newman-Keuls test.

**High-salt challenge**

One day after completion of the acute restraint stress, all rats were fed the 8% NaCl diet for one week followed by one week of recovery on standard rat chow. Control MAP and HR values were obtained for each rat by averaging all data from the day (both light- and dark-values) prior to the high-salt challenge. All data points for the entire 7-day high-salt challenge were averaged to give the experimental value for each rat. The recovery values were obtained by averaging all data points obtained during the seventh day (24 hours) of recovery. Within male and female groups, control, experimental, and recovery data were compared using 2-way ANOVA for repeated measures. In addition, day-by-day values for MAP and HR during the week of high-salt challenge were
compared for male and female groups during light or dark periods using 2-way ANOVA for repeated measures. Where appropriate *post hoc* analysis was performed using the Student-Newman-Keuls test. Rats were killed at the end of the final day of recovery from the high-salt challenge and brains were collected at this point for subsequent determination of basal CRH mRNA expression. In some rats, trunk blood was also collected for subsequent determination of plasma levels of corticosterone.

**Stress-induced CRH expression and corticosterone levels**

In a final series of experiments, naïve (no telemetry implant) NS and HS adult offspring of both sexes (NS:males – n=4; HS:males – n=4; NS:females – n=9; HS:females – n=9) were subjected to 60 minutes of restraint stress as outlined above. At the end of the stress period, the rats were removed from the restraining tube and 5 minutes later were killed by decapitation. Blood was collected for determination of plasma corticosterone and brains were prepared for subsequent *in situ* hybridization for CRH.

**Data analysis**

For all statistical analyses, a p-value less than 0.05 was considered statistically significant. In addition to the statistical analyses mentioned above, the following were also used. The female to male ratio of each litter, the ratio of live pups to total pups in each litter for HS and NS groups, and litter size between HS and NS groups were compared using the Student t test. Within male and female groups, HS and NS pup birth weight and plasma corticosterone levels during basal and stressed conditions were compared using 2-way ANOVA. All values are reported as the mean ± SEM.
Results

Maternal food and water intake and litter size

Food intake for the pregnant NS dams averaged 26.4 ± 0.6g per day for four days prior to delivery and was not significantly different from the HS average of 27.3 ± 0.9g (Student t-test). Water intake for NS dams was 45.5 ± 2.7 mL per day, compared to 150.2 ± 3.4 mL per day for HS dams (p<.001, Student t-test). Body weight on day 20 of gestation did not differ between the two groups (HS, 408 ± 21 g; NS, 418 ± 7 g, p = 0.72, t-test).

Litter sizes for NS (13.4 ± 1.4, n=8) and HS (14.0 ± 0.8, n=8) groups did not differ (p = 0.71). The percentage of live pups per litter was similar between NS (0.92 ± 0.05) and HS (0.96 ± 0.02) groups (p = 0.52).

Pup birth weight and sex

Most studies of fetal programming have focused on a low birth-weight model to program hypertension. In the present investigation, though females weighed significantly less than males at birth (6.25 ± 0.07 vs 6.5 ± 0.07 g, respectively, p = 0.002), there was no difference in birth weight between NS and HS litters (6.32 ± 0.07 vs 6.47 ± 0.07 g, respectively, p=0.11). The individual group birth weights were NS: males - 6.46 ± 0.09 g, n=54; females - 6.17 ± 0.10 g, n=45; HS: males - 6.62 ± 0.09 g, n=50; females - 6.32 ± 0.09 g, n=44. Thus, the prenatal HS diet did not produce low birth weight. There was no difference in the ratio of female to male offspring born (including dead pups) in either group (NS: 0.96 ± 0.14, HS: 0.92 ± 0.14, p=0.81).
Basal recordings

For male groups, resting blood pressure was the same (figure 1, upper panel) for HS and NS offspring during both light (F(1,24) = 0.153, p=0.70) and dark (F(1,24) = 0.314, p = 0.58) periods. Male heart rate was also the same for HS and NS groups during light (F(1,24) = 0.001, p = 0.98) and dark (F(1,24) = 0.92, p=0.35) periods. For females, resting blood pressure was the same (figure 1, lower panel) for HS and NS offspring during both light (F(1,28) = 0.063, p = 0.80) and dark (F(1,28) = 0.001, p = 0.99) periods as was heart rate for both groups (light, F(1,28) = 0.002, p = 0.96) (dark, F(1,28) = 0.011, p = 0.92). Therefore, our treatment did not program hypertension or lasting changes in basal heart rate in offspring of dams fed a HS diet.

Acute restraint stress

Baseline data collected at five-minute intervals before restraint stress revealed no differences in blood pressure values between HS and NS rats regardless of sex (males: NS -121.1 ± 3.7, HS – 120.0 ± 2.7 mm Hg; females: NS -108.2 ± 2.7, HS -107.1 ± 2.0 mm Hg). However, two-way ANOVA showed that baseline MAP for males was significantly greater than for females (p<.001). There were also no differences in pre-stress HR (males: NS - 348 ± 8, HS - 342 ± 10; females: NS - 353 ± 6, HS - 340 ± 6).

The tachycardic and pressor responses produced by the restraint stress were significantly greater in the HS females compared to the NS females (HR: diet, F(1,28) = 12.95, p = 0.001; MAP: diet, F(1,28) = 6.15, p = 0.019) across the entire 60 minutes of restraint and ensuing 60 minutes of recovery (figure 2). There was no significant interaction (diet x time) in any of the analyses. Hence, the HS female offspring had
difficulty accommodating the restraint stress. Normal salt females were able to recover to baseline values sooner than HS females.

In the males, neither the HR nor the blood pressure response produced by restraint stress (figure 3) was different between NS and HS offspring (HR: diet, $F(1,24) = 0.0005$, $p = 0.982$; MAP: diet, $F(1,24) = 0.0003$, $p = 0.986$).

**High-salt challenge**

There was no difference in the HR or MAP response to the high-salt challenge between NS and HS offspring (figure 4) in either males (HR: $F(1,22) = 0.10$, $p = 0.76$; MAP: $F(1,22) = 0.04$, $p = 0.85$) or females (HR: $F(1,28) = 0.42$, $p = 0.52$; MAP: $F(1,28) = 0.76$, $p = 0.39$). Both males and females exhibited a significant ($p<0.001$) increase in MAP during the week of high-salt diet and returned to control levels by the seventh day after returning to the standard chow. Heart rate was significantly increased by the high-salt challenge in the male group, and fell significantly below control during the recovery period in all groups ($p<0.001$). Analysis of the heart rate and MAP data on a day-by-day basis during the high-salt challenge did not reveal any significant differences between NS and HS male or female groups during light or dark periods. Male groups did exhibit a significant ($p < 0.001$) increase in MAP each day on the diet compared to the previous day until day 5 (data not shown). Female groups showed a significant increase in MAP from day 1 to day 2 ($p < 0.001$) on the diet and then no further increase for the rest of the week (data not shown).
CRH mRNA

*In situ* hybridization for CRH mRNA was performed on serial sections through the hypothalamus. The paraventricular nucleus (PVN) was the only region to exhibit a hybridization signal. For female offspring, there was a significant main effect of diet ($F(1,41) = 10.62, p = 0.002$); that is, female HS offspring had significantly higher relative CRH mRNA levels in the PVN than did NS females averaged over basal and stressed conditions (figure 5A & 5B). Since the CRH data were normalized to the NS group in both the basal and stressed conditions there was no significant main effect of stress on CRH mRNA expression. However, when absolute expression levels were compared, the CRH mRNA expression of stressed females was approximately twice that of the basal group ($10140 \pm 523 \text{ vs } 5152 \pm 723 \mu\text{Ci/g X pixel}^2, p < 0.001$). Male HS and NS CRH levels did not differ ($F(1,28) = 0.15, p = 0.70$) (figure 5A & 5C). Absolute expression of CRH mRNA was also significantly increased by stress in the male rats ($6300 \pm 215 \text{ vs basal – } 3407 \pm 345 \mu\text{Ci/g X pixel}^2, p < 0.001$).

**Plasma Corticosterone**

Plasma corticosterone levels under basal and stressed conditions in male and female offspring are shown in Table 1. In both male and female offspring there was a significant main effect of stress (males – $F(1,14) = 79.0, p < 0.001$; females – $F(1,33) = 430.7, p < 0.001$). However, there was no difference between NS and HS offspring in either sex (males – $F(1,14) = 0.96, p = 0.34$; females – $F(1,33) = 0.083, p = 0.77$).
Discussion

This is the first study, to our knowledge, to examine the programming effects of a 8% NaCl diet given to Sprague Dawley rats only during pregnancy (and 1 week prior to conception). Since an early study using a 2.3% NaCl diet during pregnancy reported no effect on blood pressure of adult offspring (18), we chose a higher concentration of salt in order to maximize the potential effect. Most prior studies have typically given the high salt also during lactation and after weaning and have produced a lasting elevation in blood pressure (2,6). We found that when the high salt was limited to pregnancy the following effects were observed. 1) In both adult male and female offspring, there was no difference in HR or MAP between the HS and NS groups. 2) In female HS rats, there was an enhanced pressor and tachycardic response during acute restraint and a delayed recovery after restraint. This was accompanied by increased expression of CRH mRNA in the PVN, under basal and stressed conditions. However, plasma corticosterone was not increased. Adult male offspring did not exhibit an enhanced cardiovascular response to stress or increased expression of CRH mRNA. 3) Prenatal high salt did not produce offspring that were salt sensitive as adults, that is, giving a high-salt challenge to the adult HS offspring did not produce hypertension.

Contreras and co workers have shown that giving female rats a high-salt diet during pregnancy and lactation and for ten days after weaning will produce a lasting increase in blood pressure in the adult offspring (8). This prolonged application of high salt does not allow determination of shorter critical periods in the programming paradigm. Preliminary experiments in our lab tested the hypothesis that a high-salt diet limited to the prenatal period would be sufficient to cause hypertension in offspring.
Initial testing using an arterial catheter to measure blood pressure 1-2 days after surgery showed that this might be the case (16). However, the increase in arterial pressure was modest and there were concerns that stress associated with the surgical implantation of the catheter or handling of the rats during blood pressure recording could have produced an artifactual hypertension.

In the present investigation, radiotelemetry probes were used to measure blood pressure in offspring from HS or NS mothers. The radiotelemetry system is an improvement over catheterization because radiotelemetry uses an implanted radio-frequency transmitter that records blood pressure and heart rate without investigator involvement. Rats are able to recover completely from surgery before data are collected. Recordings can be taken during the light and the dark cycle, giving values during both the sleeping and waking cycles. Recordings made using radiotelemetry showed that HS rats were not hypertensive (figure 1). In both male and female offspring, the heart rate and mean arterial pressure tracings of NS and HS groups were virtually superimposable during light or dark periods. This suggests that exposure to high salt only during pregnancy is not sufficient to program hypertension in adult offspring. We also found in preliminary studies, using catheterized rats, that limiting the high salt exposure to the lactation period was not sufficient to produce hypertension in the offspring (16). Apparently, the high salt must be present during lactation (and possibly for a short time after weaning) in addition to pregnancy to produce a lasting increase in arterial pressure. Rats are known to continue development of several key systems, including the renin-angiotensin system, the renal system, the hypothalamo-pituitary-adrenal system, and the brain for several weeks after birth (20,41,45). It is likely that there are multiple critical
periods during pregnancy and lactation when high salt exposure can imprint changes in subsequent development.

Most models of fetal programming involve perturbations that produce low birth weight in offspring (11,25,36,45). It is thought that the decreased growth, *in utero*, includes alterations in renal development that subsequently lead to hypertension. In the present investigation, maternal high salt limited to the prenatal period did not produce offspring with low birth weight and this could explain the absence of elevated blood pressure in later life. Though the pregnant rats eating the HS diet drank considerably more water, food intake was not different from NS mothers. Since the two diets were virtually identical except for the NaCl content, calorie intake in both groups was similar and probably accounted for the similar body weight at day 20 of gestation and similar birth weights of the offspring. Low birth weight, *per se*, is not an absolute requirement for programming of hypertension. There are isolated reports that perturbations such as limiting maternal protein to 9% throughout pregnancy (31), prenatal exposure to interleukin-6 (33), or lard feeding during pregnancy (21) can program lasting hypertension without producing low-birth-weight offspring. Likewise, low-birth-weight offspring don’t necessarily develop hypertension (43). The prenatal HS diet used in the present investigation may prove to be an excellent model for studying programming effects that aren’t confounded by low birth weight, incomplete development of the kidney, and/or subsequent catch-up weight.

Despite the absence of elevated basal blood pressure, the HS female offspring did exhibit an enhanced pressor and tachycardic response to acute restraint and/or handling stress. There have been other reports that fetal programming can produce lasting effects
on the blood pressure response to acute stress. Offspring of mothers fed 5% protein
during pregnancy exhibited an enhanced increase in MAP in response to ammonia stress
(38). Likewise, 6 month-old offspring of mothers subjected to restraint/light/heat three
times per day for the last five days of pregnancy were not hypertensive, but did exhibit an
augmented blood pressure and heart rate response during acute restraint (17). Uterine
artery ligation also produced offspring (only males were studied) that exhibit an enhanced
blood pressure and heart rate response to olfactory stress (36). Finally, prenatal hypoxia
led to offspring with hyperresponsive blood pressure increases to air jet stress (30). The
increase in MAP and HR produced by stress involves activation of the sympathetic
nervous system (18). It is likely that the enhanced cardiovascular response in the female
offspring of HS mothers was due to augmented activation of the sympathetic nervous
system by the stress. It is not known if afferent, efferent, or integrative pathways were
permanently affected by this programming model.

The expression of CRH mRNA was increased in the adult female, but not male,
offspring of HS rats under both basal and stressed conditions. This difference in HS
female rats was detected even though basal expression of CRH mRNA was determined in
rats that had a history of surgery and experimentation and stressed levels of CRH were
determined in rats without such a history. There is evidence that CRH acts centrally to
increase sympathetic nervous activity and blood pressure during stress (13,18). The
presence of both increased CRH mRNA expression and stress hyperresponsiveness in
females and the absence of these effects in males suggest the possibility of a cause- and-
effect relationship that needs further investigation. The absence of increased resting
blood pressure despite the increased basal expression of CRH mRNA was likely due to
baroreflex buffering. The enhanced pressor and tachycardic response with stress likely occurred as the baroreflex reset to higher pressures (26). An augmented blood pressure and heart rate response to acute stress, if repeated each time a stressor is encountered, has the potential to gradually lead to permanent hypertension and/or heart disease due to structural adaptations of the heart and blood vessels in response to the added load (14).

In addition to its ability to act centrally to increase sympathetic activity, CRH also functions to activate the HPA axis during stress. In adult rats, chronic increases in dorsal hindbrain corticosterone have been reported to produce an enhanced pressor response to restraint (35). However, in the present study there was no difference in either basal or stressed corticosterone levels in NS versus HS female offspring. This makes it unlikely that the enhanced pressor and heart-rate responses were due to an action of circulating corticosterone. Likewise, these data suggest that while high-salt diet during pregnancy can program increased responsiveness of the CRH-sympathetic axis during stress, it does not program a similar increased responsiveness of the HPA axis. These two parallel systems can be regulated differently (9,19) and apparently can also respond differently to the high-salt programming signal.

There are many reports of sex differences in fetal programming of hypertension. However, there is no consistent finding in this regard. For example, protein undernutrition during pregnancy has been reported to produce hypertension in male offspring only (43), or in both male and female offspring (24). Lard feeding during pregnancy has been reported to produce hypertension in female offspring, but not male offspring (21). Prenatal administration of interleukin-6 (IL-6) programmed hypertension in both male and female offspring, but the effect occurred earlier in the females (33).
Likewise, fetal programming of the cardiovascular response to stress has been reported to be sexually dimorphic. Adult female offspring of mothers subjected to prenatal stress showed an enhanced pressor response to one-time restraint compared to their male counterparts (17). Prenatal administration of IL-6 also produced a greater pressor response in females (33). In humans, low-birth-weight adult females were shown to have an enhanced pressor response to psychological stress compared to males (42). The differences in these studies are likely due to differences in timing and/or intensity of the fetal insult. In the present study, though the adult female offspring of rats fed high salt during pregnancy were not hypertensive, they did show an augmented pressor and tachycardic response to restraint. Recent evidence suggests that in female rats, the locus coeruleus (LC), an important noradrenergic brainstem region involved in the stress response, is activated by hypotensive stress substantially more than in males (9). Perhaps the restraint stress used in the present study had a similar enhanced effect on the LC. CRH originating in the PVN is thought to contribute to the activation of the LC (39), and estrogen has been shown to increase CRH expression (40). Thus, estrogen mediated increases in PVN CRH expression could be responsible for the augmented cardiovascular effects of acute stress in the female HS offspring.

Sprague Dawley rats are not considered to be salt sensitive, that is, a high-salt diet does not produce lasting hypertension (15,28). There are a few reports that fetal programming models can imprint salt sensitivity. The perinatal high-salt protocol used by Contreras et al. resulted in adult rats that exhibited a greater rise in blood pressure when fed a 3% NaCl diet for one week compared to rats that were exposed to normal salt (8). A prenatal 5% protein diet also produced offspring that were salt sensitive as adults.
(45), though others have reported that protein under-nutrition did not produce salt sensitivity (25). Finally, uterine artery ligation during the final week of pregnancy also produced adult offspring that increased blood pressure significantly when given 2% NaCl to drink (34). In the present investigation we used a high-salt challenge to test the hypothesis that prenatal high salt could also program salt sensitivity. However, there was no difference in the blood pressure response of NS and HS offspring during one week of 8% NaCl diet. Since there was only one day after the acute stress before the high-salt diet was begun, it is possible that some residual effects of the stress remained. However, this seems unlikely since baseline values for blood pressure had returned to pre-stress levels prior to the start of the high salt. Regardless of sex, the high-salt diet produced a slight, but significant, increase in MAP in both groups, whether analyzed on a day-by-day basis, or on a weekly basis. One week after returning to the standard rat chow, MAP had returned to control levels in both groups. The recovery level of MAP in both male and female HS rats appeared to be increased compared to NS groups, but the effect was not significant. Thus, high salt limited to the prenatal period did not imprint salt sensitivity in the offspring.

How prenatal high salt could program the observed stress hyperresponsiveness into the adult female is unknown. Studies by others have shown that feeding rats high salt during pregnancy has no effect on the sodium concentration in maternal plasma or amniotic fluid or fetal plasma (7,22). These observations suggest that an indirect effect of the high salt is more likely to be responsible for programming. Possible maternal alterations that could indirectly affect long-term gene expression in offspring include decreased renin-angiotensin-aldosterone levels or increased arterial pressure (4,11).
A limitation of the present study is that some of the rats used in the protocols came from the same litters. However, when the data were collapsed within litters into one average value for each time point such that each litter only contributed once, 2-way ANOVA still showed a significant difference in the MAP or HR response to stress between NS and HS female groups (F (1,10)=5.32, p=0.044 for the MAP response, and F (1,10) = 12.05, p = 0.006 for the HR response). Hence, it is unlikely that litter bias contributed to the differences observed.

It is recognized that there was a fairly large range in the age of animals used. This happened as rats from different litters (of different ages) were incorporated to reduce potential litter bias. However, the average age of each group was not different (NS male – 12.7 ± 1.2 weeks; HS male – 13.0 ± 1.6 weeks; NS female – 12.7 ± 0.7 weeks; HS female – 13.9 ± 0.7 weeks, p= 0.48, two-way ANOVA). Others who have looked at the effect of age on fetal programming of hypertension generally find no difference in offspring ranging in age from 8 weeks to 24 weeks (3,29,33). Alexander did report hypertension in female offspring that was present at 8 weeks, but gone by 12 weeks (1). Our HS female group had only one rat younger than 11 weeks and our NS female group had only two rats younger than 11 weeks. Hence, it is unlikely that the phenomena studied in the present investigation have an age-dependent component.

It should also be noted that the high-salt diet was present during the week prior to conception. We chose to allow time for the rats to acclimate to the new diet to increase the likelihood of successful mating. We cannot rule out the possibility that the differences seen in the female offspring could have resulted from changes in the dams prior to conception or blastocyst implantation. Kwong, et al. reported that maternal
protein undernutrition for the first 4.25 days after conception was sufficient to program hypertension in adult offspring (23). Likewise, we cannot rule out an effect on the breeder males who also ate the high-salt diet. It is also possible that there are multiple critical periods during the gestational period when maternal high salt could have opposite effects. Additional studies will be needed to define a narrower critical period for fetal programming by a maternal high-salt diet.

In summary, limiting maternal exposure to a high-salt diet to the prenatal period is not sufficient to program lasting hypertension or salt sensitivity in offspring. However, an enhanced pressor and tachycardic response to acute stress does persist into adulthood, at least in female offspring. This is accompanied by an increase in relative expression of CRH mRNA under basal and stressed conditions. Over time, repeated hyperresponsiveness to acute stress has the potential to produce permanent cardiovascular disease. Additionally, stress-related psychiatric disorders are more common in females (12) and the enhanced stress response observed in the present investigation in adult female offspring could also have implications related to mental health. The programmed effects do not depend on low birth weight and therefore may involve different mechanisms than many of the current models of fetal programming.
Acknowledgements

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References


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Figure Legends

Figure 1. Resting heart rate (HR) and mean arterial pressure (MAP) during seven days of day (D) and night (N) recording using radiotelemetry in adult offspring of mothers fed normal salt or high salt during pregnancy.

Figure 2. Change in HR (top panel) and MAP (bottom panel) during one hour of restraint in high salt (HS) and normal salt (NS) adult female offspring. The p values are for the diet factor (NS vs HS) in the two-way ANOVA for repeated measures.

Figure 3. Change in HR (top panel) and MAP (bottom panel) during one hour of restraint in high salt (HS) and normal salt (NS) adult male offspring. The p values are for the diet factor (NS vs HS) in the two-way ANOVA for repeated measures.

Figure 4. Effect of a high-salt challenge (HS) on HR and MAP in adult male and female offspring of mothers eating high salt or normal salt during pregnancy. The initial values represent 24 hours of HR and MAP while eating standard (ST) rat chow. The HS values represent the 7-day average HR and MAP while eating 8% NaCl. The final values represent 24 hours of HR and MAP on the 7th day after returning (R) to the standard rat chow. *p<0.001 compared to ST and HS, †p<0.001 compared to ST and R. The numbers in parentheses depict the group sizes.

Figure 5. Effect of maternal diets on relative CRH mRNA basal expression in PVN of basal and stressed adult female and male offspring. Panel A depicts representative images scanned from the autoradiographic film of rats under basal conditions. Panel B
depicts the effects in adult female offspring and panel C depicts the effects in adult male offspring.
Figure 1

Males

- Normal Salt, n=13
- High Salt, n=13

Females

- Normal Salt, n=14
- High Salt, n=16
Figure 2

**Females**

- **HR (bpm)**
  - Time (min): 0, 20, 40, 60, 80, 100, 120
  - HS Offspring, n=16
  - NS Offspring, n=14
  - P = 0.001

- **MAP (mm Hg)**
  - Time (min): 0, 20, 40, 60, 80, 100, 120
  - Restraint
  - P = 0.019

Figure 2
Figure 3

Males

ΔHR (bpm)

- 160
- 140
- 120
- 100
- 80
- 60
- 40
- 20
- 0

- 100
- 80
- 60
- 40
- 20
- 0

ΔMAP (mm Hg)

- 30
- 20
- 10
- 0
- -10

Time (min)

0 20 40 60 80 100 120

HS Offspring, n=13

NS Offspring, n=13

P = 0.982

P = 0.986

Restraint

Males

HS Offspring, n=13

NS Offspring, n=13

P = 0.982

Restraint

Figure 3
Figure 4

MAP (mm Hg)                HR (bpm)

100  110  120  130  325  350  375  400

Normal Salt Offspring  High Salt Offspring

Males  Females

Diet: ST HS R ST HS R

(11)  (13)  (16)  (14)

+  *

Figure 4
Figure 5

A

Relative O.D. x Area

0.6
0.8
1.0
1.2
1.4

NS Offspring

HS Offspring

n=13
n=14

Female Basal
Female Stressed

Female Basal
Female Stressed

B

Females

Relative O.D. x Area

NS vs HS  p = 0.002

n=13
n=14
n=9
n=9

Females

C

Males

Relative O.D. x Area

NS vs HS  p = 0.70

n=13
n=11
n=4
n=4

Males
Table 1. Plasma corticosterone levels (ng/ml) in NS and HS male and female offspring under basal and stressed conditions.

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Stressed</td>
</tr>
<tr>
<td>NS</td>
<td>44.1 ± 18.7 (5)</td>
<td>391.2 ± 46.1 (4)</td>
</tr>
<tr>
<td>HS</td>
<td>49.8 ± 10.5 (5)</td>
<td>317.8 ± 57.6 (4)</td>
</tr>
</tbody>
</table>

NS = normal salt offspring, HS = high salt offspring. The n of each group is indicated in the parentheses. For both male and female groups there was a significant main effect of stress (p < 0.001 for both), but no significant main effect of diet.