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Uteroplacental insufficiency temporally exacerbates salt-induced hypertension associated with a reduced natriuretic response in male rat offspring

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Key points summary

- Low weight at birth increases the risk of developing chronic diseases in adulthood
- A diet that is high in salt is known to elevate blood pressure, which is a major risk factor for cardiovascular and kidney diseases
- This paper demonstrates that growth restricted male rats have a heightened sensitivity to high dietary salt, in the context of raised systolic blood pressure, reduced urinary sodium excretion, and stiffer mesenteric resistance vessels
- Other salt-induced effects, such as kidney hyperfiltration, albuminuria and glomerular damage were not exacerbated by being born small
- This study demonstrates that male offspring born small have an increased cardiovascular susceptibility to high dietary salt, such that that minimizing salt intake is likely to be of particular benefit to this at-risk population

Abstract

Intrauterine growth restriction increases the risk of developing chronic diseases in adulthood. Lifestyle factors, such as poor dietary choices, may elevate this risk. We determined whether being born small increases the sensitivity to a dietary salt challenge, in the context of hypertension, kidney disease and arterial stiffness. Bilateral uterine vessel ligation or sham surgery (offspring termed Restricted and Control, respectively) was performed on 18-day pregnant WKY rats. Male offspring were allocated to receive a diet high in salt (8% sodium chloride) or remain on standard rat chow (0.52% sodium chloride) from 20-26 weeks of age for 6 weeks. Systolic blood pressure (tail-cuff), renal function (24 h urine excretions) and vascular stiffness (pressure myography) were assessed. Restricted males were born 15% lighter than Controls and remained smaller throughout the study. Salt-induced hypertension was exacerbated in Restricted offspring, reaching a peak systolic pressure of ~175 mmHg earlier than normal weight counterparts. The natriuretic response to high dietary salt in Restricted animals was less than in Controls and may explain the early rise in arterial pressure. Growth restricted males allocated to high salt diet also had increased passive arterial stiffness of mesenteric resistance arteries. Other aspects of renal function, including salt-induced hyperfiltration, albuminuria and glomerular damage were not exacerbated by uteroplacental insufficiency. This study demonstrates that male offspring exposed to uteroplacental insufficiency and born small have an increased sensitivity to salt-induced hypertension and arterial remodeling.

Introduction

Hypertension affects 30% of the adult population, and is a major risk factor for cardiovascular and kidney diseases (Mills *et al.*, 2016). Studies in both humans and laboratory animals have provided clear evidence that high dietary salt contributes to elevated arterial pressure and that a reduction in

salt intake is associated with low average pressures (He & MacGregor, 2003; Appel *et al.*, 2006; Elliott *et al.*, 2007). Blood pressure responses to dietary salt, however, are heterogeneous with some individuals having greater sensitivity than others. In individuals with hypertension, 30-50% are thought to have salt-sensitive blood pressure, which is associated with a range of risk factors including African American ethnicity, older age, obesity and diabetes (Weinberger *et al.*, 1986; Kotchen *et al.*, 2013).

Many animal studies have reported a genetic susceptibility to salt sensitivity, particularly in the context of raised blood pressure. The Dahl rat is the most studied model in the field, whereby salt-induced hypertension can be attenuated, and albuminuria prevented, upon the substitution of specific genetic material from a normotensive rat (Mattson *et al.*, 2008). Furthermore, transgenic mice with various gene deletions, including atrial natriuretic peptide and its receptor, have increased salt-sensitive blood pressure (John *et al.*, 1995). Increased sensitivity to dietary salt, however, may occur irrespective of genetic background. In the Dahl rat, the impact of maternal diet on salt-sensitive hypertension and kidney injury was recently assessed (Geurts *et al.*, 2015). Rats that had been embryo-transferred from casein-fed donors to grain-fed surrogates had attenuated salt-induced hypertension and kidney damage. The converse was true for rats conceived by grain-fed parents and transferred to a casein-fed surrogate. These findings implicate the gestational environment in determining salt sensitivity in adulthood.

Low weight at birth is a surrogate of developmental perturbations and, in such cases, organ structure and function are compromised, including that of the heart, blood vessels, kidneys, and pancreas and the risk of developing chronic diseases increases (Barker *et al.*, 1989; Barker *et al.*, 1990; White *et al.*, 2009; Clough, 2015). Uteroplacental insufficiency is the most common cause of fetal growth restriction in Western nations and affects about one in ten pregnancies (Henriksen &

Clausen, 2002). The delivery of nutrients and oxygen to the fetus is compromised because of poor placental function. We, and others, use an established rat model to mimic uteroplacental insufficiency, whereby the uterine vessels are bilaterally ligated during late gestation (Simmons *et al.*, 2001; Schreuder *et al.*, 2007; Wlodek *et al.*, 2007). This surgical procedure induces a 10-15% reduction in offspring birth weight along with life-long deficits in nephron and cardiomyocyte endowment, glomerular hypertrophy, and mesenteric and femoral arterial dysfunction in males (Wlodek *et al.*, 2008; Black *et al.*, 2012; Tare *et al.*, 2012). These can (Wlodek *et al.*, 2008), although not always (Wadley *et al.*, 2016), result in arterial hypertension at six month of age. In general, growth restricted males present with more prominent physiological changes but similar structural deficits compared with female counterparts.

We investigated the effects of a short-term dietary salt challenge on blood pressure, arterial stiffness, and kidney function in adult male rats that had been exposed to uteroplacental insufficiency during gestation. We hypothesized that low birth weight rats would have an increased sensitivity to high dietary salt, such that arterial blood pressure, vascular stiffness, and renal function would be more adversely affected compared to male rats born of a normal weight.

Methods

Animal procedures

All experiments were approved by The University of Melbourne Animal Ethics Committee prior to commencement. The experiments were carried out in accordance with the Australian Code for the care and use of animals for scientific purposes set out by the National Health and Medical Research Council (2013), and conform to the principles and regulations as described in the Editorial by Grundy (2015). Wistar Kyoto (WKY) rats, housed in an environmentally controlled room, had access to food

and tap water *ad libitum*. Rats were mated and uteroplacental insufficiency (UPI; offspring termed Restricted) or sham (offspring termed Control) surgery performed at embryonic day (E) 18, as previously described, under anesthesia (intraperitoneal injection of a mixed solution containing ketamine (50 mg/kg body weight) and ilium xylazil-20 (10 mg/kg body weight)) (*n*=8-11 per group) (Wlodek *et al.*, 2007). Rats delivered at term (E22). At postnatal day 35 (P35), offspring were weaned and provided with *ad libitum* access to standard rat chow and water. At 20 weeks of age, male offspring were allocated to one of two diets (Specialty Feeds, Glenn Forrest, WA, Australia): High salt (SF01-004, 8% sodium chloride) or Normal salt (SF01-002, 0.52% sodium chloride) with access to water *ad libitum* (1 male offspring per litter per diet, *n*=12 per group). Treatment duration was for 6 weeks and rats were euthanized at 26 weeks of age. Male offspring were used as we have previously reported their increased susceptibility to the developmental programming of high blood pressure (Wlodek *et al.*, 2008).

Blood pressure and blood glucose monitoring

Blood pressure was measured by non-invasive tail-cuff plethysmography (ADInstruments Pty. Ltd., Castle Hill, NSW, Australia) in rats that were acclimatized to the restraint procedure at 8, 12, 17, 23 and 26 weeks of age (Wlodek *et al.*, 2007; Moritz *et al.*, 2009; Gallo *et al.*, 2012a; Gallo *et al.*, 2012b). The final 5 of 10 acquired traces were recorded and averaged to determine systolic blood pressure. Blood glucose (non-fasted; tail vein sample) was monitored at 8, 12, 17, 23 and 25 weeks of age using a glucometer (Accu-Chek Performa, Roche, Mannheim, Germany) during morning day light hours.

Food and water intake, urinary excretions, and biochemistry

Rats were weighed and placed individually into metabolic cages for 24 h measurements of food and water intake and urine production at 8 weeks (pre-treatment) and 26 weeks (after 6 weeks of normal or high salt diet) of age (Moritz *et al.*, 2009; Gallo *et al.*, 2012a; Gallo *et al.*, 2012b). Rats were acclimatized to metabolic cages by placing them in for short daylight periods of 3 h and 8 h on two separate occasions to minimize stress and associated behavioral changes. Measurements of urinary albumin, total protein, sodium, potassium, and creatinine were made using commercially available kits according to the manufacturers' instructions (Cobas Integra 400, Roche Diagnostics, Burgess Hill, UK). Plasma samples collected immediately upon removal from metabolic cages, which was at *post-mortem*, were analyzed for sodium, potassium, chloride, and creatinine. Creatinine clearance ($ml.min^{-1}$) was calculated using: (urinary creatinine [$\mu mol.l^{-1}$] × 24 h urine production [ml]) / (plasma creatinine [$\mu mol.l^{-1}$] × 1440 [min]); predicted sodium filtered ($mmol.24\ h^{-1}$) was calculated using: plasma sodium [$mmol.l^{-1}$] × creatinine clearance [$l.24\ h^{-1}$]; and fractional sodium excretion (%) was calculated using: (urinary sodium [$mmol.l^{-1}$] × plasma creatinine [$mmol.l^{-1}$]) / (plasma sodium [$mmol.l^{-1}$] × urinary creatinine [$mmol.l^{-1}$]) × 100.

Post mortem tissue collection

At post mortem, at 26 weeks of age, rats were anesthetized with an intraperitoneal injection of a mixed solution containing ketamine (100 mg/kg body weight) and ilium xylazil-20 (30 mg/kg body weight). Heart, kidneys, liver, pancreas, and dorsal fat were excised and weighed. Kidneys were immersion fixed in 10% neutral buffered formalin and processed to paraffin for histological analyses. Small renal and mesenteric arteries were isolated and used for functional studies.

Arterial stiffness

Passive mechanical wall properties were assessed in mesenteric (350-380 µm) and renal (440-460 µm) resistance arteries ~3-4 mm long mounted on a pressure myograph (Living Systems Instrumentation), as described previously (Mazzuca *et al.*, 2010; Mazzuca *et al.*, 2012). Briefly, arteries were continuously superfused at ~15 ml.min^{-1} with Ca^{2+}-free, 1 mM EGTA physiological saline solution (PSS) containing (in mM): NaCl 120, KCl 5, $NaHCO_3$ 25, glucose 11, $MgSO_4$ 1.2, KH_2PO_4 1, gassed with 95% O_2 and 5% CO_2 at ~36°C. Each artery was pressurized from 0 to 110 mmHg. Arterial dimensions (length, outer diameter (OD), internal diameter (ID), and wall thickness (WT)) were measured at each 10 mmHg increment in intraluminal pressure. Wall stress and strain were derived as follows: wall stress (kPa) = (intraluminal pressure × ID) / (2 × WT); wall strain = (ID – ID extrapolated to 5 mmHg pressure) / (ID extrapolated to 5 mmHg pressure) (Wigg *et al.*, 2001; Mazzuca *et al.*, 2010). The media-to-lumen (M-L) ratio was calculated as WT / ID.

Kidney morphometry

Representative 5 µm transverse, midline sections from the kidney were collected. In sections stained with Masson's trichrome, perivascular fibrosis and interstitial fibrosis were assessed (Walton *et al.*, 2017). For perivascular fibrosis, 9 arterioles per animal were randomly selected from 3 separate, non-sequential slides, and the area of adventitial collagen was normalized to vessel lumen area and averaged for each animal. Interstitial fibrosis was quantified in 5 random cortical/outer medullary fields per animal using a point-counting technique. In each field, 121 points were counted with 11 equidistant grid lines. Points falling on interstitial fibrosis were expressed as a percentage of the total number of grid points, and averaged for each animal. In sections stained with periodic acid-

Schiff (PAS), 20 glomeruli were randomly selected to evaluate glomerulosclerosis using a semi-quantitative method (Gallo *et al.*, 2016).

Statistical analyses

Results were analyzed with GraphPad Prism (Versions 6 and above; GraphPad Software, La Jolla, USA). Data were tested for normality and a 2-way ANOVA was performed to determine main effects of diet (Normal salt 0.52% and High salt 8%) and uteroplacental insufficiency (Control and Restricted). When significant interactions were observed, individual group means were compared using the Fisher's LSD test. Stress-strain relationships were analyzed using repeated measures 2-way ANOVA followed with Tukey's *post-hoc* multiple comparison testing. $P<0.05$ was accepted as statistically significant and *n* represents the number of animals per group from different litters.

Results

Uteroplacental insufficiency did not impact salt-induced alterations in body and organ weights

Uteroplacental insufficiency reduced total litter size (Restricted: 6 ± 1 *vs* Control: 10 ± 1, $P < 0.05$) and litter average male body weight (-15%, Restricted: 3.62 ± 0.12 *vs* Control: 4.24 ± 0.09, $P < 0.05$) compared with Controls at P1. Restricted males remained ~12 % lighter than Controls throughout the study (Table 1). A diet high in salt (8% sodium chloride) reduced body weight during weeks 3, 5 and 6 of treatment, such that total weight gained over the 6-week treatment period was only 13-15 g in high salt fed rats *vs* 34-41 g in normal salt (0.52% sodium chloride) fed rats (Table 1). This reduction in weight gain was not exacerbated by uteroplacental insufficiency (Table 1).

At 8 weeks of age, Restricted offspring consumed less food but this did not persist at 26 weeks of age (Table 1). High dietary salt did not affect the amount of food or energy consumed over

a single 24 h period (Table 1) but, over the 6 weeks, total energy intake was less in rats allocated to high *vs* normal salt ($P < 0.01$; High salt Control: 12.17 ± 0.23, High salt Restricted: 12.13 ± 0.67 *vs* Normal salt Control: 14.39 ± 0.74, Normal salt Restricted: 14.40 ± 0.77 MJ/6 weeks). The amount of sodium chloride consumed per day was ~15-fold greater in rats allocated to High salt *vs* Normal salt ($P < 0.0001$) and not different between Control and Restricted rats (Table 1). The same fold difference was observed for sodium consumption independent of chloride ($P < 0.0001$; High salt Control: 0.596 ± 0.030, High salt Restricted: 0.538 ± 0.036 *vs* Normal salt Control: 0.035 ± 0.003, Normal salt Restricted: 0.038 ± 0.004 g/24 h).

A high salt diet increased relative heart and kidney weights, but liver and dorsal fat weights were lower at post-mortem (Table 1). These changes were not exacerbated by uteroplacental insufficiency (Table 1). Uteroplacental insufficiency, however, independently reduced heart, kidney, liver, and dorsal fat weights compared with Control (Table 1). Left ventricular and pancreas weights were not affected by salt intervention or uteroplacental insufficiency (Table 1). The reported organ weights were corrected for leg length which was ~2 mm shorter in Restricted males (Table 1).

Uteroplacental insufficiency hastened the development of salt-induced hypertension

Systolic blood pressure was not different between groups at diet baseline, that is, at 20 weeks of age (Fig. 1*A* and *B*). When compared to baseline, rats allocated to high dietary salt developed elevated blood pressure at 3 weeks into the diet, which was exacerbated in Restricted offspring (Restricted: +40 mmHg and Control: +27 mmHg *vs* baseline, Fig. 1*B*). Between 3 and 6 weeks of high salt feeding, systolic blood pressure continued to rise in Control rats, but plateaued in Restricted (Fig. 1*B*). When compared with normal salt, 3 weeks of a high salt diet increased systolic blood pressure by 27 mmHg in Restricted and 12 mmHg in Control (Fig. 1*C*). Following 6 weeks of high salt feeding (*i.e.* study

end), blood pressure was elevated by a similar degree regardless of birth weight (High Salt Control: 176 ± 4 mmHg, High Salt Restricted: 171 ± 4 mmHg *vs* Normal Salt Control: 144 ± 4 mmHg, Normal Salt Restricted: 148 ± 3 mmHg, Fig. 1*D*). Over the 6-week treatment period (*i.e.* 20-26 weeks of age), all rats that remained on normal chow (both Control and Restricted) did not display changes in blood pressure, highlighting no effect of ageing (Fig. 1*A*).

Uteroplacental insufficiency reduced the natriuretic response to high dietary salt

The dietary salt challenge from 20-26 weeks of age induced a 4-4.5-fold increase in urine output, which was partially balanced by increased water consumption (+2.9-fold in Control and +2.1-fold in Restricted, Fig. 2*A-B*). A positive correlation between urinary output and water intake was observed for Control (R^2=0.529), but not Restricted (R^2=0.028), offspring on the high salt diet (Fig. 2*A*). The 24 h thirst response to high dietary salt (*vs* normal salt) was significantly less in Restricted rats, *i.e.* ~35 ml of extra water *vs* ~58 ml in Control (Fig. 2*A*). Over the 6-week treatment period, however, there was no statistical difference in the amount of water consumed between Control and Restricted rats and > 2.5 L of extra water was consumed in rats allocated to high *vs* normal salt diet ($P < 0.05$, L/6 weeks; High salt Control: 4.14 ± 0.12, High salt Restricted: 3.96 ± 0.17 *vs* Normal Control: 1.43 ± 0.07, Normal salt Restricted: 1.55 ± 0.09).

As expected, high dietary salt for 6 weeks increased the filtered load and urinary excretion (total and fractional) of sodium, regardless of birth weight (Fig. 2*C-E*). However, in Restricted offspring, total and fractional sodium excretion increased by a lesser magnitude than in Controls (Fig. 2*D-E*), suggesting enhanced sodium reabsorption. Urinary excretions of potassium and sodium to potassium ratio were increased in response to high salt feeding, and there were no differences between Control and Restricted (Fig. 2*F-G*). Plasma concentrations of sodium, potassium, and

chloride were not different between Control and Restricted, nor were they affected by high salt consumption (Table 2). Non-fasted blood glucose levels were also not different between groups throughout the study (Table 2).

Uteroplacental insufficiency modestly attenuated high salt-induced albuminuria

Creatinine clearance, a surrogate of glomerular filtration rate, was increased by 20-38% following 6 weeks of high dietary salt but there was no effect of uteroplacental insufficiency (Fig. 3*A*). High salt also induced albuminuria and proteinuria in all rats, albeit the degree of albuminuria was ~30% less in Restricted *vs* Control (Fig. 3*B-D*).

High dietary salt and uteroplacental insufficiency induced mild glomerular and tubulointerstitial fibrosis, respectively

Rats allocated to the high salt diet had increased glomerulosclerosis by study end (*i.e.* 26 weeks of age), regardless of birth weight (Fig. 3*E*). Tubulointerstitial and perivascular fibrosis, however, were not affected by the salt challenge (Fig. 3*F-G*). Restricted rats had increased tubulointerstitial, but not perivascular, fibrosis compared with Controls (Fig. 3*F-G*). Representative sections of kidneys stained with Masson's trichrome and PAS are shown in Fig. 3.

High dietary salt increased mesenteric arterial wall stiffness in Restricted, but not Control, offspring

For Restricted *vs* Control rats on the normal salt diet, there was no significant difference in the passive stress-strain relationship (indicative of arterial stiffness) in mesenteric arteries at study end (*i.e.* 26 weeks of age, Fig. 4*A*). The mesenteric artery media-to-lumen ratio was also not different between Control and Restricted groups (Fig. 4*B*, Table 3).

High salt diet was without effect on the stress-strain relationship in mesenteric arteries for Control rats (Fig. 4*A*). However, in Restricted rats, the stress-strain curve was shifted to the left, reflecting significantly increased arterial wall stiffness in response to high dietary salt (Fig. 4*A*). The media-to-lumen ratio of mesenteric arteries from both Control and Restricted rats, however, was significantly increased after 6 weeks of high dietary salt (Fig. 4*B*). This salt-induced change in media-to-lumen ratio in mesenteric arteries of Control and Restricted rats was underpinned by a significant increase in wall thickness and a tendency (P=0.073) for reduced internal diameter (Table 3).

For renal arteries, there was no effect of uteroplacental insufficiency or high salt diet on the passive mechanical wall properties or arterial stiffness (Table 3, Fig. 4*C-D*).

Discussion

Cardiovascular disease is the major cause of death globally and hypertension is considered a leading preventable risk factor. High dietary salt plays a prominent role in the development of hypertension, particularly in a subset of individuals (Weinberger *et al.*, 1986; Kotchen *et al.*, 2013). Being born small increases one's risk of a range of chronic diseases in adulthood and, in general, males are considered more at risk than female counterparts. In the current study, we investigated the impact of high dietary salt on adult male rats born small due to uteroplacental insufficiency. Salt-induced hypertension developed earlier in Restricted offspring than in normal weight counterparts and the natriuretic response to high dietary salt was significantly less, which may explain their early rise in arterial pressure. Restricted rats were also more salt-sensitive in terms of passive arterial wall properties, exhibiting increased arterial stiffness in mesenteric resistance vessels. Other aspects of renal function, however, including salt-induced hyperfiltration, albuminuria, and glomerular damage

were not exacerbated by uteroplacental insufficiency. These findings suggest that males born small due to poor development *in utero* are more susceptible to lifestyle challenges that lead to adverse cardio-renal health outcomes. While female offspring were not examined in the current study, future work should ascertain whether a dietary salt challenge unmasks physiological deficits or, indeed, what renders them protected from the long-term effects of uteroplacental insufficiency.

Our growth restricted males exhibited a greater increase in blood pressure at 3 weeks into the salt challenge suggesting they were more salt sensitive than normal weight controls. By 6 weeks, Controls had achieved the same blood pressure level. A chronic salt challenge, even in healthy individuals, will ultimately increase blood pressure (Ha, 2014). The earlier rise in systolic pressure in growth restricted offspring, however, prolongs exposure time to the negative effects of arterial hypertension which promotes end-organ damage and cardiovascular disease.

Several mechanisms have been proposed to explain the effects of high dietary salt on blood pressure. Ultimately, a "natriuretic handicap", that is, a limited capacity of the kidneys to excrete sodium, is the common denominator (Kotchen *et al.*, 2013). Indeed, our Restricted offspring displayed an earlier rise in arterial pressure compared with Controls, and this was associated with a ~25-30% reduction in total and fractional sodium excretion. Impaired natriuresis may occur due to an inherent problem with the kidneys and/or factors that increase tubular reabsorption of sodium. Previously, we have reported that Restricted males have a reduction in the number of nephrons (Wlodek *et al.*, 2008) and this is thought to exacerbate salt-sensitivity in the context of raised blood pressure (Kotchen *et al.*, 2013). In rat offspring with reduced nephron endowment due to maternal low protein diet during pregnancy, blood pressure was elevated, and this was exacerbated by high dietary salt (Woods *et al.*, 2004). This was associated with reduced glomerular filtration rate (GFR), although this was only modest compared to the reduction in nephron number. In the current study,

high salt-induced creatinine clearance and urine production increased equally in Restricted and Control rats, suggesting that whole-kidney GFR was sufficient despite a known nephron loss (Wlodek *et al.*, 2008).

Hyperfiltration of existing nephrons is thought to contribute to progressive kidney disease (Brenner, 1983). Restricted rats in the present study had increased tubulointerstitial fibrosis which, when superimposed with salt-induced glomerulosclerosis and increased kidney mass, may have compromised tubular-glomerular signaling and sodium excretion. In diabetes, proximal tubular growth is thought to contribute, in part, to increased sodium reabsorption and subsequent increases in single nephron GFR (Vallon, 2011). Indeed, in rat offspring prenatally exposed to dexamethasone, proximal tubule NHE3 activity and volume absorption were increased and considered to contribute to prenatal programming of hypertension (Dagan *et al.*, 2007). Increased expression of renal sodium channels has also been demonstrated in sheep offspring that have a low nephron endowment and develop high blood pressure after exposure to prenatal glucocorticoids (Moritz *et al.*, 2011). In our model, a detailed analysis of renal tubule segment lengths and proximal tubule transport activity is required to ascertain the mechanisms underlying impaired salt-induced natriuresis. It is worth noting that, whilst high dietary salt increased albuminuria, this was surprisingly less in Restricted *vs* Control rats. The relevance of this finding requires further study but may have occurred as a consequence of the mild, non-significant reduction in 24 h urinary output (Restricted: 66 ml *vs* Control: 73 ml).

Excessive salt intake stimulates several endocrine, neural, and paracrine mechanisms that target the kidneys and blood vessels to help achieve a net sodium balance. In response to high salt intake, endogenous cardiotonic steroids that bind and inhibit the Na^+/K^+-ATPase, such as marinobufagenin, are secreted by the brain and adrenal glands (Bagrov *et al.*, 2009). In normotensive women, 6 days of high salt intake increased marinobufagenin excretion that directly

correlated with total- and fractional- sodium excretion and inversely correlated with systolic blood pressure (Anderson *et al.*, 2008). A lack of marinobufagenin-induced inhibition of Na^+/K^+-ATPase in the kidneys and/or blood vessels may therefore render one being salt sensitive (Kotchen *et al.*, 2013). Increased activity of the sympathetic nervous system may also enhance sensitivity to dietary salt. Indeed, fetal activation of the sympathetic nervous system is thought to play a role in the developmental programming of sensitivity to cardiovascular stressors (Harris & Seckl, 2011) and we recently reported enhanced respiratory-related thoracic sympathetic nerve activity in our growth restricted male offspring (Menuet *et al.*, 2016). Experimental studies assessing the development of salt sensitive hypertension also implicate the renin angiotensin system (RAS), formation of reactive oxygen species, and reduced production of nitric oxide, which are all similarly implicated in the developmental programming of cardiovascular disease (Alexander *et al.*, 2015). Previous programming studies have reported an upregulation of renal RAS in association with hypertension (Vehaskari *et al.*, 2004; Grigore *et al.*, 2007; Singh *et al.*, 2007; Walton *et al.*, 2017), although this has not been observed in our model (Wlodek *et al.*, 2008) and therefore unlikely to contribute to enhanced salt-sensitivity seen in this study.

The impact of the gestational environment *vs* genetic polymorphisms on adulthood blood pressure has been demonstrated previously through embryo transfer approaches in the spontaneously hypertensive rat (SHR) and Dahl salt sensitive rat. Salt sensitive rat embryos transferred to salt resistant dams had blood pressures that matched salt resistant rats (Kubisch *et al.*, 1998) and SHR rats exposed to a WKY uterine environment had a remarkable reduction in blood pressure (Lee & Azar, 2010). In these studies, susceptibility to postnatal dietary salt, however, was either not altered (Kubisch *et al.*, 1998) or not assessed (Lee & Azar, 2010). In the SHR, we have previously shown that the maternal environment does not, in fact, mediate salt preference (Di

Nicolantonio *et al.*, 2005) or blood pressure in adulthood (Di Nicolantonio *et al.*, 2006) and may instead be genetically determined. While embryo transfer, in theory, allows for the separation of genetic and environmental influences, it should be noted that this technique is fraught with inherent confounders that are likely to interact with the different experimental groups (Tran *et al.*, 2014). This limits our interpretation from such studies. Nevertheless, by using a single rat strain and manipulating the gestational environment in a randomized manner, we and others mentioned above have demonstrated that suboptimal exposures during early development can impact blood pressure responses, independently of genetic inheritance.

The vasculature is sensitive to perturbations in the early life environment and adaptations made in this system can predispose to later cardiovascular disease (Clough, 2015). In the present study, passive stiffness of mesenteric and renal arteries was not different between Control and Restricted male offspring. This finding contrasts with earlier studies in male growth restricted offspring by our group (Tare *et al.*, 2012) and most probably reflects differences in early life exposure to stress; that is, in our earlier study, male offspring were cross-fostered to a different dam at birth. However, mesenteric resistance vessels from Restricted males exhibited increased stiffness following a high salt diet. Excess sodium intake leads to progressive deterioration of microvascular and renal function (Weinberger & Fineberg, 1991; Sacks *et al.*, 2001; Yu *et al.*, 2004), including increased vessel stiffness and media thickening, which is independent of age and blood pressure, and mediated, in part, through alterations in the RAS and bradykinin system (Safar *et al.*, 2000). The wall thickness of mesenteric arteries of both Control and Restricted offspring was increased with the high salt diet, likely reflecting alterations in the deposition of extracellular matrix and proliferation and hypertrophy of smooth muscle cells, resulting in inward remodeling (Safar *et al.*, 2000). Despite increased wall thickness in arteries from both Control and Restricted offspring, only the arteries

from Restricted males exhibited increased stiffness following a high salt diet. These findings are similar to a previous study in the mouse showing that high salt-induced mesenteric stiffness was exacerbated by prenatal hypoxia (Walton *et al.*, 2016). Whether this reflects altered deposition or arrangement of extracellular matrix (Jalil *et al.*, 1989; Arribas *et al.*, 2008) requires further investigation. Interestingly, renal arteries from both Control and Restricted offspring were resistant to salt-induced changes observed in the mesenteric arteries, likely reflecting differences in local regulatory mechanisms between the arteries.

Rats allocated to High salt consumed ~15-fold more sodium chloride (or sodium alone) than Normal salt rats. This is greater than extreme differences in sodium intake reported in humans (~five-fold), with most adult populations falling within a two-fold range (Brown *et al.*, 2009). In this study, an 8% high salt diet was used to determine whether hypertension was salt-sensitive. Future studies should determine whether high dietary salt that is more consistent with the range reported in humans yield similar effects.

Finally, we have demonstrated that multiple organ systems controlling blood pressure, namely, the kidneys and microvasculature, are differentially affected by uteroplacental insufficiency, a postnatal high salt diet or a combination of the two. Dietary sodium intake for 6 weeks led to hypertension and signs of glomerular injury in all offspring, irrespective of growth restriction. Growth restricted males, however, exhibited mild renal fibrosis and, when challenged with a high salt diet, developed an earlier peak in arterial pressure, a reduced natriuretic response, and stiffening of the mesenteric microvasculature. These findings are pertinent given that most adults consume, on average, five to ten times the daily amount of salt that is physiologically required. The identification of individuals at cardiovascular risk following even short-term salt loading may warrant targeted dietary recommendations based on birth weight. Furthermore, the long-term effects of

these salt-induced changes and the effects of chronic high salt intake from childhood are worthy of follow-up.

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Additional information

Competing interests

The authors declare no competing interests.

Author contributions

Animal experiments were performed in the Department of Physiology, The University of Melbourne and histological experiments were performed in the School of Biomedical Sciences, The University of Queensland. All authors approved the final version of the manuscript, agree to be accountable for all aspects of the work, and qualify for authorship. KMM, MEW and HCP conceived the experiments and obtained funding. LAG, SLW, MQM and MT conducted the experimental work. LAG drafted the manuscript and all authors revised it critically for important intellectual content.

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Table 1 Body weight, food and energy intake, and organ weights.

	Normal salt (0.52%)		*High salt (8%)*		*Two-way ANOVA*	
	Control	Restricted	Control	Restricted	Diet	Restricted
Body weight (g)						
Before high salt diet - age						
P6	9.1 ± 0.3	7.5 ± 0.5	8.5 ± 0.4	7.1 ± 0.3	NS	$P < 0.05$
P14	22.3 ± 0.5	18.9 ± 1.1	20.9 ± 0.7	18.1 ± 1.6	NS	$P < 0.05$
P35	82 ± 2	72 ± 3	80 ± 2	72 ± 4	NS	$P < 0.05$
8 weeks	162 ± 4	135 ± 8	165 ± 5	133 ± 7	NS	$P < 0.05$
12 weeks	293 ± 6	261 ± 9	288 ± 4	251 ± 9	NS	$P < 0.05$
16 weeks	358 ± 6	323 ± 9	352 ± 4	305 ± 12	NS	$P < 0.05$
20 weeks	365 ± 7	336 ± 10	365 ± 5	314 ± 13	NS	$P < 0.05$
During high salt diet - age						
23 weeks	388 ± 5	348 ± 9	365 ± 6	318 ± 14	$P <$	$P < 0.05$
25 weeks	399 ± 5	368 ± 10	377 ± 6	325 ± 13	0.05	$P < 0.05$
26 weeks (*i.e.* study end)	405 ± 7	370 ± 12	380 ± 6	327 ± 13	$P <$	$P < 0.05$
Total weight gained	40.6 ± 2.8	34.0 ± 4.4	15.1 ± 2.5	13.1 ± 2.8	0.05	NS
					$P <$	
					0.05	
					$P <$	
					0.05	
Food intake ($g.24h^{-1}$)						
Before high salt diet (*i.e.* 8 weeks of age)	19.9 ± 0.9	16.4 ± 0.9	19.4 ± 1.0	17.2 ± 1.6	NS	$P < 0.05$
At six weeks of high salt diet (*i.e.* 26 weeks of age)	17.6 ± 1.3	19.1 ± 1.9	19.2 ± 1.0	17.4 ± 1.2	NS	NS
Energy intake ($kJ.24h^{-1}$)						
At six weeks of high salt diet (*i.e.* 26 weeks of age)	246 ± 18	268 ± 26	248 ± 12	224 ± 15	NS	NS

Salt intake (g.24h^{-1}) At six weeks of high salt diet (*i.e.* 26 weeks of age)	0.092 ± 0.007	0.100 ± 0.010	1.537 ± 0.077	1.388 ± 0.092	*P* < 0.05	NS
Organ weight (mg.mm leg length^{-1})						*P* < 0.05
At six weeks of high salt diet (*i.e.* 26 weeks of age)	26.2 ± 0.4	24.2 ± 0.6	28.1 ± 0.7	25.7 ± 1.0	*P* <	NS
	18.8 ± 0.6	18.4 ± 0.4	20.3 ± 0.9	19.3 ± 0.9	0.05	
Heart					NS	
Left ventricle						
Left kidney	25.4 ± 1.0	23.2 ± 0.6	28.5 ± 0.7	25.2 ± 1.2	*P* < 0.05	*P* < 0.05
Liver	222 ± 6	204 ± 5	207 ± 5	192 ± 7	*P* < 0.05	*P* < 0.05
Pancreas	12.5 ± 0.9	13.2 ± 0.9	13.9 ± 1.3	12.7 ± 1.3	NS	NS
Dorsal fat	87.3 ±	64.0 ± 6.2	57.6 ± 4.3	40.3 ± 6.9	*P* <	*P* < 0.05
Leg length (mm)	11.2	51.4 ± 0.5	53.0 ± 0.5	50.6 ± 0.7	0.05	*P* < 0.05
	53.2 ± 0.4				NS	

Control and Restricted rats were randomized to Normal salt or High salt diet for 6 weeks at 20-26 weeks of age. No significant interactions between diet and uteroplacental insufficiency (UPI) surgery by two-way ANOVA. *N*=7-11/group. NS, not significant.

Table 2 Blood and plasma biochemistry.

	Normal salt (0.52%)		*High salt (8%)*		*Two-way ANOVA*	
	Control	Restricted	Control	Restricted	Diet	Restricted
Non-fasted blood glucose (mmol.l^{-1})						
Before high salt diet - age						
8 weeks	9.2 ± 0.5	9.3 ± 0.3	8.7 ± 0.3	9.0 ± 0.3	NS	NS
12 weeks	9.8 ± 0.5	10.1 ± 0.6	9.6 ± 0.3	9.9 ± 0.6	NS	NS
16 weeks	9.7 ± 0.3	10.3 ± 0.5	9.8 ± 0.4	8.8 ± 0.4	NS	NS
During high salt diet - age						
23 weeks	11.1 ± 0.8	10.4 ± 0.3	9.7 ± 0.4	9.8 ± 0.4	NS	NS
25 weeks	10.6 ± 0.8	11.1 ± 0.3	10.6 ± 0.8	10.2 ± 0.6	NS	NS
Plasma electrolytes (mmol.l^{-1})						
At six weeks of high salt diet (*i.e.* 26 weeks of age)	141.6 ± 3.9	138.2 ± 1.5	143.6 ± 4.3	140.8 ± 1.4	NS	NS
Na^+	6.2 ± 0.3	5.9 ± 0.2	5.7 ± 0.3	5.7 ± 0.2	NS	NS
K^+	114.7 ± 2.9	112.1 ± 0.7	115.7 ± 2.8	113.9 ± 1.2	NS	NS
Cl^-						

Control and Restricted rats were randomized to Normal salt or High salt diet for 6 weeks at 20-26 weeks of age. No significant interactions between diet and uteroplacental insufficiency (UPI) surgery by two-way ANOVA. *N*=6-11/group. NS, not significant.

Table 3 Passive wall properties in arteries pressurized to 100 mmHg at six weeks of high salt diet.

	Normal salt (0.52%)		*High salt (8%)*		*Two-way ANOVA*	
	Control	Restricted	Control	Restricted	Diet	Restricted
Mesenteric artery						
OD (μm)	462 ± 18	438 ± 14	457 ± 10	427 ± 15	NS	*P* = 0.0699
ID (μm)	422 ± 17	389 ± 14	395 ± 11	362 ± 15	*P* =	*P* < 0.05
WT (μm)	19.9 ± 1.5	24.5 ± 2.4	30.9 ± 2.1	32.7 ± 1.8	0.0730	NS
M-L ratio	0.048 ±	0.065 ±	0.080 ±	0.092 ±	*P* <	*P* < 0.05
	0.004	0.008	0.007	0.007	0.0001	
Renal artery					*P* <	
OD (μm)	575 ± 27	540 ± 17	552 ± 21	573 ± 14	0.0001	NS
ID (μm)	496 ± 27	459 ± 17	466 ± 18	492 ± 14		NS
WT (μm)	39.0 ± 1.9	40.5 ± 2.2	42.7 ± 3.0	40.3 ± 1.6		NS
M-L ratio	0.081 ±	0.089 ±	0.092 ±	0.083 ±	NS	NS
	0.006	0.005	0.007	0.004	NS	
					NS	

Control and Restricted rats were randomized to Normal salt or High salt diet for 6 weeks at 20-26 weeks of age. No significant interactions between diet and uteroplacental insufficiency (UPI) surgery by two-way ANOVA. *N*=10-11/group. NS, not significant; OD, outside diameter; ID, internal diameter; WT, wall thickness; M-L, media-to-lumen.

Figures and legends

Figure 1. Systolic blood pressure during six weeks of high salt diet.

Systolic blood pressure in *A*, Normal salt diet (0.52%) and *B*, High salt diet (8%) groups at baseline (20 weeks of age), 3 weeks (23 weeks of age) and 6 weeks (26 weeks of age) of diet intervention and *C*, all groups at 3 weeks (23 weeks of age) and *D*, 6 weeks (26 weeks of age) of diet intervention. Data

are (*A-B*) mean ± SEM; *n*=7-11/group or (*C-D*) individual values with means ± SEM. **P*<0.05, ***P*<0.01 Restricted *vs* Control within diet group by Fisher's LSD test. Figs *A*, *B*: Solid lines indicate *P*<0.05 between connecting time-points and dotted lines indicate no significance between connecting time-points. UPI, uteroplacental insufficiency; NS, not significant.

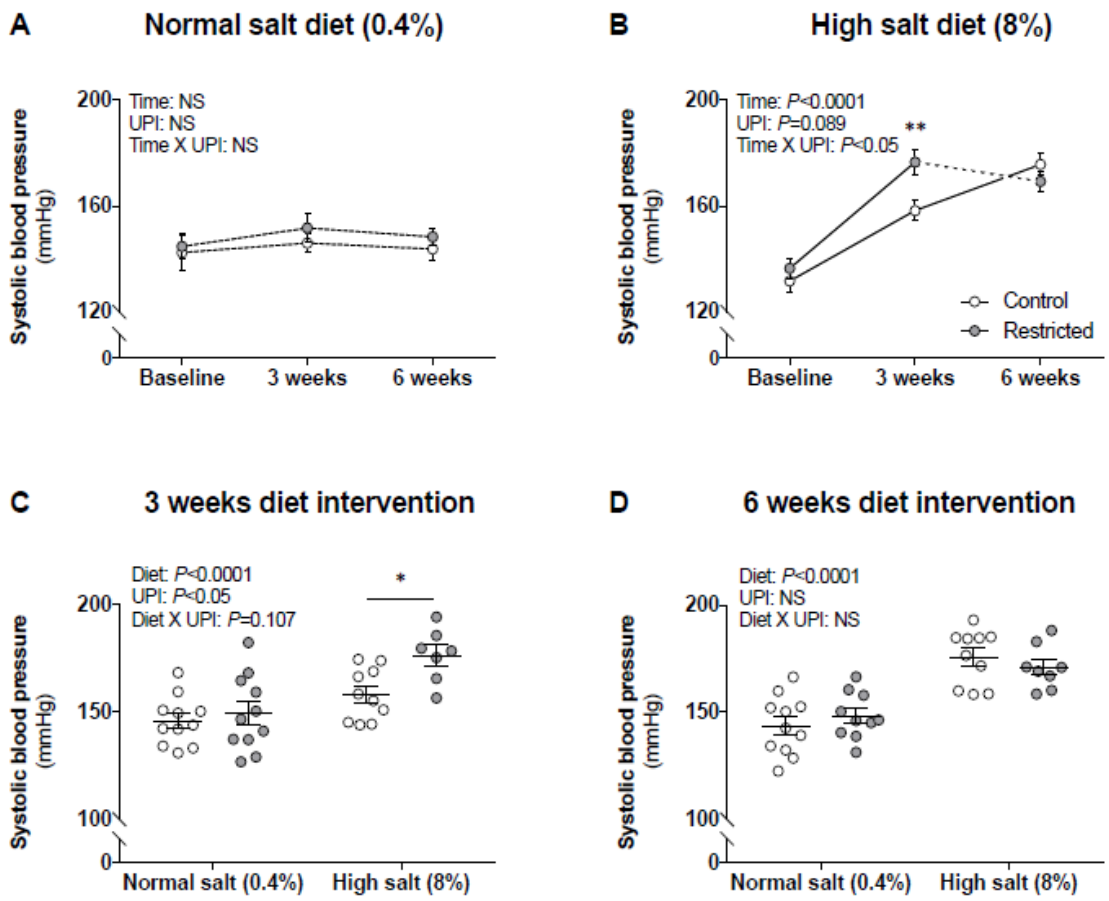

Figure 2. Fluid and electrolyte balance at six weeks of high salt diet. *A-B*, water intake in relation to urinary ouput, *C*, predicted filtered sodium, *D*, urinary sodium excretion ($U_{Na}V$), *E*, fractional sodium

excretion (FE_{Na}), *F*, urinary potassium excretion (U_KV), and *G*, sodium to potassium ratio ($U_{Na:K}V$) at 6 weeks of diet intervention (26 weeks of age). Data are individual values with mean ± SEM. Pearson's correlation performed for *A*. **P*<0.05, ***P*<0.01 Restricted *vs* Control within diet group by Fisher's LSD test. UPI, uteroplacental insufficiency; NS, not significant.

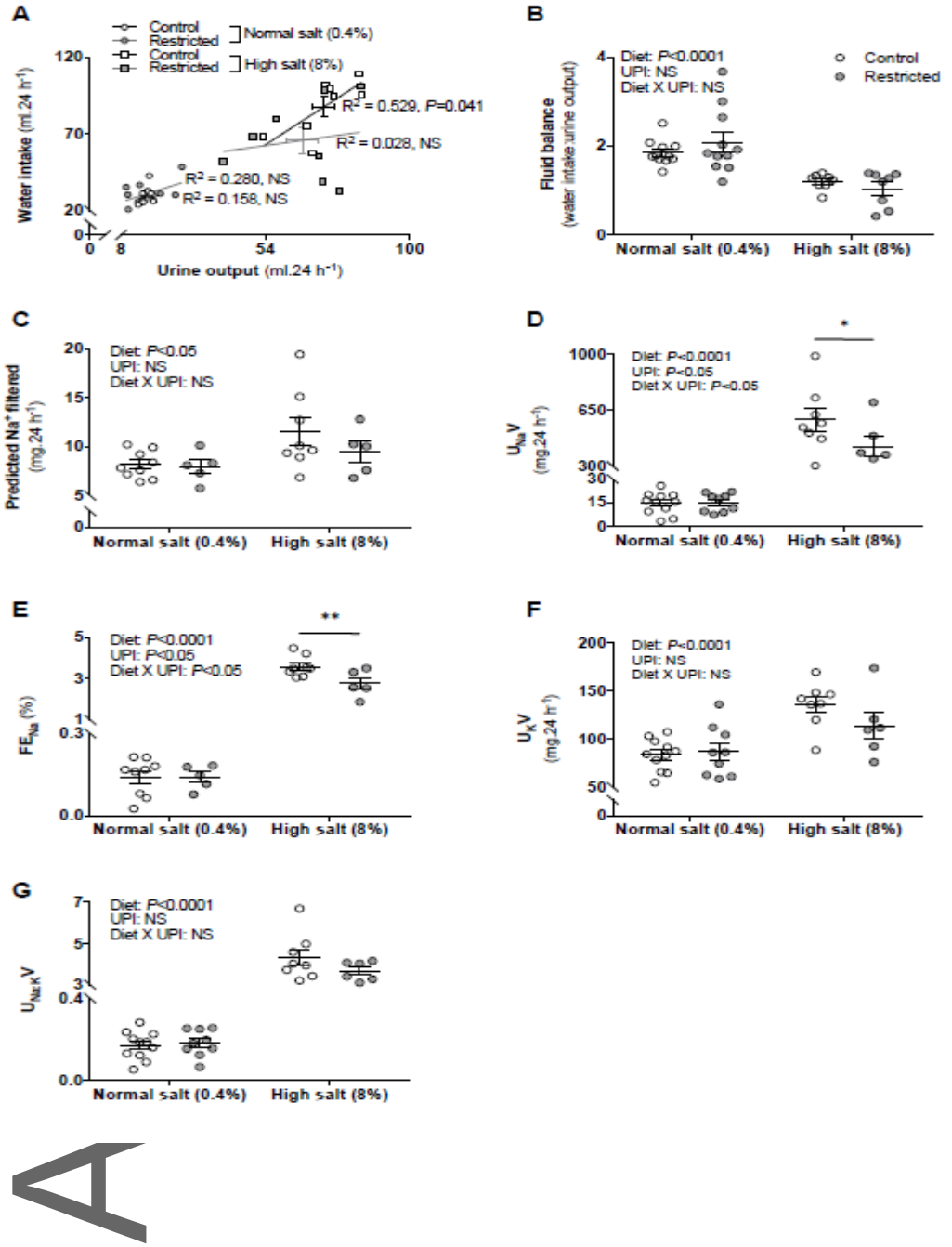

Figure 3. Kidney function and histopathology at six weeks of high salt diet.

A, creatinine clearance, *B-C*, albuminuria, *D*, proteinuria, *E*, glomerulosclerosis, *F*, tubulointerstitial fibrosis, and *G*, perivascular fibrosis at 6 weeks of diet intervention (26 weeks of age). Data are individual values with mean ± SEM. **P*<0.05, ***P*<0.01 Restricted *vs* Control within diet group by Fisher's LSD test. UPI, uteroplacental insufficiency; NS, not significant. Representative kidney sections stained with PAS and Masson's Trichrome. Scale bars represent 50 µm.

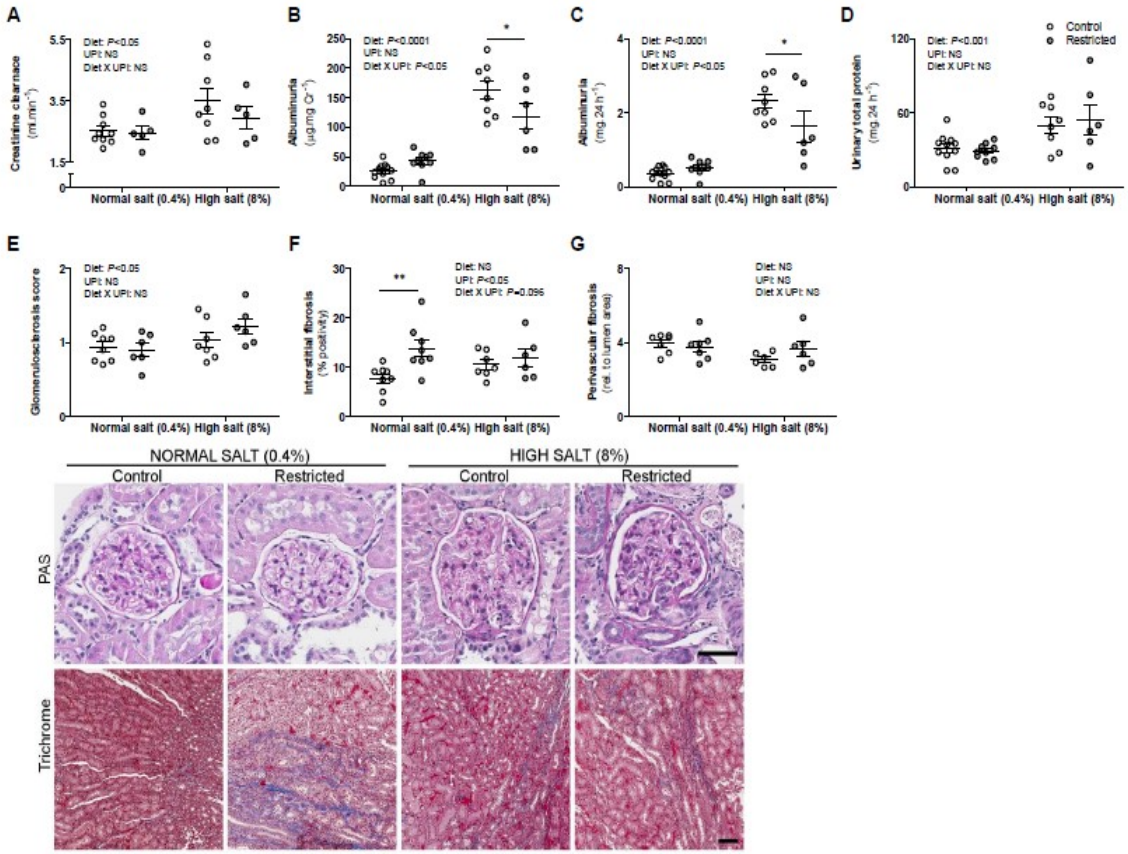

Figure 4. Passive mechanical wall properties of mesenteric and renal arteries at six weeks of high salt diet.

A,C, stress-strain relationships and *B,D,* media-to-lumen ratio for mesenteric (top) and renal (bottom) arteries at 6 weeks of diet intervention (26 weeks of age). Data are mean ± SEM; *N*=10-11/group. #*P*<0.05 High *vs* Normal salt within UPI group by Tukey's *post-hoc* test.