Deoxycorticosterone Acetate-Salt Mice Exhibit Blood Pressure–Independent Sexual Dimorphism

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Abstract—We tested the hypothesis that female and male mice differ in terms of cardiac hypertrophy after deoxycorticosterone acetate (DOCA)+salt hypertension (uninephrectomy and 1% saline in drinking water) and focused on calcineurin signaling. We excluded confounding effects of blood pressure elevation or sex-related blood pressure differences by treating DOCA-salt mice with hydralazine (250 mg/L in drinking water). We found that directly measured mean arterial blood pressure was lowered to control values with hydralazine and corroborated this finding in separate mouse groups with radiotelemetry. Male mice were more responsive to DOCA-salt–related effects. They developed more left ventricular hypertrophy and more renal hypertrophy after 6 weeks of DOCA-salt+hydralazine compared with female mice. In hearts, transcripts for calcineurin Aβ and for myocyte-enriched calcineurin interacting protein 1 were upregulated in male but not in female mice. Enhanced activity of calcineurin Aβ, as indicated by diminished phosphorylation of NFATc2 in male mice, accounted for this sex-specific difference. Stretch-related, inflammatory, and profibrotic responses were also accentuated in male mice, as shown by higher transcript levels of atrial natriuretic peptide, monocyte chemoattractant protein-1, and transforming growth factor-β. Our results support sex-specific regulation of the calcineurin pathway in response to largely blood pressure–independent mineralocorticoid action. We suggest that sex-specific calcineurin activation determines the maladaptive cardiac and renal hypertrophic responses and accompanying organ injury in male mice. (***Hypertension*****. 2008;51:1-7.)**

Key Words: heart ■ kidney ■ hypertrophy ■ inflammation ■ fibrosis ■ calcineurin ■ MCIP1 ■ NFATc2

Sex differences in cardiac[1] and renal[2] function are well established. Women have a lower overall incidence of left ventricular (LV) hypertrophy[3] and nondiabetic renal disease than men.[4] Less pronounced injury in female animals has been attributed to estradiol-mediated protective effects.[5] Male vulnerability was related to detrimental effects of androgens.[6] Gonadal steroids regulate renal and systemic hemodynamics, as well as renal sodium handling.[7] Chronic salt overload is a major cardiovascular stressor that sets in motion a series of adaptive processes in hearts and kidneys in patients with renal dysfunction.[8,9] Deoxycorticosterone acetate (DOCA)+salt acts similarly to aldosterone, promotes sodium retention and potassium excretion in the distal nephron, and serves as a "prototype" model for salt-sensitive low-renin hypertension.[10] Rats or mice with DOCA-salt hypertension develop sex-specific differences in cardiorenal target-organ damage that are mainly attributed to more delayed and less extensive blood pressure increases in females compared with males.[11] Whether sex-specific differences in cardiorenal damage still exist in the context of independent systemic blood pressure responses to salt overload has not been studied. We hypothesized that female mice have endogenous compensatory mechanisms related to cell signaling that make them less amenable to mineralocorticoid action, independent of a lesser increase in blood pressure in response to DOCA-salt. Signaling events controlled by calcineurin and its modulator, myocyte-enriched calcineurin interacting protein 1 (MCIP1), are essential regulators of myocardial hypertrophy.[12] Calcineurin is also involved in the development of target organ damage in mineralocorticoid excess.[13] We, therefore, further hypothesized that sex-specific differences in the activation of the calcineurin pathway may contribute to blood pressure–independent cardiac and renal remodeling in DOCA-salt+hydralazine mice.

Materials and Methods

Animal Model

Six-week–old male and female C57BL/6J mice, at initial weight of 19 to 21 g, (Harlan-Winkelmann) were housed with a 12:12-hour

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light-dark cycle, at a constant temperature of 24°C, and received standard mouse chow (Altromin). All of the mice underwent unilateral left nephrectomy via flank incision under isoflurane anesthesia and were divided into 4 groups (n=12 per group): male and female sham-operated uninephrectomized mice and male and female DOCA-salt mice. DOCA pellets (50 mg of DOCA per pellet) with 42-day release (Innovative Research of America) were implanted subcutaneously immediately after nephrectomy. Sham animals were given 1% NaCl for drinking (Uni-Nx+salt). Animals with DOCA-pellets received 1% NaCl with 250 mg/L of hydralazine in their drinking water. The mice were weighed and put into metabolic cages on a weekly basis for monitoring of fluid intake and urine collections. Local authorities approved all of the studies according to American Physiological Society guidelines. After 6 weeks, echocardiography and invasive blood pressure measurements were performed. Hearts and kidneys were excised, weighed, and fixed in 4% neutral buffered formalin for histology. For protein and RNA isolation, samples were snap frozen in liquid nitrogen and stored at −80°C.

Hemodynamics in Anesthetized and Conscious Rats and Echocardiography

Direct blood pressure and heart rate measurements were performed using a 1.4F catheter tip micromanometer (ARIA, Millar Instruments) placed in the carotid artery in mice anesthetized with isoflurane. After implantation, the catheter was connected to the transducer for hemodynamic measurement. Baseline of blood pressure was recorded for 10 minutes.

The telemetric technique was described in detail previously.[14] In brief, 5 additional female mice and 5 additional male mice were anesthetized with isoflurane. The pressure-sensing catheter of the TA11PA-C10 BP device (Data Sciences International) was advanced via the carotid artery into the ascending aorta, and the transmitter was placed in a subcutaneous pocket. All of the mice were allowed 11 days recovery from surgery before baseline blood pressure (BP) and heart rate (HR) values were recorded for 3 days. By this time, the mice had regained their circadian BP and HR rhythm, and the surgery and anesthesia-dependent initial changes in systolic BP, diastolic BP, mean arterial BP (MAP), and HR were followed. After uninephrectomy and implantation of the DOCA pellet, mice were given 1% NaCl to drink. After 8 days with DOCA+salt, mice additionally received hydralazine for 10 days. All of the data were sampled every 5 minutes for 10 seconds continuously day and night with a sampling rate of 1000 Hz. Systolic BP, diastolic BP, and HR were recorded using the DATAQUEST software (A.R.T. 2.1, Data Sciences International). HR was computed from the pulse intervals of the BP recordings. For statistical analysis, we used 3 days of baseline values, the days 6 to 8 under DOCA+salt and the days 8 to 10 under DOCA+salt combined with hydralazine.

For echocardiographic studies, 2D short-axis and long-axis views of the left ventricle were obtained with a 30-MHz transducer in anesthetized mice. M-mode tracings were recorded and used to determine the interventricular septum in systole and diastole, the diameter of the left ventricle at the end of the systole and diastole, as well as the LV posterior wall thickness.

Cardiac and Renal Pathology, Immunohistochemistry, and Morphometry

Paraffin serial sections (2-μm thick) were stained by hematoxylin and eosin and periodic acid-Schiff. Myocyte cross-sectional areas were calculated from ≥200 myocytes from a minimum of 4 animals per group in randomly selected high-power fields. Maximum kidney distal tubular diameters (basolateral to basolateral side) were determined in ≥250 tubules from ≥6 different animals per group in randomly selected high-power fields in the renal cortex and the medulla. The means for 6 to 8 mice at any time point were grouped together to obtain a final mean±SD.

Gene Expression Analysis

For reverse transcription, total RNA from deep frozen tissue (hearts and kidneys) was prepared according to standard methods. RNA was quantified and integrity was proven. After synthesis of cDNA (PCR Core Kit, Applied Biosystems; oligo-dt primers, TIB Molbiol) we used the Light Cycler PCR and detection system (Roche) for amplification and online quantification following the manufacturer's instructions. The primer sequences (TIB Molbiol) and annealing temperatures are available on request. Run data were analyzed by "second derivative maximum" with the quantification program Quant versions 2.7 and 3.0.

Immunoprecipitation and Western Blot Analysis for Ser 213/217/221 Phosphorylated NFATc2

For immunoprecipitation, 500 μg of tissue protein extract in 500 μL of lysis buffer (250 mmol/L of NaCl, 0.1% Nonidet P-40, 50 mmol/L of HEPES, 5 mmol/L of EDTA, 0.5 mmol/L of dithiothreitol, and complete protease inhibitor mixture [Roche, Applied Biosystems]) was incubated with an antibody against total NFATc2 (clone 4G6–65, Santa Cruz) after preclearing with protein A/G Sepharose (Santa Cruz). Immune complexes were precipitated with protein A/G Sepharose beads, and the pellet was resuspended in Laemmli buffer after washing 3 times. SDS gel electrophoresis and Western blotting were performed according to standard methods using 8% polyacrylamide gels. Membranes were blocked in 5% BSA and incubated with the primary antibody (anti-pNFATc2 Ser 213/217/221, 1:10 000, Santa Cruz) for 3 hours at room temperature. After washing 3 times with Tris-buffered saline Tween-20, the signal was detected using an appropriate peroxidase-labeled secondary antibody (Dako Cytomation) and ECL substrate (Amersham). Membranes were stripped and reprobed with a polyclonal anti–total-NFATc2 antibody (Santa Cruz) for control of equal protein loading.

Statistical Analysis

The results between groups were compared using nonparametric Mann-Whitney U test. A $P<0.05$ was considered significant. Statistics was performed using SPSS 10.0 for Windows.

Results

We first studied uninephrectomized male and female mice given 1% saline as drinking water, without (Uni-Nx+salt) or with DOCA pellet (DOCA+salt). To eliminate the effects of BP, groups were included that received hydralazine (250 mg/L) in drinking water (DOCA+salt+hydralazine; Figure 1). Figure 1A shows MAP measurements in anesthetized mice. DOCA+salt increased BP, which was avoided by hydralazine in both sexes (n=12 per group). Figure 1B shows direct HR measurements corresponding with MAP in Figure 1A. Females had higher HRs without hydralazine treatment. We then included telemetric measurements of BP to assure ourselves that the direct MAP values under anesthesia were correct. Figure 1C shows telemetric measurements in conscious mice. MAP values recorded by telemetry in conscious mice were higher compared with catheter recordings because of the lack of anesthesia-related effects. The males had higher BPs than females with DOCA+salt. We also found that hydralazine was highly effective in lowering BP to basal values in this model (n=5 per group). Figure 1D shows the telemetric HRs corresponding with MAP in Figure 1C. Females again had baseline faster HRs than males, corroborating the direct measurements. We focused particularly on the comparison between Uni-Nx+salt and DOCA+salt+hydralazine treatment in our study, because we sought to avoid the confounding influence of BP differences between

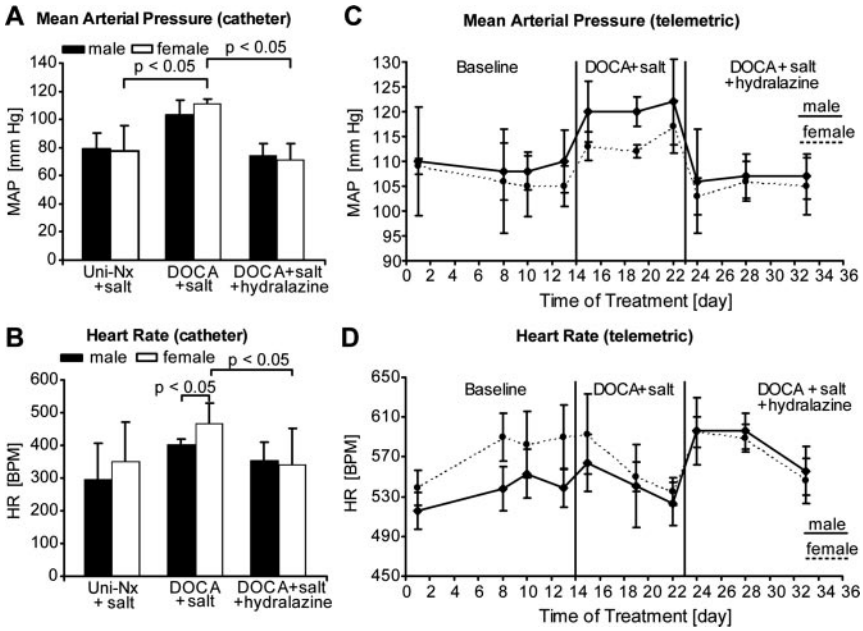

Figure 1. A, Direct mean arterial pressure (MAP) measurements in anesthetized mice. DOCA-salt increases blood pressure, which can be avoided by hydralazine in both sexes (n=12 per group). B, Direct HR measurements corresponding with MAP in A. Females had higher HRs without treatment. C, Telemetric measurements in conscious mice show that males had higher blood pressures than females with DOCA-salt that can be avoided by hydralazine (n=5 per group). D, Telemetric HRs corresponding with MAP in C. Females again had faster HRs than males.

the sexes and BP-related effects in general. The figures below draw attention to these groups.

To exclude possible confounding effects of differences in potassium handling between the sexes, we measured serum and urine potassium values (Figure 2A through 2C). Baseline serum potassium was higher in male Uni-Nx+salt mice than females. With DOCA+salt+hydralazine, however, potassium decreased in both sexes, and no differences were observed. Urine potassium excretion increased in both sexes with DOCA+salt+hydralazine to a similar degree. We observed an increase in urine volume with DOCA+salt+hydralazine, without sex differences. We interpret these data as indicating no confounding effects on potassium homeostasis.

We observed a greater degree of target organ hypertrophy in males than in females (Figure 3). In male but not in female mice, DOCA+salt+hydralazine treatment resulted in a heart weight:body weight ratio that was significantly greater than in control counterparts. Figure 3A shows that male mice developed a 26% increase in this ratio, whereas females had no increase compared with Uni-Nx+salt mice. We also sought to determine whether the heart weight:body weight ratio was reflective of differences in functional echocardiographically determined hypertrophy indices (Table). Although in both female and male hearts individual indices did not show significant differences between male and female DOCA+salt+hydralazine mice, morphometric evaluation revealed that both DOCA+salt+hydralazine male and female hearts display microscopic features of cardiac hypertrophy, as we observed a significant increase in myocyte cross-sectional area compared with Uni-Nx+salt female mice, shown in Figure 3B. Male DOCA+salt+hydralazine mice showed a substantially larger myocyte cross-sectional area compared with female counterparts. We further observed sex-specific differences in the compensatory growth of the remaining kidney after unilateral nephrectomy in Uni-Nx+salt and DOCA+salt+hydralazine animals. Female Uni-Nx+salt mice showed a higher kidney weight:body weight ratio after unilateral nephrectomy compared with male Uni-Nx+salt mice that was not attributed to differences in the diameter of distal tubules, as shown in Figure 3C and 3D. Both DOCA+salt+hydralazine–treated male and female mice had an increase in the kidney weight:body weight ratio; however, this effect was associated with enlargement of distal tubule only in male mice.

We observed significant upregulation of calcineurin Aβ and MCIP1 transcripts in male DOCA+salt+hydralazine mice compared with control counterparts (Figure 4A and 4B). Female mice showed a trend in downregulation of both transcripts that did not reach statistical significance. Western blot analysis with phosphospecific antibodies against NFATc2 phosphorylated at Ser 213/217/221 after immunoprecipitation of heart protein extracts for total NFATc2 revealed abolished NFATc2 phosphorylation in male DOCA+salt+hydralazine–treated mice (Figure 4C). Uni-Nx+salt male and female mice had equal levels of phosphorylated NFATc2 as female DOCA+salt+hydralazine–treated mice. These data show that upregulation of calcineurin Aβ

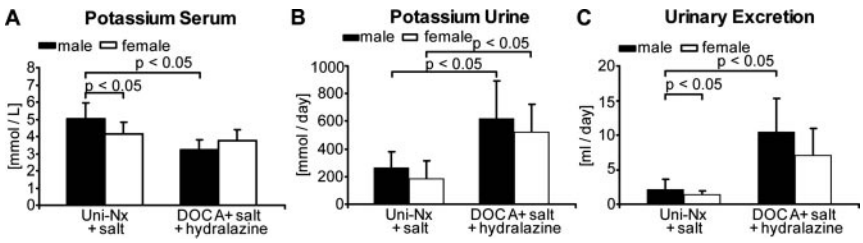

Figure 2. A, Serum potassium concentrations were higher in male Uni-Nx+salt mice than females. DOCA+salt+hydralazine lowered serum potassium without sex differences. B, Urine potassium excretion was higher with DOCA+salt+hydralazine, albeit without sex differences. C, Urine volume increased with DOCA+salt+hydralazine treatment without sex differences.

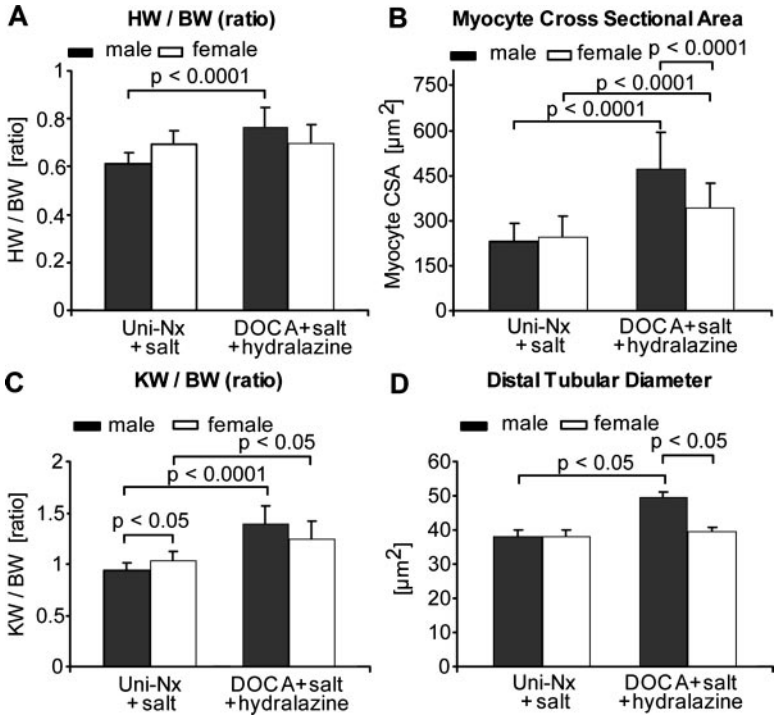

Figure 3. Shown are Uni-Nx+salt controls compared with DOCA-salt mice that were treated with hydralazine to remove confounding effects of blood pressure elevation. A, Heart weight:body weight ratio was increased with DOCA-salt even with hydralazine but only in males and not in females. B, Myocyte cross-sectional area increased in both males and females but more so in males. C, Kidney weight:body weight ratio increased in both males and females, albeit more in males. D, Distal tubular diameter increased but only in males and not in females.

and MCIP1 in male DOCA+salt+hydralazine–treated mice results in enhanced activity of calcineurin Aβ as indicated by diminished phosphorylation of NFATc2.

To evaluate the hypertrophic stimulus-related changes in male hearts compared with female hearts, we measured transcripts of injury response including the cardiac fetal gene program induced by myocardial stretch, proinflammatory genes, and profibrotic genes that are altered by a maladaptive response to a hypertrophic stimulus. Atrial natriuretic peptide (Figure 5A) but not brain natriuretic peptide (Figure 5B) transcripts were significantly upregulated in male and female DOCA+salt+hydralazine mice, although the increase was more pronounced in males compared with females. We observed significantly higher transcript levels of the proinflammatory chemokine monocyte chemoattractant protein-1 (MCP-1) in hearts and kidneys of male DOCA+salt+hydralazine mice compared with hearts (Figure 5C) and kidneys (Figure 5D) of female DOCA+salt+hydralazine mice and male Uni-Nx+salt mice. Transcript levels of transforming growth factor-β were significantly upregulated in hearts (Figure 5E) and kidneys (Figure 5F) of DOCA+salt+hydralazine males compared with Uni-Nx+salt males and DOCA+salt+hydralazine females. In contrast, transforming growth factor-β transcripts in hearts and kidneys of DOCA+salt+hydralazine females remained unchanged as compared with hearts and kidneys of sham females.

Discussion

Our major findings were clear-cut, sex-specific differences in cardiac and renal functional adaptation and injury in response to DOCA+salt+hydralazine in mice. These effects occurred independent of BP differences between the sexes and possibly independent of substantial BP increases. Sex-specific activation of the calcineurin pathway accounted for the differences in cardiac adaptation to injury and BP-independent hypertrophic stimulus. Female hearts and kid-

Table. Echocardiographic Assessment of Left Ventricular Chamber (Mean±SD)

Echo Dimensions, mm	Male Uni-Nx+salt	Male DOCA+Salt+ Hydralazine	Female Uni-Nx+Salt	Female DOCA+Salt+ Hydralazine
IVSs	0.96±0.13	1.41±0.27*	1.05±0.08	1.25±0.24
IVSd	0.74±0.09	0.85±0.10*	0.79±0.05	0.80±0.12
LVIDs	3.70±0.20	2.62±0.54*	3.39±0.18	2.65±0.35*
LVIDd	4.38±0.18	4.22±0.34	4.12±0.12	4.15±0.25
LVPWs	0.79±0.10	1.29±0.22†	0.92±0.07	1.22±0.26*
LVPWd	0.67±0.08	0.83±0.07*	0.72±0.04	0.80±0.14

Males developed more hypertrophy than females. M-mode transthoracic echocardiography measurements were recorded from sham-operated and DOCA+salt+hydralazine–treated male and female mice. IVSs and IVSd, end-systolic and end-diastolic intraventricular septal thickness; LVIDs and LVIDd, end-systolic and end-diastolic LV internal dimensions; LVPWs and LVPWd, end-systolic and end-diastolic LV posterior wall thickness. Data are represented as means±SDs.

*$P<0.05$ and †$P<0.001$ vs corresponding sex-matched Uni-Nx+salt control group.

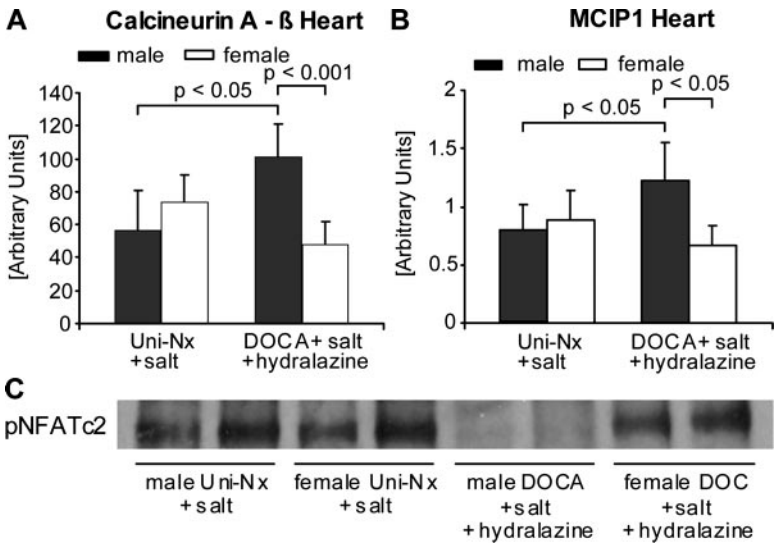

Figure 4. Shown are Uni-Nx+salt controls compared with DOCA-salt mice that were treated with hydralazine to remove confounding effects of blood pressure elevation. A, RT-PCR for calcineurin Aβ-isoform in the heart showed an increase with DOCA+salt+hydralazine but only in males. B, RT-PCR for modulating calcineurin and inhibiting protein 1 (MCIP1) showed increase with DOCA+salt+hydralazine but only in males. C, Immunoprecipitation experiment and subsequent Western blot for phosphorylated nuclear factor of activated T cells c2 (pNFATc2) are shown. Male DOCA+salt+hydralazine mice showed markedly decreased phosphorylation compared with females.

neys showed adaptive features of both cardiac and renal hypertrophy in response to mineralocorticoid effects that did not translate into increased proinflammatory and profibrotic responses.

The relationship between sex and DOCA-salt–induced injury is complex and possibly involves sex-related differences in BP and sympathetic tone, as well as responses to mineralocorticoid excess and salt retention. Although we have minimized differences in BP as a possible confounder, we cannot be absolutely certain about the predominant stimulus that led to sex-related differences in the activation of calcineurin and its downstream targets. Hydralazine is a vasodilator, which effectively lowers BP and at the same time activates the baroreflex resulting in increased HR. Prevention of hypertension with hydralazine could have influenced sympathetic tone in a sex-specific manner. At baseline, the HR was actually higher in female mice, suggesting stronger activation of sympathetic tone in female mice. Increased renal sympathetic tone in the DOCA-salt rat facilitates sodium retention and is necessary for the development of hypertension.[15] Sex differences in autonomic cardiovascular regulation are described in both animals and humans, characterized by reduced responsiveness of female individuals to challenges of the baroreflex, such as orthostasis and vasoactive

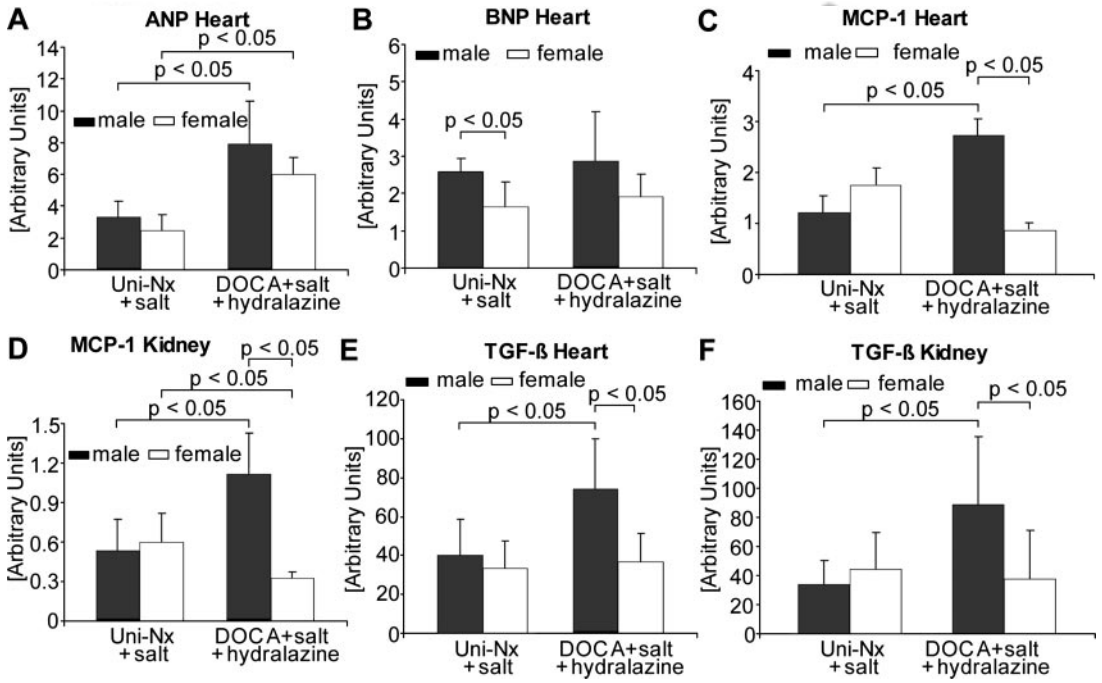

Figure 5. Shown are UniNx+salt controls compared with DOCA-salt mice that were treated with hydralazine to remove confounding effects of blood pressure elevation. A and B, RT-PCR for atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in heart. ANP increased more in males than females. BNP increased in males but remained unchanged in females. C and D, MCP-1 in heart and kidney. This chemokine markedly increased in males. E and F, transforming growth factor-β (TGF-β) in heart and kidney. A similar pattern with a substantial increase in males but not in females was observed.

drugs.[16,17] These differences may be because of stimulation of estrogen receptors in central autonomic nuclei[18] or sex differences in norepinephrine transporter function.[19] However, hydralazine treatment eliminated sex differences in HR, both with direct measurement under anesthesia or with radiotelemetry. In the rat aortocaval shunt-induced chronic volume overload model, female hearts developed significant concentric hypertrophy, without impairment in cardiac function or myocardial compliance.[20] The DOCA-salt model features total body salt retention, although the effects on volume compartments are less clear.[21]

We suggest that features of target-organ damage were likely related to receptor- and nonreceptor-mediated mineralocorticoid actions. Mineralocorticoids promote cardiac hypertrophy in normotensive 1-renin gene mice in part by the activation of angiotensin type 1 receptors.[22] Hypokalemia and metabolic alkalosis as consequences of mineralocorticoid actions may also contribute to the development of cardiac and renal hypertrophy and cardiac dysfunction under the condition of mineralocorticoid and salt excess.[23] Although male mice started with higher potassium values in our study, they had no worse hypokalemia compared with females at the end of the study. We do not believe that hypokalemia contributed to sex-related differences in our model.

Our study was not specifically designed to determine the relative contribution of individual BP-independent hypertrophic stimuli for the activation of calcineurin and downstream regulated pathways. Nevertheless, sympathetic effects mediated by adrenergic receptor activation, as well as receptor-dependent and -independent mineralocorticoid action, converge onto alterations in calcium cycling and sensing. This state of affairs underscores the importance of our major finding, namely, a previously undescribed sex-specific difference in the cardiac induction of the calcineurin pathway, possibly in a BP-independent manner. We were not able to measure BP and HR by radiotelemetry in every mouse in our study. However, for a 10-day telemetry measurement period, we found no difference in BP between DOCA+salt+hydralazine female and male mice. We conclude that downstream events controlled by calcineurin are possibly shared among pathophysiologic forms of cardiac hypertrophy but not in physiological hypertrophy.[24]

Numerous studies have established the pivotal role of calcineurin/nuclear factor of activated T cells signaling in transgenic and pressure-induced models of cardiac hypertrophy.[25] Constitutive activation of calcineurin in mouse hearts by a transgenic strategy is sufficient to induce massive cardiac enlargement and heart failure.[26] Calcineurin inhibition by MCIP1 overexpression blunted the hypertrophic response in models of LV remodeling after myocardial infarction.[27] Remarkably little is known regarding the calcineurin pathway in pressure-independent forms of hypertrophy or the role of sex-specific differences. Estradiol limits agonist-induced cardiac hypertrophy at least in part by upregulating MCIP1 and subsequent repression of calcineurin activity.[28] This action possibly explains the milder hypertrophic phenotype observed in female mice.

Changes in atrial natriuretic peptide and brain natriuretic peptide occur with nuclear factor of activated T-cell activation.[29] Aside from involvement in hypertrophic responses, the calcineurin-dependent pathway mediates MCP-1 expression in vascular smooth muscle cells and promotes vascular inflammation.[30] We observed higher MCP-1 expression in both hearts and kidneys of male mice that we would attribute to higher calcineurin activity, at least in the heart. Consistent with our findings, cardiomyocytes of rats with chronic volume overload–induced heart failure also overexpress MCP-1.[31] Increased transforming growth factor-β transcripts and changes in collagen content (data not shown) cannot be directly explained through activation of the calcineurin pathway. Instead, these responses may be related to sex-specific sensitivity to nongenomic mineralocorticoid effects. Aldosterone is implicated in the induction of cardiac fibrosis through multiple mechanisms.[32] Aldosterone can stimulate calcineurin activity in cortical collecting ducts in the rat kidney via nongenomic effects.[33] Such effects may explain the larger inner tubular diameters in the distal nephron of male mice. Tubular enlargement may, together with described proinflammatory signals, translate into tubulointerstitial remodeling, the most reliable marker of renal progressive disease severity.[34] We have not characterized the distal tubular dilatation in our male mice in detail and have not mapped the finding specifically. Nevertheless, the fact that this dilatation could specifically involve the aldosterone-sensitive distal nephron has not escaped us.

Our study highlights the importance of sex differences in calcineurin activation in the BP-independent DOCA+salt+hydralazine model. Future studies, including animals deficient in estrogen and androgen receptors, will address the relative importance of gonadal steroid receptors for our observations. Our results add to the increasing complexity of sex-related differences in the cardiac and renal adaptation and stress an importance of the calcineurin pathway in pathophysiologic forms of cardiac hypertrophy.

Perspectives

The calcineurin pathway offers several therapeutic avenues. Treatment with calcineurin inhibitors would at first glance appear unappealing because of the BP increases and toxicities reported with cyclosporine or tacrolimus. However, the pathway interacts with numerous other signaling mechanisms. For instance, the novel mitogen "lacritin" was found recently to target the downstream nuclear factor of activated T cell and the mammalian target of rapamycin via a protein kinase C-α–dependent mechanism.[35] These complex signaling pathways open numerous potential therapeutic targets for our findings.

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Disclosures

None.

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