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Androgens drive divergent responses to salt stress in male versus female rat kidneys

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Abstract

Dahl-Iwai (DI) salt-sensitive rats were studied using microarrays to identify sex-specific differences in the kidney, both basal differences and differences in responses to a high-salt diet. In DI rat kidneys, gene expression profiles demonstrated inflammatory and fibrotic responses selectively in females. Gonadectomy of DI rats abrogated sex differences in gene expression. Gonadectomized female and gonadectomized male DI rats both responded to high salt with the same spectrum of gene expression changes as intact female DI rats. Androgens dominated the sex-selective responses to salt. Several androgen-responsive genes with roles potentiating the differential responses to salt were identified, including increased male expression of angiotensin-vasopressin receptor and prolactin receptor, decreased 5α-reductase, and mixed increases and decreases in expression of *Cyp4a* genes that can produce eicosanoid hormones. These sex differences potentiate sodium retention by males and increase kidney function during gestation in females.

Keywords: Kidney; Hypertension; Rat; Sex; Gene expression; Microarrays; Angiotensin–vasopressin receptor; Prolactin receptor; 5α-Reductase

Surveys of nonreproductive organs have revealed profound male/female differences in gene expression, differences that underlie physiological dimorphisms. Sex differences in gene expression arise through at least three known mechanisms, epigenetic, genetic, and developmental. For example, androgens and estrogens act on the developing rodent brain, altering DNA methylation near key genes, thus triggering persistent sexually dimorphic gene expression. Genetic rather than epigenetic mechanisms are also thought to contribute to sexually dimorphic brain development [1]. In a second example, sex steroids act on the hypothalamus to regulate growth hormone (GH) release into the blood from the pituitary in an ~4-h cycle in males vs. an ~30-min cycle in females. These disparate GH regimens in turn differentially regulate at least six cytochrome P450 genes *Cyp* genes) in livers of male or female rats and mice [2,3]. Whereas several *Cyp* genes are also expressed with sex biases in rat kidney, these genes are regulated by androgens/estrogens acting directly on the rodent kidney, not by GH pulsatility [3]. Male and female rats respond in profoundly different ways to uninephrectomy. While the remnant kidneys of both sexes increase in mass, the male kidney undergoes hypertrophy, whereas the female kidney undergoes hyperplasia [4].

Hypertension is a complex disease that may arise from dysfunction of blood vessels, central nervous system regulation, hormonal homeostasis, or kidney functions. Hypertension and

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the accompanying complications stroke, heart disease, and kidney disease, arise from interactions between genetic risk factors, environmental risk factors, and sex-specific hormonal effectors. Genetic risk factors in humans include a mutation in the preangiotensinogen gene that is thought to give rise to the "nonmodulating" phenotype, a blunted response to angiotensinogen II under high-salt conditions observed most frequently in males [5]. Environmental risk factors include high-salt and high protein diets. Men show an increased risk for hypertension and renal disease compared to women, a risk that interacts with the nonmodulating phenotype and with diet [6].

In many human patients and in diverse circumstances researchers have observed that "hypertension follows the kidney," suggesting the kidney is often a source of the hypertensive phenotype. For example, human kidney transplant recipients display significantly higher blood pressure if the donor was hypertensive or had hypertensive relatives [7]. Conversely, normotensive kidney donors commonly normalize blood pressure in hypertensive recipients [7]. Sexually dimorphic risk factors do not follow this rule, since male rats maintain higher blood pressure than female rats. In spontaneously hypertensive rats (SHR), for example, female rats transplanted with male kidneys are normotensive, whereas male rats with female kidneys show elevated blood pressure characteristic of the normal male rats [8]. This suggests that sex differences in blood pressure arise from male/female hormonal regimens.

Available evidence from studies of humans and rats indicates that males suffer increased blood pressure and accelerated kidney failure [8]. For example, male rats have higher blood pressure than females in the SHR, Dahl salt-sensitive (DSS), deoxycorticosterone salt-hypertensive, and New Zealand hypertensive rat strains [9]. Male rats and humans show a characteristic resistance to sodium excretion following increased blood flow through the glomerulus, called "blunting of pressure natriuresis". Castration of young male rats attenuates the development of hypertension in high-salt-fed SHR and DS rats and in male rats with reduced renal mass. Similarly, the androgen receptor antagonist flutamide attenuates blood pressure in male SHR to the level seen in female SHR. Testosterone treatment reverses this effect in SHR, raising blood pressure in both castrated males and ovariectomized females [8]. In contrast, evidence is conflicting regarding whether estrogens suppress blood pressure and kidney failure or are neutral. These studies implicate androgens in elevation of blood pressure.

Dahl-Iwai (DI) rats are salt-sensitive, insulin-resistant, and hyperlipidemic and develop proteinuria and glomerulosclerosis during progression of hypertension. The DI rat phenotype resembles the salt-sensitive hypertension prominent in the African American population.

The vascular-renal physiology of DSS rats and nine other rat strains was characterized in detail by H. Jacobs and colleagues. The DSS substrain was derived from the same Dahl colony as the DI substrain, so it can be expected to share some hypertensive susceptibility genes with DI substrain rats. The DSS rats were characterized by low renin activity, severe proteinuria, and relatively high responses in mean arterial pressure in response to angiotensin II or norepinephrine (see http://pga.mcw.edu/). These traits indicate physiological similarities with low-renin essential hypertension in the human population.

Results and discussion

Dahl-Iwai experiment

The DI salt-sensitive rat model was studied as a model of chronic progressive kidney disease in human essential hypertension. The chronic nature of this model enabled us to ask how the kidney responds to salt stress, how it alters its transcriptional program to reach a new homeostasis. This model was also used to examine how kidneys of male versus female animals differ and how they react differently to salt stress. The initial experiment integrated measurements of gene expression profiles with measurements of kidney dysfunction and kidney histology in male and female DI rats on high- and low-salt diets. This experiment gave rise to a second experiment of similar design to determine whether these sex differences were specific to the DI strain or common to wild-type Sprague–Dawley rats. A third iterative experiment addressed whether sex-specific responses to a high-salt diet are modified by gonadectomy (GX-DI rats): orchidectomy of male rats or ovariectomy of females. Measurements of kidney function, kidney histopathology, gene expression microarrays, and immunohistology were performed on the same groups of rats to enable synthesis of physiology and gene regulation data.

Blood pressure in DI rats

Male and female 8-week-old DI rats were put on low- or high-salt diets, three rats per treatment group. At 14 weeks, rats were sacrificed for microarray studies, genotyping, and blood chemistry. Mean arterial pressure was measured on a separate set of DI rats implanted with blood pressure monitoring equipment (Fig. 1). In response to a high-salt diet, mean arterial pressure increased rapidly from 100 to 110 mm Hg in DI rats of both sexes. In contrast, mean arterial pressure remained steady in wild-type Sprague–Dawley rats switched to a high-salt diet [14]. In male DI rats blood pressure rose steadily during 14 weeks in response to the high-salt diet; from 110 to 145 mm Hg, whereas in female DI rats blood pressure remained steady over the 14 weeks. This male-selective salt sensitivity is also typical of several other salt-sensitive rat strains [9].

Reproducibility of the kidney gene expression profiles was assessed by estimating the false discovery rate or FDR using data permutations subjected to ANOVA testing. The FDR estimate for the 453 genes was 11%, an indication that the microarray data are relatively reproducible and that these animals were relatively genetically similar. DI rats showed salt-sensitive hypertension relative to the Sprague–Dawley parental strain but did not suffer mortality within the 14-week studies as reported for salt-sensitive rats from the original Dahl-Iwai colony [15]. Along with the limited variability observed, this suggests a "genetic bottleneck" has reduced the number of genes contributing to hypertension in this DI colony.

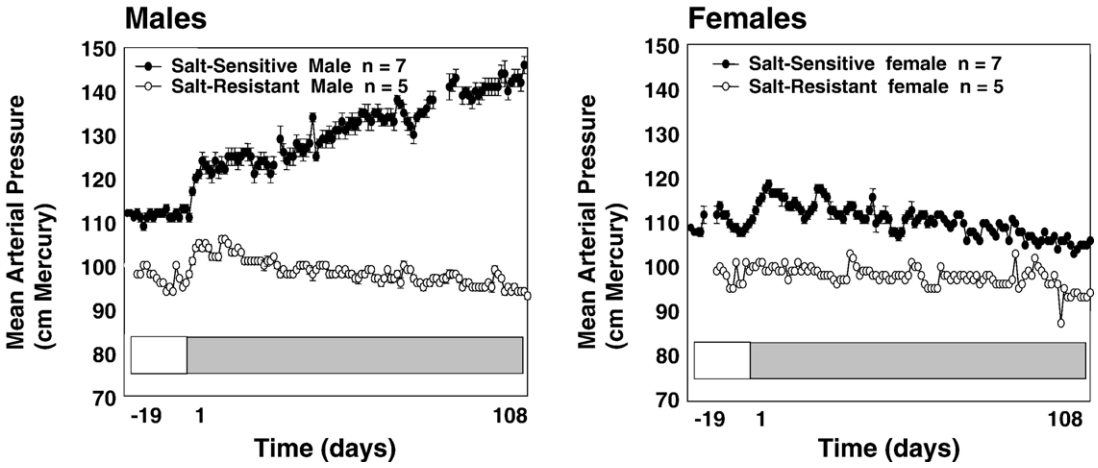

Fig. 1. Effects of high-salt diet on mean arterial blood pressure in Dahl-Iwai rats. Pressures were measured in a dedicated set of 12 rats, 3 per treatment group, by implantation of monitoring devices. After 19 days on a 0.1% NaCl diet, half of the male and female rats were switched to an 8% NaCl diet for 108 days.

Kidney histology

Kidneys were weighed, and histopathology was examined in kidney tissue slices from each rat at the 14-week endpoint (Table 1). Kidney weights were higher for rats on high-salt diets than for rats on low-salt diets regardless of strain, sex, or gonadectomy, reflecting water retention in response to high salt. Kidneys were scored 0–5 for glomerulosclerosis, tubular dilatation, tubular basophilia, interstitial fibrosis, and cellular infiltrate and received an overall grade for nephropathy if three or more conditions were observed. Pathology in each category was slight and sporadic or nonexistent in wild-type animals, similar or slightly more pronounced in GX-DI animals, and considerably greater in DI salt-sensitive rats of both sexes and diets. In the DI group, the high-salt diet increased the frequency and severity of tubular dilatation, tubular basophilia, interstitial fibrosis, and cellular infiltration, but not glomerulosclerosis. Care should be taken not to overinterpret these histological differences between treatment groups given the small sample sizes from this study. To gain a more detailed picture of biochemical events in these kidneys gene expression microarray analysis and immunohistochemical staining for CD44 were performed.

Microarray profiling

Kidneys were harvested, divided into outer medulla (OM) and cortex (CX) regions, and analyzed for expression of ~8800 genes using Affymetrix RG-U34A microarrays. While OM and CX gene expression profiles from a given treatment showed considerable overlap, enrichment of genes in OM or CX helped to narrow the cellular localization of each gene. Regulated expression in OM may indicate localized regulation in particular regions of the ascending or descending loop of Henle or in the attendant vasculature that is more abundant in medulla. Conversely, enrichment in cortex may indicate regulated expression in the glomerulus or in the proximal or distal convoluted tubules.

Expression values, along with statistical significance levels, were calculated with the SAFER algorithm for all of the 18 treatment ratios described under Materials and methods. An expression ratio was considered reliably and consistently

Table 1
Kidney histopathology

Treatment group	Kidney (% of body wt)	Nephropathy	Glomerulo-sclerosis	Tubular dilatation	Tubular basophilia	Interstitial fibrosis	Cellular infiltration	Cortico-medullary mineralization
SDF low	0.60	0.0	0.0	1.0	0.0	0.3	0.0	1.0
SDF hi	0.69	0.0	0.0	1.0	0.0	0.3	0.0	0.7
SDM low	0.61	0.3	0.3	0.7	1.0	1.0	0.0	0.7
SDM hi	0.70	0.0	0.0	1.0	0.0	0.0	0.0	0.0
DIF low	0.65	1.0	1.0	1.7	1.0	1.0	1.0	1.3
DIF hi	0.85	2.0	1.0	2.5	2.0	1.5	1.5	1.5
DIM low	0.60	1.0	1.0	1.0	1.0	1.0	0.3	0.0
DIM hi	0.75	2.0	1.3	2.3	2.0	2.0	1.3	0.0
GXF low	0.52	0.0	0.3	1.7	0.7	0.3	0.0	1.0
GXF hi	0.61	1.3	0.7	1.7	1.3	0.3	0.0	1.3
GXM low	0.49	0.3	0.0	1.0	0.3	1.0	0.0	0.0
GXM hi	0.64	0.7	0.7	2.0	1.0	0.7	0.0	0.0

Kidney histopathology was scored for each category from 0, no pathology, to 5, marked pathology. The average is presented for the three animals in each treatment group. Kidney wet weights are also presented as % of body weight.

"significant" if: (1) the ratio was more than 2 (up-regulation) or less than ½ (down-regulation), (2) the p value associated with the ratio was less than the significance level of 0.01, and (3) the average expression value in at least one of the two treatment samples was more than 40. To minimize the number of false positive differential expression values between treatment groups, a gene was selected only if it had a significant expression ratio in at least one of the 18 experiments. This filtering process resulted in a final set of 453 genes. A conservative estimate of the false discovery rate, using the mean of 5000 permutations, suggests that the FDR was approximately $1 \times 51/453 = 0.11$. Thus, we expect that about 51 of our 453 genes, or ~11%, are false positives.

To identify groups of coordinated genes influencing sex-specific kidney remodeling, these 453 selected genes were clustered using UPGMA, a hierarchical clustering algorithm, based on data from the 18 treatment ratios. Algorithm and metric were selected to generate nine discrete clusters that can each be intuitively described by a qualitative pattern of up, down, or no change at each of the 18 ratios. The dendrogram in Fig. 2 illustrates these relationships in detail, and colored bars mark discrete clusters. For clarity, the dendrogram branches were pruned. Genes were ordered into nine discrete clusters, A–J, each cluster representing a canonical profile of gene regulation according to rat strain, sex, and dietary salt. Supplemental Table 3 details expression ratio data for representative genes from these clusters.

To uncover subtle gene expression profile relationships between pairs of treatment groups, a Spearman's correlation coefficient was calculated for each possible pair of ratios represented in Fig. 2, considering the 453 genes showing significant changes. Table 2 lists these correlations. Correlations were most informative in the OM region, where the majority of gene responses to salt were observed.

Pairwise comparisons were examined to ascertain whether sex differences in DI rats' responses to salt comprised responses to androgens in males or responses to estrogens in females. To answer this question, salt response profiles of GX-DI males and GX-DI females were compared to salt response profiles of intact male DI or female DI rats. Table 2 indicates that the GX-DI female OM salt response profile resembled the salt response profile of DI female OM ($\rho = 0.83$), rather than the profile of male DI rats ($\rho = -0.27$). This robust correlation ($\rho = 0.83$) indicates that after 14 weeks of ovariectomy, female rats retained nearly the same gamut of gene responses to high salt seen in the intact DI females. Furthermore, the GX-DI male OM salt response profile also resembled the salt response profile of DI female OM ($\rho = 0.58$) rather than the salt response profile of male DI OM ($\rho = -0.11$). Fig. 3 shows relevant scatter plots for expression ratios across significantly related treatment pairs. These contrasts indicated that the overwhelming majority of genes in GX-DI male or GX-DI female rat kidney OMs responded to salt like those in DI females rather than DI males; hence, these sex differences arose predominately from the effects of androgens. Correlation coefficients for most other pairs were unremarkable (Table 2).

Male-selective gene expression in low-salt-fed DI rats

Female and male Dahl-Iwai rat kidneys exhibited many sex-specific differences in gene expression even on low-salt diets. Sixteen Cluster F and 58 Cluster I genes were male-selective in expression in both DI and SD rats fed low-salt diets. Male-selective expression was confirmed using TaqMan quantitative RT-PCR for *Agxt2, Gamt, Cyp2d10,* and *Prlr.* Cluster F genes were expressed in a male-selective manner in both OM and CX, whereas Cluster I genes showed male selectivity only in OM. Most of the genes that were sex-selective in DI rats were also sex-selective in SD rats (Fig. 2). The highly similar gene expression patterns between DI and SD rats attest to experimental consistency between the two rat experiments and between the microarray measurements. This similarity also demonstrates that the two rat strains utilize similar homeostatic gene expression programs under low-salt dietary conditions. Male-selective expression was observed predominantly in outer medulla rather than in cortex, suggesting a sex difference manifested in the loop of Henle and/or in blood vessels, structures enriched in outer medulla. This male-selective canon could indicate gene expression stimulated by male hormones or suppression by female hormones.

Why do kidneys of males have a different gene expression profile compared to those of females? This sex difference could be a manifestation of the blunting of pressure natriuresis that conserves sodium and raises blood pressure in male rats and humans. Alternatively, it could be a prospective adaptation to the increased demand on the kidneys in gestating female rats. At the histological level, sex differences have been observed in proximal tubule epithelial cells, differences that are altered by gonadectomy [16,17]. The functional significance of these histological observations is unclear.

Several of these genes that differentiate male and female rats may execute functions relevant to the blunting of pressure natriuresis that characterizes male rats and humans. For example, the angiotensin II/vasopressin receptor *Nalp6* mRNA was expressed at higher levels in male than in female OM (Fig. 2). This male-selective expression was marked in SD rats (9.6-fold) and moderate in DI rats (1.7-fold). Angiotensin and vasopressin signaling via the NALP6 causes vasoconstriction and reduced natriuresis. This male selectivity is consistent with greater activation of the renin-angiotensin system observed in male humans and some rat strains [9]. The moderated male specificity observed in DI compared to SD rats may be a compensation for the increased sensitivity to angiotensin II observed in Dahl salt-sensitive rats (see Physgen Web site: http://canadeo.brc.mcw.edu/pga/). The response of the *Nalp6* gene to male hormones has been previously observed. Testosterone was shown to restore expression of the vasopressin receptor in medullary collecting ducts of the aging rat [18]. Testosterone elevation of *Nalp6* expression is a possible mechanism by which males maintain blunting of pressure natriuresis.

Like Cluster I, Cluster F genes exhibited male-selective expression in OM, but differed in also exhibiting male-specific expression in CX. Like *Nalp6*, proangiotensinogen *Agt* mRNA

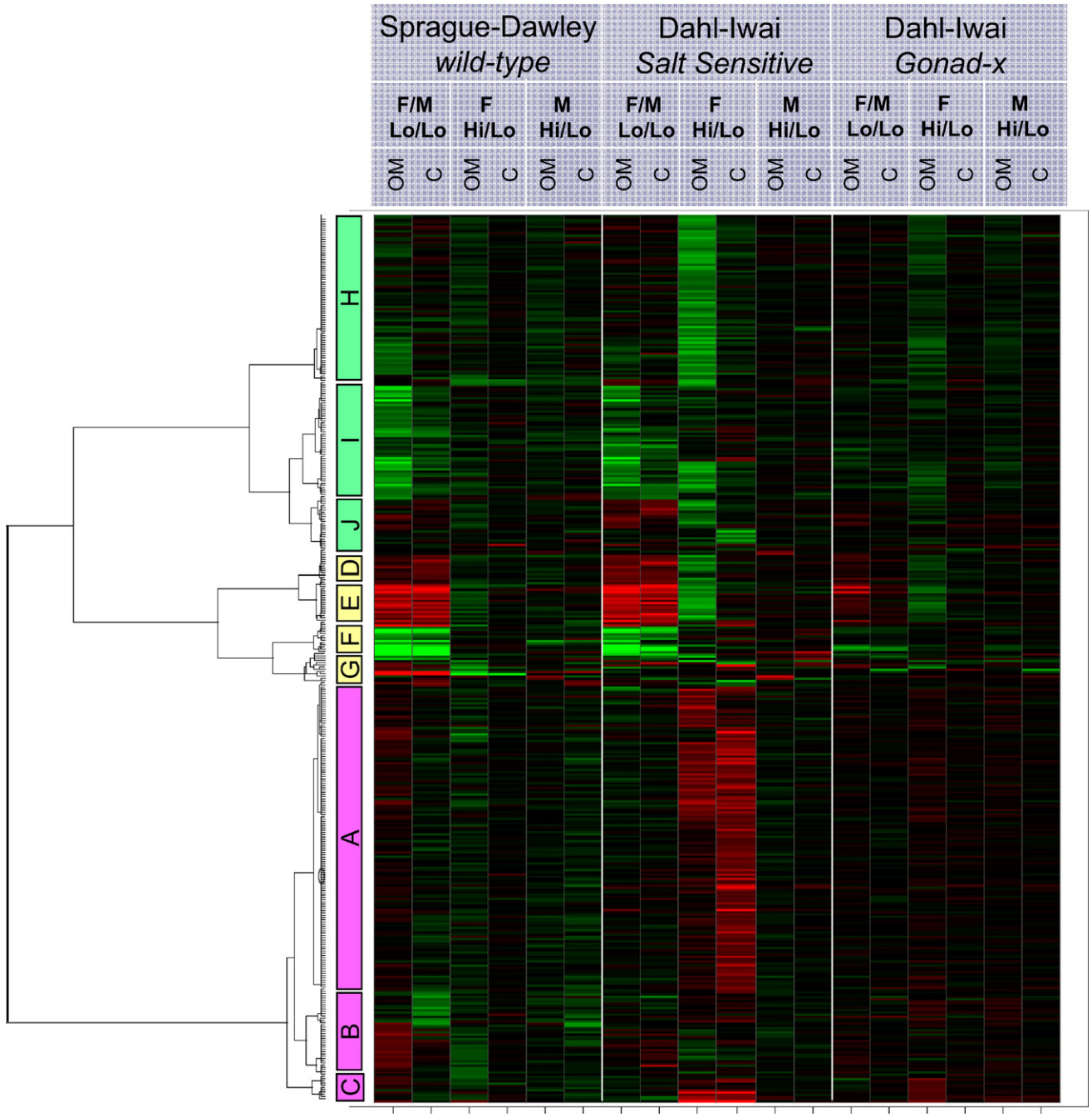

Fig. 2. Gene expression ratios from rat kidney tissues are shown for 453 genes that exhibited $p < 0.01$ and > 2-fold regulation in one or more ratios. Red indicates a ratio > 1, green indicates a ratio and <1, and black indicates a ratio approximately = 1. Brighter colors reflect greater deviance from ratio of 1 up to saturation at 10-fold. Note that the SAFER error model calculates expression values more conservatively than the standard Affymetrix "Average Difference" algorithm. Female/male expression ratios in rats on low-salt diets are represented by columns 1, 2, 7, 8, 13, and 14. Odd column numbers represent outer medulla tissue, whereas even column numbers represent cortex tissue. Key: OM, outer medulla tissue; C, cortex tissue; Hi/Lo, expression ratio of high-salt fed rats to low-salt fed rats; F, female rats; M, male rats.

expression was detected differentially in male kidneys, suggesting a higher capacity for angiotensin II hormone production in males.

Prolactin receptor *Prlr* transcripts, both long- and short-splice forms of *Prlr,* were expressed differentially in male DI animals. Two probe sets, representing prolactin receptor short-splice form transcript L48060 and medium-splice form transcript M19304, appeared in Cluster I with male-selective expression in the OM of DI and SD rats. Two probe sets representing long-splice forms of prolactin receptor with common 3′ sequences appear in Cluster F, indicating male-selective expression of the long form in DI and SD rats in both OM and CX. Additionally, both *Prlr* long forms were down-regulated in males fed the high-salt diet in SD but not in DI rats. Prolactin regulates multiple functions in mammals, including lactation, testicular testosterone secretion, and immune functions [19]. Hyperprolactinemia was observed to increase aldosterone secretion by the rat kidney [20],

Table 2
Spearman correlation coefficients comparing responses of pairs of treatment groups to high-salt diet

Treatment group	SD F	SD M	DI F	DI M	GX-DI F	GX-DI M
SD F		*−0.01*	*0.13*	*0.02*	*0.23*	*0.13*
SD M	0.22		*−0.39*	*−0.12*	*−0.10*	*0.01*
DI F	0.24	0.30		*0.01*	*0.21*	*0.17*
DI M	0.09	0.03	−0.29		*0.17*	*0.12*
GX-DI F	0.17	0.23	**0.83**	−0.27		*0.37*
GX-DI M	0.02	0.34	**0.58**	−0.11	**0.65**	

Each entry represents the correlation between changes in 453 genes between the pair of treatment groups designated by row or column. The lower left part contains correlations related to high-salt versus low-salt diet responses in kidney samples from outer medulla. The italicized numbers in the upper right portion are correlation coefficients in cortex. All statistically significant correlations (p value < 0.001) with an absolute value greater than 0.5 are shown in bold. F, female; M, male.

suggesting that male-selective prolactin receptor expression is a possible mediator of blunting of pressure natriuresis in males. Although some researchers reported no effects of prolactin in mammalian kidney function, Bussieres et al. provided evidence that prolactin stimulates N^+/K^+ ATPase activity directly in the distal nephron [21]. Kau et al. [20] observed that aldosterone levels increased in kidneys of ovariectomized rats implanted with anterior pituitary cells. They suggested that this was a result of prolactin secreted by the transplanted pituitary cells, resulting in increased blood pressure [20]. Although a role for prolactin osmoregulation of the mammalian kidney is not firmly established, an evolutionary precedent suggests such a role. Prolactin and its receptor play a major osmoregulatory role in fishes that move between salt and fresh water, where they decrease water uptake and increase Na^+ and Cl^- retention [22]. The precedent in fishes and these expression data support a role for prolactin in mediating the blunting of pressure natriuresis selectively in male rats. Additionally, the failure of salt-sensitive male DI rats to down-regulate *Prlr* long-form genes, as SD males did, suggests *Prlr* may play a role downstream of a salt-sensitivity gene in male DI rats. These observations call for further investigation of the role of the prolactin receptor in regulating kidney function.

A variety of phase II drug-conjugating enzyme genes were differentially expressed in male SD and DI rats, including *Ugt1a1, Gsta1, Mgst1,* and *Sult1b1*. Since these genes are known to be regulated collectively by oxidative stress, these observations suggest an oxidative stress response in males [23].

Cyp2c11 was expressed in a male-selective pattern in both OM and CX of DI and SD rats (Fig. 2). *Cyp2c11* was induced by high-salt diet in male DI but not male SD rats. *Cyp2c11* is thought to metabolize testosterone, but also to form epoxyeicosatrienoic acids, or EETs, from arachidonic acid-derived precursors [24]. These EETs are generally dilatory and natriuretic in the nephron. Thus, *Cyp2c11* may play a role in modulating salt stress to the male kidney.

A2m, encoding α2-macroglobulin, was expressed selectively in OM of male rats.α2-Macroglobulin is a circulating proteinase inhibitor. It is present in blood and urine at much higher levels in male rats than in female rats, and A2M is produced in higher amounts during the acute-phase response. As *A2m* has been shown to sequester TGFβ, inhibit collagenases [25], and reduce cardiac fibrosis [26], it may function to inhibit fibrosis in kidneys of male rats.

Female-selective gene expression in low-salt-fed DI rats

Clusters D and E comprised 15 and 21 genes, respectively, genes expressed selectively in females in both DI and SD rats fed low-salt diets. Female-selective expression was confirmed using TaqMan quantitative RT-PCR for *Akr1b4, Igfbp1,* and *Sod3*. Cluster D genes were expressed selectively in females in OM and CX of DI rats and in CX but not OM of SD rats. Cluster E genes were expressed selectively in females in OM

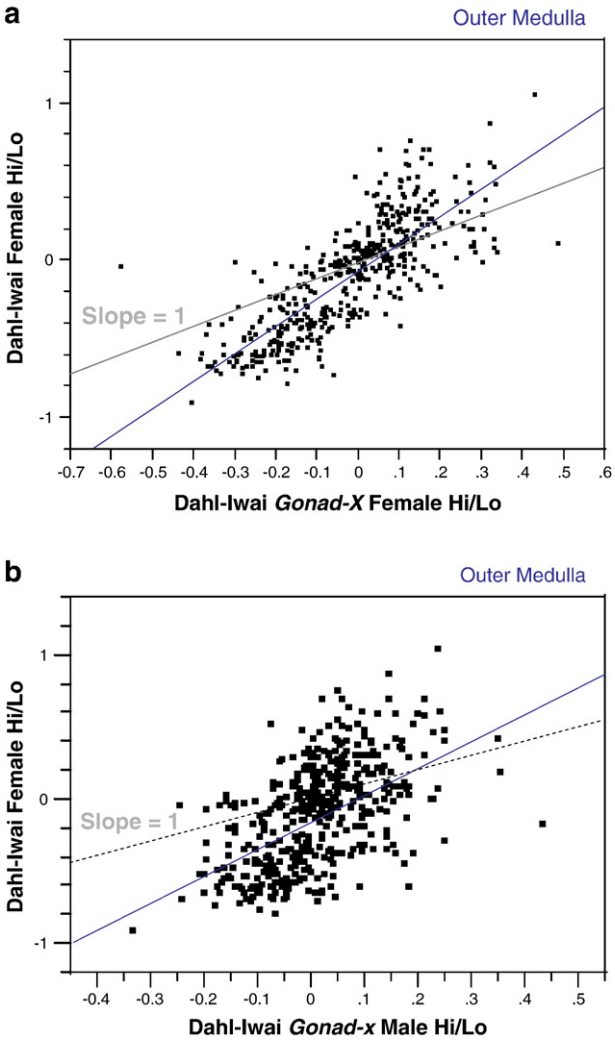

Fig. 3. Scatter plots for expression ratios (in logarithmic scale) across significantly related treatment pairs from the outer medulla region. The dark diagonal shows a best-fit line, and the dotted line with slope = 1 is shown as a reference. The best-fit lines with slopes > 1 indicate greater overall responses to high salt in the female DI rats than in gonadectomized males or females. (a) Female Hi/Lo Dahl-Iwai salt-sensitive vs Gonad-x female Hi/Lo Dahl-Iwai; (b) female Hi/Lo Dahl-Iwai salt-sensitive vs. Gonad-x male Hi/Lo Dahl-Iwai. "Hi/Lo" indicates expression ratio of high-salt-fed rats to low-salt-fed rats.

and CX of DI rats and in OM and CX of SD rats. Genes from both Clusters D and E were down-regulated by high-salt diet in female DI rats.

No less than five *Cyp4* gene subfamily members were significantly regulated in these experiments. All five were down-regulated by high-salt diet in OM of female DI rats. Four of these genes were also down-regulated by high-salt diet in OM of gonadectomized female DI rats, *Cyp4a22, -a3, -a12,* and *-f14,* albeit *Cyp4a12* was not statistically significant. CYP4A22 and other *Cyp4* gene products produce hydroxyeicosatetraenoic acids, principally 20-HETE. 20-HETE is an autacoid hormone that limits glomerular filtration in CX by constricting afferent arterioles. 20-HETE also inhibits Na^+/K^+ ATPase and $Na^+/K^+/2Cl^-$ cotransporters in proximal tubules and the thick ascending loop of Henle, promoting natriuresis [24]. 20-HETE synthesis increases during gestation in pregnant rats [27], suggesting that higher *Cyp4a22* expression in females may be a preadaptation to pregnancy. Despite these similarities, differences in regulation placed the five *Cyp4* genes in five different clusters. *Cyp4a22* and *Cyp4b1* were expressed selectively in females of both DI and SD strains. *Cyp4b1* was unique in being up-regulated by high salt in female DI rats in CX. *Cyp4a12* expression was female selective in CX of DI rats. *Cyp4a14*, by contrast, was expressed selectively in males of both DI and SD strains. There is precedent for sex-selective regulation of *Cyp4a* genes. For example, androgen treatment caused increases in *Cyp4a12* and decreases in *Cyp4a22* mRNAs, increased biosynthesis of 20-HETE, and increased systolic blood pressure in male and female rats [28]. The concerted down-regulation of these genes in females suggests decreased production of 20-HETE and possibly other eicosanoid hormones. Decreased 20-HETE in turn suggests adaptive decreased renal arterial pressure and increase glomerular filtration rate, but also increased recovery of sodium and other cations [24]. Decreased blood pressure in female rats relative to males is consistent with observations in various salt-sensitive rat strains. We speculate that Cyp4a down-regulation by high-salt diet in female rats may be a protective adaptation to facilitate increased filtration load during gestation. Although a comprehensive picture of CYP4 enzymatic products, localization, and adaptive gene regulation is lacking, these data are consistent with an intricate role for *Cyp4* genes in regulation of filtration and ion balance in the kidney.

Steroid 5α-reductase (*Srd5a1,* Cluster A) expression was increased five- to sixfold by high-salt diet in the CX of female kidney, but not male kidney. SRD5A1 converts testosterone irreversibly to the more potent androgen dihydrotestosterone [8]. This androgen may trigger vasoconstriction via regulating *Cyp4a22* and *Cyp4a12* as described above. Alternatively, SRD5A1 is capable of inactivating corticosterone, a mineralocorticoid that exerts an antinatriuretic action by increasing transcription and activity of Na^+/K^+ ATPase [29]. Corticosterone plays a critical role in rats, because among mineralocorticoids, rat adrenals secrete almost exclusively corticosterone, whereas human adrenals secrete ~7:1 cortisol:corticosterone plus aldosterone, dehydroepiandrosterone, and androstenedione [30]. By inactivating corticosterone SRD5A1 would increase natriuresis in females. Thus, increased SRD5A1 expression may be a mechanism by which females minimize hypertension relative to males.

The female-selective expression of 36 Cluster D and E genes was dramatically reduced or eliminated by gonadectomy with the exception of 5 genes: *Adh1, Slc10a2, Angpt2, Ly6a,* and *Ly6b.* None of these genes are sex linked. *Adh1* encodes alcohol dehydrogenase 1; *Ibat* encodes intestinal bile acid transporter. *Ly6*-family genes encode small signaling proteins anchored in the cytoplasmic membranes that play roles in the differentiation of hemopoietic cells and in T cell activation. It is unknown whether these genes maintain sex-selective expression 14 weeks postgonadectomy due to an irreversible developmental effect of sex hormones or due to a persistent epigenetic effect.

Female-specific response to salt in DI rats

Cluster A is a group of genes that were up-regulated in female DI rats fed a high-salt diet in OM or CX or both. Female DI rat kidneys carried out a complex change in gene expression in response to high-salt diet, a change comprising significantly altered expression of 156 genes (Fig. 2, lanes 9 and 10). This global response was entirely absent from male DI rats on the high-salt diet (Fig. 2, lanes 11 and 12). These gene expression changes were consistent between rats in the DI groups, but were not observed in SD rats (Fig. 2, lanes 3 and 4) or in gonadectomized male or female DI rats (Fig. 2, lanes 15–18). Since the gonadectomized DI rats were studied in a separate experiment from the DI rats, albeit from the same colony, we cannot be sure that the genetic risk factors for salt sensitivity were identical between studies.

Immune response

Clusters A and C comprising the female-specific response to salt were examined to determine the nature of the response. Cluster A and C genes were induced by the high-salt diet in female DI rats, but not in male DI rats nor SD rats (Fig. 2, lanes 9 and 10). Such clusters were highly enriched for genes playing roles in immune responses and in extracellular matrix remodeling and cell-matrix signaling. The immune response-related genes included several genes that are known to be induced by interferons. These include the genes *Cxcl10/Mob1, Mx1, EST X61381, Irf1, Irf7,* and *Irf8,* encoding cytokine MOB-1, interferon-induced GTP-binding protein MX-1, interferon-induced mRNA, and interferon regulatory factors -1, -7, and -8. Interferon regulatory factors -1, -7, and -8 are transcription factors that bind to the interferon-stimulated regulatory element in promoters of interferon-regulated genes [31]. The immune response-related genes included complement components *C1qb* and *C4a*, *Major histocompatibility complex I* and *II* (*RT1@, Hladma*), *Ly6c,* B cell attractant chemokine *Cxcl13, Aif1, Il18, Tnfsf13, Cd53,* and *Spp1* encoding osteopontin. These genes are collectively characteristic of infiltration of B cells, T cells, and macrophages into tubulointerstitial spaces in the complex cascade that triggers fibrosis. Tubulointerstitial fibrosis is thought to originate with IL-1 and TNFα production by

lymphocytes. These lymphocytes in turn activate tubule epithelial cells that produce chemokines and cell adhesion molecules that attract and activate macrophages. Both the tubule epithelial cells and the macrophages then make cytokines that activate fibroblasts and myofibroblasts, which in turn produce collagen and noncollagen matrix that comprises fibrosis [32].

Remodeling response

Cluster A and Cluster C, containing the immune response genes, also include genes for components of extracellular matrix (ECM) glycoproteins, cell–matrix and cell–cell signaling, cell growth, and cellular differentiation, collectively indicating remodeling of the kidneys of female DI rats. The similar regulations of immune response and remodeling genes observed in these experiments support a cause–effect relationship between the immune response and the remodeling response. In an analogous study, characterization of interstitial fibrosis in a mouse model of Alport syndrome demonstrated that macrophage infiltration preceded ECM remodeling [33]. It is notable that Cluster A, the female-specific response of DI rats to high salt, shared many orthologous immune response genes and remodeling genes with the interstitial fibrosis characterized in the Alport mouse. Indeed activated inflammatory cells are known to stimulate ECM synthesis via secretion of eicosanoids, TGFβ, and interleukin-1. Extracellular matrix genes induced in female salt-challenged DI rats as well as the Alport model included genes for collagen Ia1, *Col1a1;* collagen IIIa1, *Col3a1;* and fibronectin, *Fn1;* and ECM-modifying genes encoding lysyl oxidase, *Lox,* and tissue inhibitor of metalloproteinase 1, *Timp1*. These are well-characterized markers for interstitial fibrosis, and most are known to be produced by interstitial myofibroblasts or by proximal tubule epithelial cells that can differentiate into myofibroblasts [33]. The cluster also includes cell–matrix and cell–cell signaling genes. Cell–cell signaling genes induced included kidney injury molecule-1 (*KIM-1*), indicative of regeneration of epithelial cells of the proximal tubule [34], and *Connexin-26, Gjb* a component of intercellular gap junctions. Cell–matrix signaling genes include *Megalin/Lrp2,* a receptor that mediates endocytosis; *Glycam1,* and *Integrin α1.* Induction of *Cytokeratin8, Krt8; Vascular cell adhesion molecule-1, Vcam;* and *Intercellular adhesion molecule-1, Icam1;* indicated recruitment and infiltration of vasculature by inflammatory cells [35]. As a whole, these gene inductions describe an inflammatory and remodeling response including matrix remodeling, and cell replacement.

Like Cluster A genes, 8 of 12 Cluster C genes were up-regulated in DI females by a high-salt diet. Cluster C differs from Cluster A in that all 12 genes are definitively up-regulated by a high-salt diet in OM of female GX-DI rats as well as in female DI rats. This result indicates that these genes somehow "remember" that they are in female kidneys despite 14 weeks without the female hormonal milieu. This effect appears to be specific to the outer medulla and may arise from a sex-specific developmental event or a persistent epigenetic feature such as DNA methylation. Several of these genes are part of the inflammation/remodeling cascade described for Cluster A, including *Tgfb1i4,* encoding a TGFβ-inducible transcription factor that regulates the C-type natriuretic peptide gene promoter [36]; *Timp1,* encoding tissue inhibitor of metalloproteinase 1 [37]; *Irf1,* encoding interferon regulatory factor-1; and *Col1a1* and *Col1a2,* encoding collagens 1A1 and 1A2.

Eicosanoid response

A variety of genes that regulate eicosanoid hormone production or breakdown responded to high-salt diet in DI rats. Subsets of eicosanoid hormones are known to be natriuretic and vasodilatory (EETs) or antinatriuretic and vasoconstrictive (HETEs). Cytochrome P450 genes capable of generating EETs, *Cyp2c23, Cyp2c11,* and *Cyp2e1,* were up-regulated by salt in male DI rats. This response in males suggests increased production of EETs as an adaptation to salt stress. In contrast, female DI rats responded to salt by down-regulating CYP genes for HETE production, *Cyp4a14, Cyp4a8,* and *Cyp4b1,* as well as the genes for EET production (described above). It is unclear whether this response in females is an adaptation to stress or simply part of the global decrease in metabolism described below.

Redox response

Female DI rats responded to high salt by a mixed induction and repression of genes responsible for scavenging oxygen radicals and reactive organic compounds. Fig. 2 illustrates induction of genes for metallothionein 1 (*Mt1*) and glutathione peroxidase 2 *Gpx2*. In contrast, extracellular superoxide dismutase (*Sod3*), plasma glutathione peroxidase *Gpx3*, glutathione synthetase *Gss*, and flavin-containing monooxygenase 1 (*Fmo1*) were all suppressed by the high-salt diet in female DI rats. These induction and suppression events were not observed in male DI rats on a high-salt diet. It is interesting to note, however, that *Sod3* mRNA was expressed at high levels in OM and CX of female, but not male, rats on low-salt diets. *Sod3* encodes the superoxide dismutase present on the vascular endothelium. This *Sod3* expression may reflect a higher rate of NO production in kidney vasculature of female vs male DI and SD rats, a possible contributor to the lower arterial pressure observed in female DI rats.

Metabolic response

Genes in Clusters H, D, and E and subsets of Clusters I and J were down-regulated in female DI rats fed a high-salt diet, almost exclusively in OM (Fig. 2). This response was also seen in OM of female GX-DI rats, but was nearly absent in wild-type SD rats. Kidney medulla tissue primarily uses glucose and stored glycogen for energy via anaerobic and aerobic glycolysis [38]. This apparent suppression of energy metabolism may be a consequence of the inflammation and fibrosis occurring in these OM tissues. This pattern indicates metabolic suppression in response to injury in salt-sensitive rats that is blocked by androgens.

These genes are predominantly energy metabolism genes, including down-regulation of five peroxisomal genes encoding

fatty acid oxidation enzymes: *Slc27a2,* very-long-chain acyl-CoA synthetase; *Acaa1,* ketoacyl-CoA thiolase; *Ehhadh,* bifunctional enzyme; *Hao2,* long chain α-hydroxy acid oxidase; and *Ech1,* enoyl hydratase-like protein. This observation indicates a down-regulation of peroxisomal fatty acid catabolism.

This response also includes down-regulation of gluconeogenic genes *Aldob,* fructose bisphosphate aldolase B; *Fbp1,* fructose bisphosphatase 1; *G6P,* glucose 6-phosphatase; and *Pck1,* phosphoenolpyruvate carboxykinase. Down-regulation of these four genes indicates decreased gluconeogenesis in high-salt fed DI females. Winiarska et al. demonstrated a close reciprocal relationship between the rate of gluconeogenesis and the reduced/oxidized glutathione ratio in rabbit kidney tubule primary cells [39]. They showed that stimulation of gluconeogenesis resulted in increased activity of the pentose phosphate pathway (PPP). The PPP generates NADPH needed for reduction of glutathione. Conversely, a high glucose supply suppressed gluconeogenesis, PPP, and glutathione reduction, as well as glutathione synthesis via γ-glutamyl cysteine synthetase. Genes encoding steps in glutathione synthesis, *Gclc*,γ-glutamyl cysteine synthetase; *Gclm*, γ-glutamyl cysteine synthetase regulatory subunit; and *Gss,* glutathione synthetase; as well as *Ggt1*, γ-glutamyl transpeptidase, which functions to maintain intracellular reduced glutathione levels, were down-regulated in female high-salt kidneys. This relationship and the similar regulation patterns of genes for gluconeogenesis and glutathione suggested a potential depletion of reduced glutathione [39], possibly resulting in the redox stress response described above.

Amino acid metabolic genes were also down-regulated in female DI rat OM. These include *Gnmt,* glycine methyltransferase, and *Gamt,* guanidinoacetate methyltransferase; *Ahcy, S*-adenosylhomocysteine; *Cth,* cystathionine γ-lyase; *Csad,* cysteine sulfinic acid decarboxylase; *Cdo1,* L-cysteine oxygen dioxygenase; *Pah,* phenylalanine hydroxylase; *Abat*, β-alanine oxoglutarate aminotransferase; *Psat1,* phosphoserine aminotransferase; and *Ubp1*, β-alanine synthase.

The *Odc1* gene encoding ornithine decarboxylase was sharply down-regulated in high-salt-fed SD and DI female rats. Interestingly, *Odc1* was down-regulated in both OM and CX of SD rats but only in OM of DI rats. Ornithine decarboxylase is rate limiting for polyamine biosynthesis. Polyamines are regulators of cell growth that in the kidney promote kidney proximal tubular hypertrophy [40]. Down-regulation of *Odc1* suggests that the female rats in this study do not undergo proximal tubule hypertrophy.

Guca2a, guanylin, was strongly up-regulated in female DI OM by high-salt diet. Guanylin is a hormone that triggers natriuresis and diuresis by activating cGMP formation [41]. Thus, *Guca2a* up-regulation may be a component of female resistance to hypertension by increasing natriuresis.

Male responses to salt in DI rats

Male DI rats responded to high-salt diet with a small number of gene expression changes, despite the precipitous rise in blood pressure (Fig. 1) and moderate histological damage. Thus while female DI rats adapt to salt insult by remodeling, the males apparently tolerate insult and escalating blood pressure. This global difference between male and female DI kidney responses to salt insult raises the question of whether the remodeling response is enabled by female hormones, or is suppressed by male hormones, or whether sex hormones irreversibly influence this response during development. Previous studies showed that castrated males of various salt-sensitive strains modulated blood pressure to the levels of females [9]. Studies with ovariectomized females have been equivocal regarding whether female hormones modulate blood pressure or are neutral [9]. Thus we compared salt-sensitive DI responses to salt-resistant SD responses to identify which gene expression changes are normal adaptations to salt (SD rats) versus responses to insults that include kidney damage (male DI rats) and adaptive kidney remodeling (female DI rats).

Sex differences in wild-type rats

Wild-type SD rats were examined to determine how their kidneys react to a high-salt diet. Male and female rats were fed high- or low-salt diets for 14 weeks following the same protocol as the DI rats. SD rats did not show steady increases in blood pressure as observed in male DI rats [14]. Gene expression profiles were assayed in parallel for all the SD rats such that the four treatment groups could be compared as ratios. With the low-salt diet, sex differences in SD rats were nearly identical to sex differences in DI rats (compare Fig. 2, lanes 1 and 2 to lanes 7 and 8). This similarity indicated that rats of both sexes and both DI and SD strains were relatively unstressed on low-salt diets. This similarity also provided an internal control, establishing the consistency and reliability of the gene expression profile data, the consistency of the kidney dissections, and the consistency of sex differences between SD and DI strains.

Cluster B is a group of 44 genes expressed selectively in OM of female vs male SD rats, much more strongly than in OM of female vs male DI rats. Sex selectivity was not observed for Cluster B genes in GX-DI rats. Most Cluster B genes were modulated in expression by a high-salt diet.

Female responses to salt in wild-type rats

Female SD rats reacted to the high-salt diet with a small subset of the changes seen in female DI rats on the high-salt diet (compare Fig. 2, lanes 3 and 4 to lanes 9 and 10). Only *Stearoyl-CoA desaturase 2* or *Scd2* was up-regulated by the high-salt diet in female SD rats, whereas several genes were down-regulated. Down-regulated genes included *Ornithine decarboxylase, Odc1;* and *Alcohol dehydrogenase 1, Adh1;* genes that were also down-regulated by salt in DI rats, suggesting they were part of the adaptation to salt stress in normal and salt-sensitive animals. The small number of gene changes seen in female SD rats in response to the high-salt diet reflects the salt-resistant phenotype of SD rats, requiring fewer adaptations to salt stress.

Male responses to salt in wild-type SD rats

Male SD rats fed high- versus low-salt diets yielded a small number of gene expression changes, changes that may have been adaptations to the high-salt diet to maintain filtration and constant blood pressure. These adaptive changes in male SD rats had few genes in common with the adaptations to salt by female SD rats, an indication that even salt-resistant rats react differently to salt according to sex. For example, it was noted that *Prlr* was not only expressed in a male-selective pattern in SD and DI rats, but also down-regulated by high-salt diet in SD but not DI rats. This expression pattern further suggests a role for *Prlr* in male-specific blunting of pressure natriuresis, with adaptive reduction of expression in response to high-salt diet in wt SD males, but not in DI males. This difference suggests a mechanism underlying male blunting of pressure natriuresis, a phenomenon common to various rat strains and humans [8].

The adaptive changes by male SD rats also have few genes in common with the adaptations to salt by male DI rats (Table 2, $\rho = 0.03$; compare Fig. 2, lanes 5 and 6 to lanes 11 and 12). These strain differences indicate that salt-sensitive male DI rats responded to insults to their kidneys, insults that include escalating whole-body blood pressure and moderate histological damage.

Male/female differences in gonadectomized DI rats

Male and female DI rats were gonadectomized to examine how this would affect both baseline sex differences in gene expression and the responses of males and females to a high-salt diet. Fig. 2 (lanes 13 and 14) shows that gonadectomy reduced the sex differences relative to kidneys of intact male versus female DI rats (lanes 7 and 8). Interestingly, kidney gene expression 14 weeks postgonadectomy did retain a small number of sex differences seen in intact DI rats. These differences include female-selective expression of *Fibrinogen α, Fga; Insulin-like growth factor binding protein 1, Igfbp1; Angiopoietin-2, Angpt2; 7Ileal sodium-dependent bile acid transporter, Slc10a2;* and *Alcohol dehydrogenase, Adh1.* Male-selective expression was maintained in GX-DI rat kidneys of *Organic cation transporter, Slc22a2; transferrin, Tf; Histamine N-methyltransferase, Hnmt; Cyp2d10;* and *Type 13 α-hydroxysteroid dehydrogenase/LOC307095.* These may represent developmental differences that persisted for 14 weeks postgonadectomy or genes that were epigenetically programmed under the influence of sex hormones.

Female responses to salt in GX-DI rats

Gonadectomized females were examined to determine whether the loss of female hormones would abrogate the female DI response to high salt. Female DI rats lacking ovaries maintained a gene expression response to a high-salt diet that was highly correlated with the response of intact females (Fig. 2b, $\rho = 0.83$). Specifically, the inflammation and remodeling genes that were up-regulated by salt in intact female DI rats were also up-regulated following ovariectomy. Similarly, many of the genes that were down-regulated by high salt in intact female DI rats were also down-regulated in ovariectomized rats. Fig. 3a indicates that the overall response of intact female GI rats was maintained in OM of GX-female DI rats although the gene expression responses were uniformly less pronounced in the GX-DI rats, resulting in few genes achieving statistical significance in GX females. Whether this moderated response in GX females arose from female hormones potentiating the inflammation and remodeling response in intact DI rat kidneys or from some experimental variability between the two experiments is unknown.

Sex differences between SD and DI rats on the low-salt diet

Cluster G is a group of 15 genes expressed with different sex biases in SD compared to DI rats on the low-salt diet, as well as different responses to salt in SD compared to DI rats. *Igfbp1* was the sole gene that was significantly up-regulated by the high-salt diet in both male and female DI rats (Cluster G). This gene was also expressed in a female-biased manner in low-salt-fed DI rats. *Igfbp1* was not significantly regulated by salt in male or female SD rats. This indicated that *Igfbp1* expression was increased as a response to salt stress in salt-sensitive DI rats of both sexes. While *Igfbp1* up-regulation by salt in DI rats occurred in both OM and CX, up-regulation by salt was maintained by both male and female GX-DI rats selectively in the OM. Female-selective basal expression and induction by high salt in males were similarly retained in GX-DI rats, again preferentially in OM. In a variety of cell types IGF1 promotes cell growth and promotes anabolic activity. IGFBPs bind and sequester IGF1 within blood vessels, but also serve to direct IGF1 to specific cell types. An increased ratio of IGFBP1 to IGFBP3 expression in the kidney is thought to promote increased partitioning of IGF1 to the nephron and increased action of IGF1 in target kidney cells. In human liver cirrhotic patients, the increased IGF1:IGFBP1 availability in the nephron correlated with increased renal blood flow and increased sodium retention [42]. In a model of rat renal toxicity, increased *Igfbp*1 expression was associated with renal tissue remodeling [43]. The presumed sodium retentive effect of IGFBP1 suggests a causative hypertensive effect in DI salt-sensitive rats; however, this study does not resolve whether IGFBP1 promotes hypertension or is a homeostatic effect of salt or hormones, possibly promoting kidney remodeling.

Slc12a3 and *Ephx2* also displayed very different regulation in DI compared to SD rats. *Slc12a3* encodes the thiazide-sensitive Na^+/Cl^- transporter that recovers sodium from the kidney filtrate [44]. *Ephx2* encodes cytosolic epoxide hydrolase, which is thought to metabolize vasoconstrictive antinatriuretic HETE eicosanoid hormones. These genes were expressed in a female-selective pattern in SD rats, suggesting they are components of the reduced hypertension observed in female rats. This female-biased expression was noted previously for SLC12A3 protein [45] but expression in a sex-biased manner

was not examined in DI rats. Both genes were up-regulated by high salt in male SD rats, suggesting they are a component of male blunting of pressure natriuresis. Both *Slc12a3* and *Ephx2* were down-regulated by high salt in female DI rats, suggesting an adaptation to increased pressure promoting natriuresis in females.

Male responses to salt in GX-DI rats

Since published data indicate a role for androgens in raising blood pressure and potentiating blunting of pressure natriuresis, we asked how orchidectomized males would respond to a high-salt diet. Orchidectomized male GX-DI rat kidneys' response to a high-salt diet was highly correlated with that of female DI kidneys ($r=0.58$ for OM) rather than that of male DI kidneys ($r=-0.11$ for OM). Thus, orchidectomized male GX-DI rat kidneys responded to a high-salt diet much like female DI kidneys. The dot plot in Fig. 3b illustrates this correlation and reveals that the magnitude of gene expression changes was generally smaller in the GX-DI males compared to intact DI females. Since gonadectomy also reduced the responses in GX female DI rats, this reduced response may have stemmed from potentiation by female hormones or from variation between the two experiments.

Immunohistochemistry

At 14 weeks histopathology showed only a moderate pathology in any of the treatment groups (Table 1), whereas gene expression data indicated transcriptional induction of a variety of genes characteristic of immune activation and fibrosis (Fig. 2). To resolve the extent of inflammation and remodeling, we investigated expression of CD44 protein using immunohistochemistry. CD44 is a well-characterized indicator of kidney inflammation and remodeling. Specifically, CD44 serves as an indicator of glomerular damage and of tubulo-interstitial fibrosis corresponding to expression in these regions [46]. CD44 protein is known to be induced in rat and human kidney in inflammatory diseases. CD44 protein plays roles in cell–cell and cell–matrix interaction, lymphocyte recruitment and activation, and binding and presentation of growth factors. Phenotypic investigation of a *Cd44*$^{-/-}$ "knockout" mouse in a model of obstructive nephropathy indicated a profibrotic role for CD44 and a protective function for renal tubules [46]. In this study, only small increases in *Cd44* mRNA were observed in female DI rats' response to high-salt diet (Table 2), and this response was not statistically significant. It is likely that *Cd44* mRNA levels in kidney were below the detection limit for microarrays (unpublished data), such that increases were underrepresented. Thus we evaluated

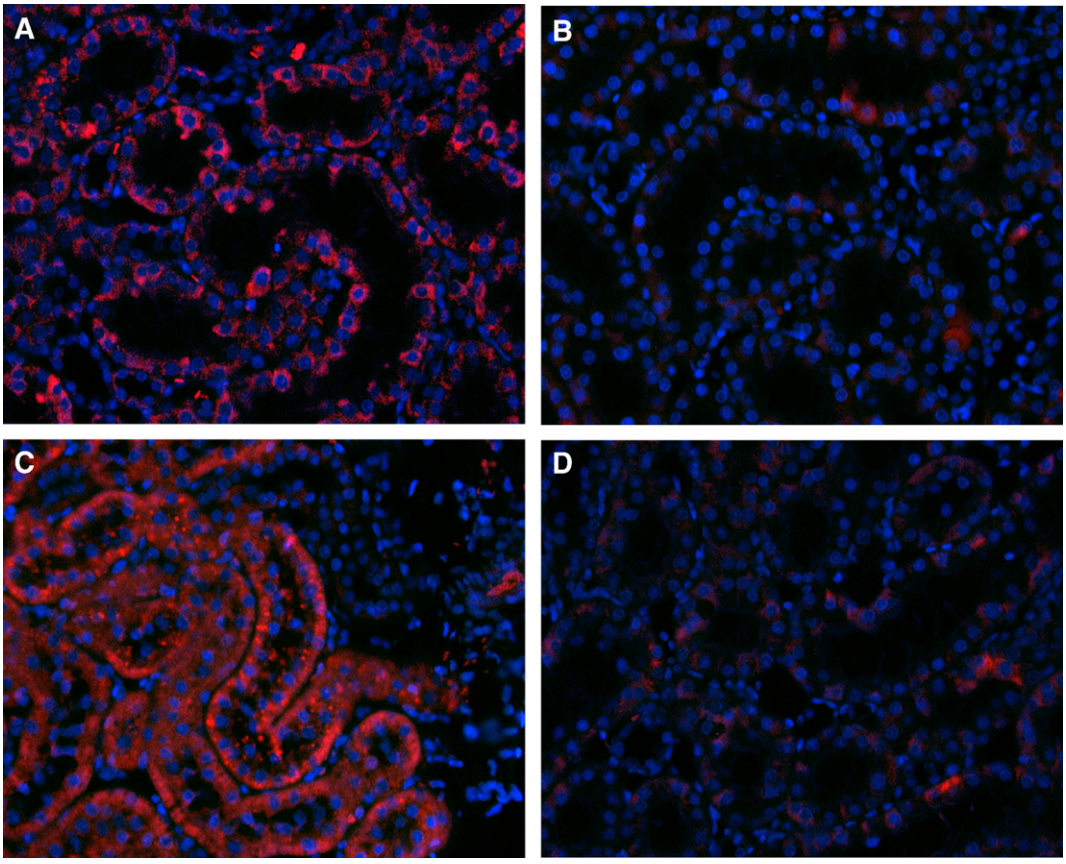

Fig. 4. Immunostaining of CD44 in kidney outer medulla tissue sections from DI rats. CD44 protein was stained with anti-CD44 antibodies (red). Nuclei were counterstained with DAPI (blue). Kidney samples were from (A) female low-salt-fed rats, (B) male low-salt-fed rats, (C) female high-salt-fed rats, and (D) male high-salt-fed rats.

CD44 protein expression. Using antibody staining we observed that CD44 protein expression was low in low-salt-fed rats in all three experiments and in high-salt-fed SD rats and male DI rats (Fig. 4). In contrast, CD44 protein was strongly expressed in high-salt female DI rats. This result confirmed the indication from the microarray data that inflammation was indeed occurring primarily in female DI high-salt-fed rats. CD44 protein expression appeared to be most abundant in epithelial cells lining collecting ducts in the outer medulla. Comparison of Fig. 4A to Fig. 4C suggests that CD44 protein is found throughout the cytoplasm of low-salt-fed DI females, but found in high-salt-fed DI females differentially at the basal side and at epithelial cell-cell junctions [47].

Conclusions

Transcriptional data indicated profound differences in homeostatic gene expression and physiology between male and female rat kidneys. While gene expression in kidneys was marginally affected by an 8% salt diet in *wild-type* rat kidneys, numerous responses were observed in salt-sensitive DI rats. These adaptations to a high-salt diet were profoundly different in kidneys of male *versus* female rats. Surprisingly, kidney adaptation to high-salt diet in female DI rats involved regulation of hundreds of genes compared to only 13 observed in male DI rats. Female responses to a high-salt diet included many genes indicative of inflammation and fibrosis, as well as decreased metabolic processes: gluconeogenesis, amino acid metabolism, and fatty acid catabolism.

Repetition of high- and low-salt feeding using gonadectomized rats indicated that androgens dominated both baseline sex differences in gene expression and sex-specific gene expression responses to dietary salt. This is not surprising, since androgens are generally observed to cause physiological sex-specific responses to dietary salt [8]. Comparison of gene expression responses to salt with published gene functions suggested several mechanisms by which androgens regulate salt sensitivity and adaptation to a high-salt diet. Fig. 5 summarizes such mechanisms suggested by these studies.

Sex-selective expression of several genes may give insights into physiological sex differences. Selective expression of prolactin receptors in male kidneys suggested a role for prolactin or prolactin-related hormones in regulating blood pressure or kidney function in male rats. Increased expression of angiotensinogen and angiotensin–vasopressin receptor NALP6 in male rat kidneys supported the increased activity of the renin-angiotensin system in male vs female rats [9].

Female-selective expression of *Srd5a1* implied selective inactivation of corticosterone in females, implying increased natriuresis. Conversely, male-selective expression of A2M, a circulating factor that neutralizes cytokines, suggested how males suppressed the inflammatory and fibrotic response cascade that was prominent in female DI kidneys under the high-salt dietary condition.

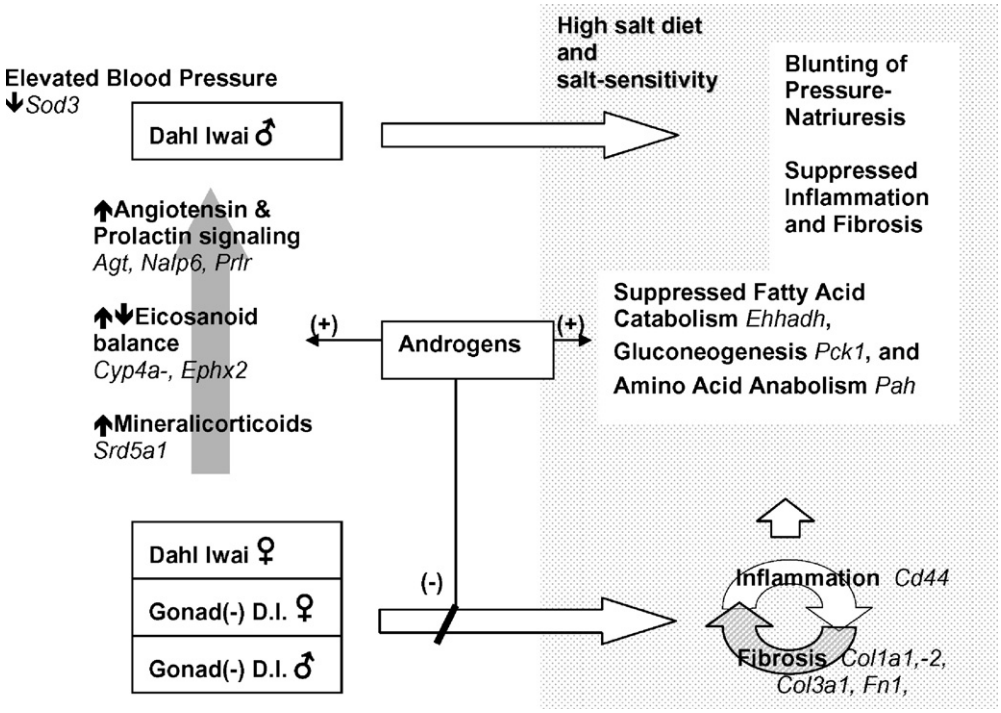

Fig. 5. A model that posits gene expression mechanisms by which androgens regulate kidney physiology. Effects of a high-salt diet in salt-sensitive Dahl-Iwai rats are indicated by text within the stippled area. Effects promoted by androgens, or blocked by androgens, are indicated by the thin arrows with (+) or (−) symbols, respectively. Gene abbreviations are indicated for example genes underlying each physiological event: *Sod3,* extracellular superoxide dismutase 3; *Agt,* angiotensin; *Nalp6,* angiotensin–vasopressin receptor; *Prlr,* prolactin receptor; *Cyp4a-,* cytochrome P450 4a-subfamily genes; *Ephx2,* cytoplasmic epoxide hydrolase; *Srd5a1,* steroid 5α-reductase 1; *Ehhadh,* peroxisomal bifunctional enzyme type 1; *Pck1,* phosphoenolpyruvate carboxykinase 1; *Pah,* phenylalanine hydroxylase; *Cd44,* hyaluronate receptor; *Col1a1, -2,3,* collagens 1a1, -1a2 and -3a1; *Fn1*, fibronectin.

A complex pattern of cytochromes P450 regulation was observed. Five *Cyp4* genes were down-regulated by the high-salt diet in female DI kidneys, but showed highly diverse regulatory responses to salt and sex under other conditions. *Cyp2c11* (and *10*) genes were expressed selectively in males. *Cyp2* and *Cyp4* families of genes are thought to play roles in metabolizing EET and HETE eicosanoid hormones, respectively, to modulate natriuresis and vascular tone in the kidney.

The published literature describes a variety of sex-selective physiological adaptations to dietary salt in rats and in humans. These include male-biased increased blood pressure, blunting of pressure natriuresis, and increased renin-angiotensin system activity. Our gene expression data suggest mechanisms for androgen mediation of these physiological events. The female-selective inflammation and fibrosis under a high-salt diet was unexpected and may be a characteristic of this genetic model. Future work will be required to determine whether these sex-selective responses to salt are generalizable to other hypertensive rat strains, and to humans, or are characteristic of DI rats. Following on these 14-week studies, it will also be useful to examine which gene expression responses to salt in DI rats are short-term effects and which are long-term effects.

Materials and methods

Animal experiments

Dahl-Iwai and Sprague–Dawley rats were purchased from Taconic Farms (Germantown, NY, USA). Animal treatments were reviewed and approved by the Institutional Animal Care and Use Committee. Rats were watered and fed chow containing low salt or high salt (0.1 or 8% NaCl, respectively) ad libitum for 14 weeks. No significant weight differences were noted between rats on the two diets. Kidneys were dissected into outer medulla and cortex with remaining tissue and preserved in RNAlater (Ambion, Inc., Austin, TX, USA) at −70°C. Blood pressures were measured using telemetry monitors [10] in a separate cohort of Dahl-Iwai rats to avoid complications to other measurements.

RNA preparation

Tissues were homogenized in Trizol (Life Technologies, Gaithersburg, MD, USA) using a Polytron (Omni International, Warrenton, VT, USA), and total RNA was isolated from each sample according to the manufacturer's instructions. Total RNA was reprecipitated using RNAmate (Biochain, San Leandro, CA, USA); and RNA integrity was evaluated using capillary electrophoresis (Bioanalyzer 2100; Agilent, Inc., Palo Alto, CA, USA) to ensure a 28S rRNA to 18S rRNA intensity ratio >1.

Microarrays

Biotinyl–RNA samples were prepared and hybridized to a rat U94A Affymetrix GeneChip array per Affymetrix technical manual P/N 700220 as previously described [11]. One microarray was used for each CX or OM kidney sample. Triplicate animals were used to generate mean expression values for each treatment group. Microarray data are available in the GEO database as accession # GSE5285. For each gene, expression ratios across pairs of experiments and their statistical significance were determined using the SAFER algorithm, which provides a robust and resistant measure of gene expression. The SAFER algorithm normalizes between arrays, ranks the probe pairs for each gene according to reproducibility of responses between samples, and down-weights the probe pairs that exhibit the most variability in performance [12]. ANOVA p values describing the reproducibility of differences between pairs of treatment groups were derived for each gene. Expression ratios were calculated for samples from male and female rats on high- vs low-salt diet and between male and female rats on low-salt diet. These three ratios were determined for all strains (SD, DI, and GX-DI) and for outer medulla and cortex regions of the kidney.

Cluster analysis

Gene expression data were clustered using the hierarchical algorithm UPGMA (unweighted pair group method with arithmetic mean) [13] and visualized in Spotfire 7.3 (Spotfire, Inc., Somerville, MA, USA). Algorithm and metric were selected to generate nine discrete clusters that can each be intuitively described by a qualitative pattern of up, down, or no change at each of the 18 ratios. To measure distance between genes X and Y with expression ratios of (x_1, x_2, …, x_{18}) and (y_1, y_2, …, y_{18}), a modified Euclidean metric was used,

$$\|X-Y\| = \left[\sum_{i=1}^{18}(x_i-y_i) + k^2\{H(|x_i|-\log 2) * \mathrm{sgn}(x_i) - H(|y_i|-\log 2) * \mathrm{sgn}(y_i)\}^2\right]^{1/2}$$

where H is the Heaviside step function with the property $H(z)=0$ if $z<0$ and $H(z)=1$ if $z>0$, sgn(z) is the sign function satisfying sgn(z)=1 if $z>0$ and sgn(z)=0 if $z<0$, and k is a dendrogram tuning parameter (introduced in [13]). The only purpose of parameter k is to aid visual representation of primary branches of the cluster dendrogram, and it has no impact on cluster membership. Fig. 2 uses a k of 5.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2007.01.009.

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