Defective Renal Dopamine Function and Sodium Sensitive Hypertension in Adult Ovariectomized Wistar Rats: Role of the Cytochrome P450 Pathway

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Running head:

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LADC, experimental work; PJA, experimental work and discussion; CC, experimental work; GL, catecholamine determinations and expertise concerning dopamine; EMO, female hormones expertise and discussion; EEA, discussion and writing; SN, CYP expertise, discussion and writing the manuscript; FRI, experimental idea and design, discussion and writing the manuscript.

ABSTRACT

We have previously shown that ovariectomy in adult Wistar rats under normal sodium (NS) intake results in an overexpression of total Na^+, K^+-ATPase α 1subunit (NKA). Upon high sodium (HS) intake ovariectomized (oVx) rats developed a defective NKA phosphorylation, a decrease in sodium excretion, and an increment in mean blood pressure (MBP). Since NKA phosphorylation is modulated by dopamine (DA), the aim of this study was to compare the intracellular response of the renal DA system leading to NKA phosphorylation upon sodium challenge in intact female (IF) and oVx rats.

In IF rats HS caused an increase in urinary DA and sodium, in NKA phosphorylation state, in Cytochrome P4504A (CYP4A) expression and in 20-HETE production, while MBP kept normal. Blockade of D1R with the D1-like receptor antagonist SCH23390 in IFHS rats shifted NKA into a more dephosphorylated state, decreased sodium excretion by 50 % and increased MBP. In oVxNS rats, D1R expression was reduced and D3R expression was increased, and under HS intake sodium excretion was lower and MBP higher than in IFHS rats (both $p<0.05$), NKA was more dephosphorylated than in IFHS and CYP4A expression or 20-HETE production did not change. Blockade of D1R in oVxHS rats changed neither NKA phosphorylation state nor sodium excretion or MBP. D2R and PKCα expression did not vary among groups.

The alteration of the renal dopamine system produced by ovariectomy could account for the defective NKA phosphorylation, the inefficient excretion of sodium load and the development of salt sensitive hypertension.

Key words:

Female hormones, ovariectomy, Dopamine D1 Receptor, Na^+, K^+-ATPase phosphorylation, salt-sensitive hypertension, CYP4A20-HETE.

INTRODUCTION

Sexual hormones play an important role in the regulation of water and sodium balance (34). In previous studies we have reported that ovariectomy results in a higher renal plasma flow (RPF) and a lower renal vascular resistance together with lower blood pressure if compared to control intact female (IF) rats (4). Further results showed that ovariectomy causes an overexpression of total Na^+, K^+-ATPase alpha 1 subunit (t-NKA) in tubular epithelial cells as well as an altered balance between the phosphorylation/dephosphorylation of NKA to a more dephosphorylated (more active) state. As a consequence, when ovariectomized (oVx) rats were shifted from a normal sodium diet (NS) to a high sodium diet (HS), they were unable to achieve a normal sodium balance and developed salt sensitive hypertension (13).

It is well documented that abnormalities in intrarenal dopamine (DA) production or DA-receptor signaling can predispose to salt-sensitive hypertension (21, 47). Renal DA is involved in the regulation of sodium excretion under both low (12) and high (1, 6, 30) sodium intake. In this last scenario it is responsible for almost 50-60% of urinary sodium excretion (11, 20), being the decrease of tubular sodium reabsorption the main mechanism responsible for its natriuretic effect. In addition to its action on other tubular sodium transporters (2), DA inhibits NKA activity (7, 9). This latter effect is, in part, the consequence of NKA phosphorylation by Protein Kinase C (PKC) at the Ser 23 residue, which renders a less active enzyme (18, 31, 40).

Based on data from other authors and on our previous findings, that, 1- an increase in renal DA production and its normal tubular function is necessary to eliminate the sodium overload under HS in intact rats (1), 2- stimulation of DA receptors activates PKC which phosphorylates NKA causing the inhibition of its activity (31, 32), and 3- an altered NKA phosphorylation at the PKC site was found in oVx rats on HS intake

together with the development of salt sensitive hypertension (13), we hypothesized that the renal DA system could be involved in the altered sodium balance in oVx rats on HS. Thus, the aim of the present study was to explore the role of the renal dopamine production, and the known dopamine- triggered intracellular pathways that might be responsible for the abnormal Na^+, K^+-ATPase phosphorylation state in ovariectomized rats under high sodium intake, and to assess its role on sodium excretion and blood pressure regulation as compared to intact female rats.

MATERIALS AND METHODS

Animals: Female Wistar rats aged 150 days, from the Animal Breeding Facility of Instituto de Investigaciones Médicas A. Lanari, University of Buenos Aires, were used in this study. All protocols were performed according to the guidelines recommended by the National Institutes of Health and were reviewed by the Institutional Committee for Animal Welfare of the Faculty of Medicine, University of Buenos Aires (CICUAL). They were housed at 22±2.2°C with a 12:12 h dark/light cycle.

At the age of 60 days rats were anesthetized with a combination of ketamine (40 mg/kg bwt) and xylazine (5 mg/kg i.p). Half of the animals were ovariectomized (oVx) while the others were subjected to identical surgical procedure but ovaries were left intact (intact female, IF). After surgery IF and oVx rats had free access to tap water and to a standard normal sodium diet (0.24 % NaCl) from Alimentos Cooperación (San Nicolás, Buenos Aires, Argentina). At the age of 145 days rats from both groups were randomly assigned to a group on a normal sodium intake (NS) which continued on standard diet and tap water, or a group on a high sodium intake (HS) which continued on standard diet and received NaCl 1 % in drinking water. On day 150, animals from the four experimental groups (IF-NS, IF-HS, oVx-NS and oVx-HS) were placed in individual cages for 24h urine collections. In order to minimize the effect of stress all rats were previously acclimatized to individual cages.

Glomerular filtration rate and blood pressure recording: Two days before clearance studies subgroups of HS rats (IF and oVx) were treated with the D1-like receptor antagonist SCH23390 (R (+)-SCH-23390 hydrochloride, Sigma-Aldrich, St. Louis, MO, USA) (1 mg/kg bwt s.c. twice a day) or with vehicle (normal saline). Animals were anesthetized with Inactin (50 mg/kg bwt, i.p.) and surgically prepared for a renal clearance experiment as described previously (13). The trachea was cannulated to

facilitate breathing. Catheters were placed in the carotid artery for blood pressure measurements and blood sampling and jugular vein for i.v. infusions. Enough inulin (Fresenius Kabi, Graz, Austria) to provide plasmatic concentrations of 0.2 mg/ml was given as a prime and a sustaining infusion diluted in saline was administered at 0.035 ml/min. The depth of anesthesia was controlled by testing the lack of response to stimulation of posterior limbs and by visual observation of a stable and regular breathing. After a 45 min stabilization period, 3 consecutive 30 min urine samples were collected: 1-basal period, 2- DA or the D1-like receptor agonist fenoldopam (Sigma-Aldrich, St. Louis, MO, USA) infusion (1 μg/kg bwt/min for both), and 3- recovery (after the infusion was ceased). Volume of blood samples and fluid losses during surgery were replaced by corresponding amounts of normal saline solution.

Analysis of urine and plasma samples: Diuresis and urine sodium concentration were determined by gravimetry and flame photometry, respectively, whereas Inulin was determined in plasma and urine samples by conventional methods (46). Urinary sodium excretion was calculated as $U_{Na+}V$ for each period. For DA analysis 24-h urine samples were collected on 500μL 6N HCl to prevent DA degradation. Dopamine was extracted from urine samples using alumina, separated by reverse-phase high-pressure liquid chromatography using a 4.6x150 mm, 5 μm C18 column (Agilent Life Sciences and Chemical Analysis, Santa Clara, CA, USA) and quantified amperometrically by a triple-electrode system (ESA, Bedford, MA, USA) (14).

Renal tissue samples: Animals were sacrificed at the end of the study and kidneys were removed, homogenates from cortex and outer medulla were prepared in protein homogenization buffer (in mM: TRIS 20, EGTA 2, EDTA 2, PMSF 1, β-mercaptoethanol 10 and aprotinin 100 KIU/ml), pH 7.4 and were kept at -70°C. Protein concentration was measured using the Bradford method.

Western Blot: Proteins (15 μg) were separated on a SDS-PAGE gel and transferred to PVDF membranes (PVDF Transfer Membranes, Pierce Biotechnology, Inc. Rockford, IL, USA). Blots were probed with the following primary antibodies: D1R, D2R, D3R, PKCα and CYP4A. To normalize for protein loading, membranes were stripped, washed, and reblotted with mouse anti- β-actin antibody.

Phosphorylation of Na^+-K^+-ATPase α1 subunit was detected with a dephosphorylation degree-specific monoclonal antibody McK-1 which binds NKA at Ser-23 when this residue is dephosphorylated and not when it is phosphorylated by PKC. Thus, immunosignal from McK1 increases when PKC site Ser 23 becomes more dephosphorylated (8, 17, 23, 29). Total NKA α1 subunit was immunodetected by a regular monoclonal antibody which recognizes NKA α1 subunit independently of its phospho-state. The phosphorylation state of NKA α1 subunit is expressed in density units from the ratio dephosphorylated NKAα1/total NKAα1 (d-NKA/t-NKA). Immunoreactive proteins were visualized using peroxidase-conjugated antibodies and an ECL Western Blotting substrate detection system (Pierce ECL Western Blotting Substrate, Pierce Biotechnology, Inc., Rockford, IL, USA). Densitometric analysis of bands was performed by ImageJ 1.34s NIH, USA software.

Antibodies: The following antibodies were used: mouse anti α1 NKA sc-21712 (1:7000, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA); mouse McK1 (1:1000, a kind gift from Dr K J Sweadner); mouse anti-dopamine D1R monoclonal antibody MAB 5290 (1:500, Millipore AB. Solna, Sweden), goat polyclonal anti D2R sc-7522 (1:2500, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA); rabbit polyclonal anti-dopamine D3 sc-9114 (1:500, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA); rabbit polyclonal anti PKCα (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); rabbit polyclonal antibody to cytochrome P450 4A (1:1500, Abcam, Cambridge,

UK) and mouse anti β Actin sc-47778 (1:2000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Peroxidase conjugated anti goat and peroxidase conjugated anti mouse antibodies (Vector Laboratories, Burlingame, CA, USA) and peroxidase conjugated anti rabbit antibody (Abcam, Cambridge, MA, USA).

cAMP assay: Adenosine 3',5' cyclic monophosphate (cAMP) levels were assessed in renal cortical slices from IF and oVx rats under NS or HS diet, incubated with or without DA (10^{-6} M) in modified Hank's solution, pH 7.4 in the presence of phosphodiesterase inhibitor IBMX (10^{-3} M). cAMP was determined using the Cyclic AMP EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer´s protocol. cAMP levels in each sample were assayed in triplicate and normalized by mg of tissue.

ω-hydroxylase activity:

Renal cortical homogenates (0.5mg protein) were incubated for 60 min at 37°C in 1 ml of 100 mM potassium phosphate buffer (pH 7.4), containing 10 mM $MgCl_2$ and 500 mM EDTA, 1 mM reduced NADPH, and 40 μM cold arachidonic acid in a shaking bath, with 100% O_2 superfusion. Reactions were terminated by acidification to pH 3.5 with formic acid. Samples were extracted twice with 1 ml of ethyl acetate, and the concentration of 20-HETE was determined by an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer´s instructions.

Statistical analysis: The results are reported as mean±SEM. For three or more groups, one- or two-way ANOVA followed by LSD's post hoc test was used. Two sided unpaired *Student's t-test* was used for two-group comparisons. A $p< 0.05$ was considered as significant. The statistical analysis was performed using the SPSS statistics 17.0 program (Illinois, USA).

RESULTS

Body weight was 288±5 g in oVx and 267±7 g in IF rats ($p<0.05$). Figure 1 shows that under NS daily urinary DA excretion was similar between IF and oVx rats. When challenged with HS both groups increased DA excretion to a similar extent over their respective NS values ($p<0.05$ vs NS for both).

The natriuretic response to DA or the D1-like receptor agonist fenoldopam infusion was then tested in IF and oVx rats under NS or HS intake. Baseline sodium excretion was similar between IF and oVX rats. DA elicited a significant increase in sodium excretion only in IF rats either on NS or HS intake. On the contrary, in oVx rats the effect of exogenous DA on natriuresis was negligible (Figure 2). A similar pattern of response was observed when the D1-like receptor agonist fenoldopam was infused (*n*=4, per group). In IF rats under NS or HS fenoldopam significantly increased urinary sodium excretion (µmol/30 min/100g bwt) from 8.4±3.8 to 29.7±6.8 and from 15.0±2.2 to 29.0±6.4, respectively, both $p<0.05$. Instead, in oVx rats, fenoldopam did not change $U_{Na+}V$ under NS or HS intake, from 7.5±4.1 to 10.3±5.0 and from 14.0±5.4 to 10.4±4.4, respectively, both p *NS*.

We next examined whether ovariectomy affects renal expression of DA receptors. Analysis of the expression of D1R is shown in Figure 3. Two bands were visualized at 75 and 55 kDa in both renal cortex and renal medulla. Under NS, the expression of the two bands in renal cortex was significantly lower in oVx if compared to IF rats (Figure 3 A), and, at the 75 kDa band the two way ANOVA showed a significant interaction oVx*sodium intake, $p=0.047$. The same difference was observed in renal medulla for the band at 55 kDa (Figure 3B) with a significant interaction oVx*sodium intake, $p=0.044$. High salt intake induced a reduction in the expression of the 75 kDa band only

in renal cortex of IF rats. No changes were observed after HS in the other analyzed bands.

The expression of D2R in renal cortex and medulla was similar in IF and oVx rats under either NS or HS (Figure 4), while D3R abundance in renal cortex and medulla was increased in oVxNS rats compared to IFNS rats (Figure 5). HS intake did not modify D3R expression in IF or oVx rats.

Table 1 depicts the functional response brought about by D1R blockade with the D1-like receptor antagonist SCH23390. Treatment with SCH23390 did not affect diuresis, sodium excretion, MBP or glomerular filtration rate in IF or oVx rats under normal sodium intake. The challenge with high sodium intake resulted in a lower sodium excretion together with increased levels of MBP in oVxHS compared to IFHS rats. Moreover, while in IFHS rats D1R blockade caused a marked decrease in diuresis and natriuresis together with a significant increment in MBP, in oVxHS rats it did not change any of these parameters. Glomerular filtration rate was similar between IFHS and oVxHS rats whether treated with D1-like receptor antagonist SCH 23390 or not.

Basal cAMP content was similar in outer cortical slices isolated from IF and oVx rats on NS and incubated with normal saline (0.69±0.16 and 0.52±0.11 pmol/mg, respectively, n=5). Incubation with DA resulted in an equivalent increase in cAMP in cortical slices from IF and oVx rats (1.09±0.10 and 0.91±0.17 pmol/mg, respectively, n=5, $p<0.05$ vs. their respective basal values for both).

The expression of total Na^+, K^+-ATPase α 1 subunit (t-NKA) and its phosphorylation state were next explored. NKA α 1 subunit is phosphorylated at Ser 23 residue by PKC. It has been previously shown that NKA is less active when phosphorylated and more active when the dephosphorylated state (d-NKA) predominates (8, 29, 31, 40). Figure 6 analyzes the role of D1R in the regulation of NKA α 1 subunit total expression and

phosphorylation in IF and in oVx rats on HS intake. There was no difference in the expression of t-NKA in renal cortex and medulla in IF or oVx rats, and it was not modified by the administration of the D1-like receptor antagonist SCH 23390 to either IF or oVx rats. On the contrary, d-NKA signal in renal cortex and medulla was significantly lower in IF rats than in oVx rats. Therefore, NKA α 1 subunit is significantly more phosphorylated in IF than in oVx rats. Since D1R plays an important role in DA- induced phosphorylation of NKA α 1 subunit at Ser 23 residue, we tested whether the blockade of D1R may alter NKA phosphorylation state. Treatment with SCH 23390 caused a marked and significant increase in d-NKA immunosignal in IFHS rats in renal cortex and medulla ($p< 0.05$). This indicates that NKA turned more dephosphorylated as a consequence of D1R blockade in IFHS rats. In oVxHS rats, on the other hand, NKA Ser 23 phosphorylation state was not altered by treatment with SCH 23390 in either renal cortex or medulla.

Since NKA α 1 subunit is phosphorylated at the Ser 23 residue by PKC α we next explored the expression of PKC α in renal homogenates from IF and oVx rats. Figure 7 shows that the expression of PKC α in renal cortex and renal medulla was similar between rats on NS or HS irrespective of whether they were IF or oVx.

The catalytic activity of PKC α is regulated by the CYP4A-20-HETE pathway. Therefore, we analyzed the expression of CYP4A in renal cortex and medulla isolated from IF and oVx rats on NS or HS intake (Figure 8). CYP4A expression in both cortex and medulla was significantly increased under HS intake only in IF rats whereas it was not increased by sodium intake in oVx rats. Moreover, a significant decrease in CYP4A expression was observed in renal medulla from oVx rats. The renal cortical formation of 20-HETE, the product of CYP4A ω-hydroxylase activity, was similar between IFNS

and oVxNS rats. In line with changes in CYP4A expression, HS intake resulted in a significant increase in 20-HETE production only in IF but not in OVX rats (Figure 9).

DISCUSSION

The results of this investigation point to an important role of the renal dopamine system in the development of salt sensitive hypertension in ovariectomized rats on a high salt intake. This conclusion is based on the abnormal dopamine signaling pathway in ovariectomized rats that leads to the defective Na^+,K^+-ATPase phosphorylation state, which in its turn results in the inability to handle a high sodium intake.

In the epithelial cells of proximal tubules of normal rats DA is formed by the enzymatic decarboxylation of l-Dopa ultrafiltered from plasma. DA release, mainly into tubular lumen, is triggered among other stimuli by sodium load (6, 41). Indeed, the natriuretic effect of DA is dependent on the state of Na^+ balance. Under Na^+-depletion, D1R–mediated natriuresis does not occur, whereas under Na^+-repletion the stimulation of D1R induces a robust natriuretic response (36). Recent studies confirmed the importance of an intact dopaminergic system in preventing the increase of blood pressure in mice fed on a high salt diet (47).

In our hands, the increase in sodium intake was associated with a similar increase in renal DA excretion in both, intact and ovariectomized rats, suggesting an intact tubular capacity for DA synthesis in oVx rats. However, despite the similar DA excretion, sodium output after a high sodium intake was lower in oVx than in control rats, and this was followed by the increase in blood pressure in oVx animals. The lack of changes in GFR among groups suggests that the lower sodium excretion in oVx rats may be mainly due to a defective tubular transport in these animals. Interestingly, treatment of IFNS with the D1-like receptor antagonist SCH 23390 had no effect on sodium excretion and blood pressure whereas it significantly reduced sodium excretion and increased blood pressure in IFHS rats. On the other hand none of these parameters was changed by administration of SCH 23390 to oVx rats even under HS intake. This prompted us to

speculate that ovariectomy results in an alteration in D1R function. In addition, the infusion of DA or the D1-like receptor agonist fenoldopam resulted in an increased sodium excretion in IF rats under either normal or high salt intake but not in oVx rats, strengthening the hypothesis that D1R function is deficient in oVx rats.

While the expression of D2R was similar among all groups, the expression of D1R (75 and 55 kDa bands) was lower in renal cortex from oVxNS when compared to IFNS rats. A reduction in D1R protein abundance at the 75 kDa band was observed under high salt intake only in renal cortex from IF rats. The 75 kDa band has been reported to be the most important one since it is placed in the lipid rafts at the plasma membrane (45). Our results are in line with previous observations from other authors who reported a lower expression of D1R in hypertensive patients (5, 10) or animal models of experimental hypertension (42). Regarding sodium intake, while some reports describe that high salt intake does not modify D1R abundance (42), a decrease in D1R expression during a high salt intake has been reported (5, 10).

In our hands, under normal salt intake, D3R expression was higher in oVx than in IF rats. However, this difference was no longer kept under high sodium intake. Other authors have reported that D3R may have a compensatory role in pathological conditions where D1R expression is decreased (10, 42). Thus, in oVxNS rats the increment in D3R abundance might help to maintain sodium excretion and blood pressure within normal levels under normal sodium intake, being this compensatory mechanism lost after sodium challenge.

Further experiments performed on rats on high sodium intake showed a different phosphorylation state of NKA when IF were compared to oVx rats. It is well known that upon PKC- phosphorylation NKA becomes less active resulting in lower tubular sodium reabsorption (3, 8). In IF rats on high sodium intake, a tonic PKC

phosphorylation of NKA was evidenced by the low d-NKA/t-NKA ratio in renal cortex and medulla. Administration of the D1-like receptor antagonist SCH 23390 to IF rats on high sodium intake increased the d-NKA/t-NKA ratio compared to non treated IFHS animals, thus confirming that stimulation of D1 receptors is necessary to maintain the phosphorylated state of NKA under high sodium intake. On the contrary, in oVx rats on high sodium intake phosphorylation of NKA was significantly lower than in IFHS rats (higher d-NKA/t-NKA ratio) and was not further modified by SCH 23390.

It is well known that stimulation of D1R in tubule cells is linked to both activation of adenylyl cyclase and phospholipase C. The activation of adenylyl cyclase leads to an increase in cAMP levels and the subsequent activation of protein kinase A, whereas activation of phospholipase C leads to the generation of inositol triphosphate and diacylglycerol (DAG), a physiological PKC activator (18). Additionally, DA induces receptor- mediated stimulation of phospholipase A2, which releases arachidonic acid from membrane phospholipids (32). We have previously shown that the main product of arachidonic acid metabolism by Cytochrome P450 4A (CYP4A) 20-hydroxyeicosatetraenoic acid (20-HETE) synergizes with DAG for the activation of PKC α, which in turn phosphorylates NKA (26, 31). 20-HETE inhibits sodium transport in the proximal tubule (35) and in the thick ascending limb of Henle (TALH) (15). Moreover, previous results from our laboratory have demonstrated *in vivo* the contribution of 20-HETE to the inhibition of sodium transport at the proximal tubule and TALH levels (16). As shown by our present results, neither the content of cAMP nor the expression of PKC α was affected by hormonal status. Therefore, we explored CYP4A pathway, which has an important role in the modulation of PKC activity through the generation of 20-HETE. In IF rats, CYP4A expression and activity were increased by sodium intake. This result is in line with previous observations by other

authors using Sprague-Dawley (38) or Dahl salt resistant rats (39). In oVx rats instead, neither CYP4A expression nor its activity in renal cortex were changed by high sodium intake. Thus, we may speculate that the molecular mechanisms responsible for the sodium- related CYP4A overexpression are hormone- dependent. The expression of CYP4A is under the transcriptional control of the peroxisome proliferator activated alpha receptors (PPARα) that are ligand-activated transcription factors of the nuclear hormone receptor superfamily. PPARα modulates transcription via binding to a specific DNA sequence element called a peroxisome proliferator response element (PPRE) (25). Interestingly, it has been shown that estrogens deficiency induced by ovariectomy reduces the expression of PPARα in rat liver and skeletal muscle (33, 24). Therefore, if a similar regulatory mechanism to the one previously reported in other tissues was also present in the kidney, the lower expression of PPRAα in ovariectomized rats might serve as an explanation for the inability of oVx rats to over express CYP4A in response to high sodium intake, as shown by our present results.

In line, Yanes and colleagues have reported a lower renal microsomal CYP4A protein expression and activity together with an increase in blood pressure in postmenopausal spontaneously hypertensive rats if compared to young matches. The authors speculate that a decrease in 20-HETE synthesis in renal tubules would be pro-hypertensive due to its inhibitory effect on sodium reabsorption (44). Accordingly, the inability to upregulate CYP4A expression or to increase 20-HETE production in response to a high salt diet derived in the development of salt- sensitive hypertension in rats (22, 39) and humans (27). Thus, we may hypothesize that a lack of increment in CYP4A expression in response to a high sodium intake in oVx rats may have contributed to sodium retention and development of hypertension in our experimental model.

Taken together these findings suggest that either a reduction in D1R or a defective response of CYP4A to sodium load, or the combination of both may account for an altered PKC phosphorylation of NKA, leading to the oVx rats' inability to increase sodium excretion.

To our knowledge, this is the first report of studies performed *in vivo* showing evidence that connect D1R stimulation to changes in NKA phosphorylation state and its implication in sodium excretion during a high sodium intake. Previous studies have emphasized the role of nitric oxide and angiotensin II in gender and age related alterations in renal function (34). This work shows that the renal dopamine system can also be compromised in the salt sensitivity pattern described in menopausal and postmenopausal states. The presence or alteration of female hormones seems to be relevant to this pathophysiological condition. Indeed, the decrease in estrogen levels in aging female SHR has been shown to be associated with a decrease in eNOS expression and NO production, resulting in a poor vasodilation and higher blood pressure levels compared with adult rats (43). Besides, other researchers have shown that in old uninephrectomized-ovariectomized SHR, estrogens supplementation ameliorates the renal damage observed in sham treated rats (19). The results of the present work are in line with these reports, highlighting the importance of estrogens, or in opposition, of their lack, in keeping an efficient tubular dopamine pathway to assure a normal renal function and blood pressure levels.

It is worth noting that oVx rats, even on NS intake, already had a reduced abundance of D1R together with an increase in D3R expression, and a lack of response to infused dopamine. In line, other authors have described that ovariectomy induces a decrease in the density of striatal D1 receptors, that was corrected by chronic treatment with 17β-estradiol (28). This observation was confirmed by other authors who demonstrated that

estrogens induce the up-regulation of D1 gene transcription and provide a mechanism for modulation of central dopaminergic functions (37). If a similar modulatory mechanism was operative in the kidney, we may hypothesize that the lack of estrogens may account at least in part for the decreased D1R expression in our experimental model. Thus, our findings may indicate the presence of a prehypertensive condition in oVx rats, that was uncovered by sodium load.

The schematic diagram shown in Figure 10 illustrates the similarities and differences in the response of the dopamine system to a high sodium intake in IF and oVx rats.

In summary, our results show that with the exception of renal dopamine generation, all the other components of the renal dopaminergic system analyzed in this study are completely asymmetric when IF rats are compared to oVx rats. Renal dopamine system is greatly distorted in ovary hormones deprived rats and, as a consequence, they are unable to handle a high sodium intake. Then, a normotensive rat strain develops salt sensitive hypertension. Data support the crucial role of gonadal steroids in regulating renal function and systemic hemodynamics as well as renal sodium handling, particularly under a high salt intake. A better understanding of the renal dopamine system in postmenopausal women as related to renal disease and blood pressure control could provide insights into new preventive and therapeutic strategies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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FIGURE LEGENDS

Figure 1:

Twenty-four-hour urinary dopamine excretion in intact females (IF, white bars) and ovariectomized rats (oVx, black bars) under normal salt (NS) or high salt (HS) intake. Results are expressed as mean ± SEM (*n*= 6 rats/group). One way ANOVA. Asterisks denote $p<0.05$ vs respective NS rats.

Figure 2:

Urinary sodium excretion before (basal) infusion, after 30 min DA infusion (DA), and after 30 min DA infusion interruption (recovery) in intact female (IF, white bars) and ovariectomized (oVx, black bars) rats. Results are shown as mean ± SEM, in percentage over basal. *n*=4 per group. Top graph normal sodium intake (NS) and bottom graph high sodium intake (HS). ANOVA for repeated measurements, * denotes $p<0.05$ DA infusion IFNS and IFHS vs basal.

Figure 3:

Dopamine 1 receptor expression (D1R) in intact female (IF, white bars) and ovariectomized rats (oVx, black bars) under normal sodium (NS) or high sodium (HS) intake. (A) Renal cortex and (B) Renal medulla. Top panels show a representative blot for D1R and corresponding β-actin from six independent experiments. Bottom columns show the density units ratio D1R/β-actin for the 75 and 55 kDa D1R associated bands. Results are shown as mean ± SEM. Statistical analysis was performed by two way ANOVA. ** $p<0.017$ IFNS vs all other groups, * IFNS vs oVx NS, $p=0.010$. Symbol # denotes IFNS and IFHS vs oVxNS, $p=0.017$.

Figure 4:

Dopamine 2 receptor expression (D2R) in intact female (IF, white bars) and ovariectomized rats (oVx, black bars) under normal sodium (NS) or high sodium (HS) intake. (A) Renal cortex and (B) Renal medulla. Top panels show a representative blot for D2R (55 kDa band) and the corresponding β-actin from six independent experiments. Bottom columns show the density units ratio D1R/β-actin. Results are shown as mean ± SEM. No differences were found among groups.

Figure 5:

Dopamine 3 receptor expression (D3R) in intact female (IF, white bars) and ovariectomized rats (oVx, black bars) under normal sodium (NS) or high sodium (HS) intake. A. Renal cortex and B. Renal medulla. Top panels show a representative blot for D3R and corresponding β-actin from 4-5 independent experiments. Bottom columns show the density units ratio D3R/β-actin. Results are shown as mean ± SEM. Statistical analysis was performed by two way ANOVA. A significative interaction oVx*Na intake was found in renal cortex ($p<0.012$) and medulla, ($p<0.049$). Asterisks denote $p<0.03$ oVxNS vs IFNS in renal cortex and $p<0.02$ oVxNS vs IFNS in renal medulla.

Figure 6:

Expression of dephosphorylated NKA (d-NKA) and total NKA (t-NKA) in intact female rats (IF, open bars) and ovariectomized rats (oVx, black bars) under high sodium intake (HS), with or without treatment with D1-like receptor antagonist SCH 23390 (SCH). Top panels show a representative blot from 8 independent experiments. (A) Renal cortex and (B) Renal medulla. Bottom columns show the density units ratio d-NKA/t-NKA. Results are shown as mean ± SEM. Statistical analysis was performed by

two way ANOVA. A significant interaction oVx*SCH 23390 was found, p=0.034. ** IFHS vs oVxHS p< 0.01; * IFHS vs IFHS+SCH p<0.01; # IFHS vs all other groups, p<0.006.

Figure 7:

PKC α expression in intact female (IF, open bars) and ovariectomized rats (oVx, black bars) under normal sodium (NS) or high sodium (HS) intake. (A) Renal cortex and (B) Renal medulla. Top panels show a representative blot for PKC α and β-actin from 4-5 independent experiments. Bottom columns show the density units ratio PKC α/β-actin. Results are shown as mean ± SEM. No significant differences were found.

Figure 8:

Cytochrome P4504A (CYP4A) expression in intact female (IF, open bars) and ovariectomized (oVx, black bars) rats under normal sodium (NS) or high sodium (HS) intake. (A) Renal cortex and (B) Renal medulla. Top panels show a representative blot for CYP4A and β-actin from 4-5 independent experiments. Bottom columns show the density units ratio CYP4A/β-actin. Results are shown as mean ± SEM. * p<0.05; ** p<0.025, unpaired student´s *t-test*.

Figure 9:

Renal cortical 20-HETE production in intact females (IF, white bars) and ovariectomized rats (oVx, black bars) under normal salt (NS) or high salt (HS) intake. Results are expressed as mean ± SEM (*n*= 4 rats/group). One way ANOVA. Asterisk denotes p< 0.041 IF HS vs IF NS and oVx NS rats and p< 0.012 IF HS vs oVx HS rats.

Figure 10:

Schematic illustration of the hypothetical model for changes in intracellular pathways triggered by switching sodium from normal to high intake in tubule cells. Left panel shows the response in intact female rats and right panel in ovariectomized rats. Thick arrows (↑) indicate pathways which were upregulated by high sodium intake in intact rats while were unchanged (=) in ovariectomized animals, as suggested by present experiments. Pathways between brackets refer to changes described by other authors. 20-HETE, 20-hydroxyeicosatetraenoic acid; AA, arachidonic acid; AC, adenylyl cyclase; cAMP cyclic AMP; CYP4A, cytochrome 450 isoform 4A; D1R and D2R, D1 and D2 receptors respectively; DAG, diacylclycerol; E, estrogen; NKA α1, Ser 23, serine residue at position 23 of the α1 subunit of Na+, K+-ATPase; P, phosphorylated state of Na+,K+-ATPase; PKC, protein kinase C; PLA2, phospholipase 2; PLC, phospholipase C; PPARα, peroxisome proliferator activated alpha receptor.

The cascade of events in IF rats starts by stimulating dopamine receptors which downstream signal to increase CYP4A expression and activity. Then, production of 20 HETE is increased and this results in NKA inhibition by its phosphorylation at Ser 23 PKC site, thus allowing an increment in sodium excretion. Ovariectomized rats instead, showed a failure at different points of the renal dopamine system pathway when challenged with high salt. D1R are decreased in expression and activity, CYP4A-20 HETE pathway does not change with high sodium intake. Thus, NKA is not properly phosphorylated and, as a consequence, oVx rats have sodium retention and develop salt sensitive hypertension.

Table 1 Renal function and systemic hemodynamic parameters of rats on high and normal sodium intake

	Parameter			
Groups	Diuresis (ml/day/100g bwt)	$U_{Na+}V$ (mmol/day/100g bwt)	MBP (mmHg)	GFR (ml/min/100g bwt)
High sodium				
IFHS	11.51±0.60	3.14±0.03	112±2	0.67±0.06
IFHS+SCH	6.13±0.19#	1.65±0.09#	140±2*	0.71±0.11
oVxHS	8.67±1.28	2.08±0.03*	135±4*	0.64±0.06
oVxHS+SCH	9.31±2.26	2.41±0.56	138±4*	0.69±0.08
Normal sodium				
IFNS	3.94±0.24	0.43±0.04	105±4	0.63±0.07
IFNS+SCH	3.48±0.39	0.39±0.04	106±8	0.55±0.02
oVxNS	4.32±0.16	0.40±0.02	99±8	0.63±0.08
oVxNS+SCH	4.00±0.28	0.39±0.02	105±4	0.57±0.07

Intact female (IF) and ovariectomized (oVx) rats under high sodium (HS) or normal sodium (NS) intake treated with the D1-like receptor antagonist SCH 23390 (SCH) or not. Results are expressed as mean±SEM. N= 20 rats per group (diuresis, sodium excretion and mean blood pressure) and 5 rats per group (GFR). $UNa^{+}V$, sodium excretion; GFR, glomerular filtration rate; and MBP, mean blood pressure. One way ANOVA. * $p<0.05$ and # $p<0.01$ vs. IFHS group.

In normal sodium groups, values of diuresis, sodium excretion, MBP and GFR treated with the D1-like receptor antagonist SCH 23390 or not, did not show significant differences among them. oVxNS group had the lowest MBP. Diuresis and natriuresis in

NS groups were, however, always significantly lower than in HS groups ($p<0.01$ for both).