CALL FOR PAPERS | Sex and Gender Differences in Renal Physiology

Ovarian hormones and prolactin increase renal NaCl cotransporter phosphorylation

Lorena Rojas-Vega,1,2 Luis A. Reyes-Castro,3 Victoria Ramírez,1,2 Rocío Bautista-Pérez,4 Chloé Rafael,5,6 María Castañeda-Bueno,1,2 Patricia Meade,1,2 Paola de los Heros,7 Isidora Arroyo-Garza,1,2 Valérie Bernard,8 Nadine Binart,5,9 Norma A. Bobadilla,1,2 Juliette Hadchouel,5,6 Elena Zambrano,3 and Gerardo Gamba1,2

1Molecular Physiology Unit, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico; 2Department of Nephrology and Mineral Metabolism, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico; 3Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico; 4Department of Nephrology, Instituto Nacional de Cardiología Ignacio Chávez, Mexico City, Mexico; 5INSERM UMR970, Paris Cardiovascular Research Center, Paris, France; 6University Paris-Descartes, Sorbonne Paris Cité, Faculty of Medicine, Paris, France; 7Escuela de Medicina, Universidad Panamericana, Mexico City, Mexico; 8INSERM U693, Le Kremlin-Bicêtre Université Paris-Sud, Faculté de Médecine Paris-Sud, UMR-S693, Le Kremlin-Bicêtre, France; and 9Assistance Publique-Hôpitaux de Paris, Hôpital de Bicêtre, Service d’Endocrinologie et des Maladies de la Reproduction, Le Kremlin Bicêtre, France

Submitted 8 August 2014; accepted in final form 9 January 2015

Rojas-Vega L, Reyes-Castro LA, Ramirez V, Bautista-Pérez R, Rafael C, Castañeda-Bueno M, Meade P, de los Heros P, Arroyo-Garza I, Bernard V, Binart N, Bobadilla NA, Hadchouel J, Zambrano E, Gamba G. Ovarian hormones and prolactin increase renal NaCl cotransporter phosphorylation. Am J Physiol Renal Physiol 308: F799–F808, 2015. First published January 13, 2015; doi:10.1152/ajprenal.00447.2014.—Unique situations in female physiology require volume retention. Accordingly, a dimorphic regulation of the thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter (NCC) has been reported, with a higher activity in females than in males. However, little is known about the hormones and mechanisms involved. Here, we present evidence that estrogens, progesterone, and prolactin stimulate NCC expression and phosphorylation. The sex difference in NCC abundance, however, is species dependent. In rats, NCC phosphorylation is higher in females than in males, while in mice both NCC expression and phosphorylation is higher in females, and this is associated with increased expression and phosphorylation of full-length STE-20 proline-alanine-rich kinase (SPAK). Higher expression/phosphorylation of NCC was corroborated in humans by urinary exosome analysis. Ovariectomy in rats resulted in decreased expression and phosphorylation of the cotransporter and promoted the shift of SPAK isoforms toward the short inhibitory variant SPAK2. Conversely, estradiol or progesterone administration to ovariectomized rats restored NCC phosphorylation levels and shifted SPAK expression and phosphorylation towards the full-length isoform. Estradiol administration to male rats induced a significant increase in NCC phosphorylation. NCC is also modulated by prolactin. Administration of this peptide hormone to male rats induced increased phosphorylation of NCC, an effect that was observed even using the ex vivo kidney perfusion strategy. Our results indicate that estradiol, progesterone, and prolactin, the hormones that are involved in sexual cycle, pregnancy and lactation, upregulate the activity of NCC.

Address for reprints and other correspondence: G. Gamba, Vasco de Quiroga No. 15, Tlalpan, 14000 Mexico City, Mexico (e-mail: gamba@biomedicas.unam.mx).
leading to lower urine volume and solute excretion compared with control subjects (5).

Here, we present evidence that NCC expression and phosphorylation are greater in female than in male rats, mice, and humans and that NCC phosphorylation is promoted by estrogens, progesterone, and PRL. Because it is known that NCC phosphorylation is a surrogate for increased NCC activity (34, 43), these observations suggest that the three female hormones contribute to increased NCC activity during physiological states unique to women.

METHODS

All experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996) and were approved by the Animal Care and Use Committee at our institutions.

Animals. Adult female and male rats and mice were randomly assigned to sham surgery or to gonadectomy, and when indicated, they were treated with vehicle, estradiol, progesterone, or PRL. Kidneys of PRLR−/− and PRLR+/+ female and male mice were obtained for Western blot analysis (32). Hormone serum levels were measured by specific radioimmunoassay in blood samples obtained the day of the euthanasia.

Diuretic response. To study the urinary response to bendroflu- methiazide (BFZT) in metabolic cages, acclimated male and female rats were treated with a single dose of an intraperitoneal (ip) injection of a 20% DMSO solution with or without BFZT, and urine was collected during an extra 2-h period. Urine electrolytes were measured with a NOVA4 electrolyte analyzer (NOVA Biomedical, Waltham, MA), and creatinine was measured with an autoanalyzer (Beckman Instruments, Brea, CA).

Gonadectomy. Bilateral ovariectomy (or sham operation) was performed as follows. Rats were anesthetized with an intraperitoneal injection of 30 mg/kg pentobarbital sodium and then shaved over the dorsal lumbar region and cleaned with benzil followed by an alcohol rinse. A 2-cm skin incision along the dorsal midline and through the abdominal musculature was made; the ovaries were then exposed and removed. The sham operation was performed using the above steps without removal of the ovaries.

NCC protein expression analysis. Kidney proteins were homogenized using a lysis buffer containing 50 mM Tris·HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyro-phosphate, 1 mM sodium orthovanadate, 1% (wt/vol) Nonidet P-40, 0.27 M sucrose, 0.1% (vol/vol) 2-mercaptoethanol, and protease inhibitors (Complete tablets; Roche). Protein extracts (50 μg) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1.5 h in 5% (wt/vol) nonfat milk dissolved in Tris-buffered saline (TBS)-TWEEN 20. Antibodies were diluted in TBS-Tween 20 containing 5% (wt/vol) nonfat milk. Membranes were incubated with primary antibodies overnight at 4°C and with horseradish peroxidase (HRP)-coupled secondary antibodies at ambient temperature for 1.5 h. Immobilized antigens were detected by chemiluminescence using the Luminata Western chemiluminescence reagents (Merck Millipore). Western blots in Figs. 1, A and B, 2B, 3A, and 4A were exposed to X-ray film, and the densitometries were made on a single exposure. The rest of the Western blot signals were detected and quantitated with a C-DiGit Blot Scanner (Li-COR) and accompanying software. Values were normalized to the mean intensity measured in the male, nongonadectomized or vehicle groups defined as 1.0. All comparisons were performed between samples run on the same blot/membrane.

Immunofluorescence microscopy. Mouse kidneys were used for this analysis with the following antibodies: polyclonal anti-NCC antibody raised in sheep (recognizing residues 906–925 of human NCC, “CHTKKFEDMIAPFRRLNDGFKD”, S965B) (39), polyclonal anti-PER-1 antibody, recognizing a C-terminal peptide raised in rabbit (9), monoclonal anti-parvalbumin antibody raised in mouse (Swant, PV 235), donkey anti-rabbit Alexa-Fluor 488, donkey anti-mouse Alexa-Fluor 594, and donkey anti-sheep Alexa-Fluor 488 (Life Technologies).

Exosome extraction. To assess NCC expression/phosphorylation in humans, urinary exosomes were isolated as reported previously (36, 51). The amount of sample loaded during immunoblotting was normalized by the spot urinary creatinine concentration. Urinary samples from women were taken at day 0 of the cycle, and none of them were using contraceptives.

Hormone treatment. At 12 wk of age, rats underwent an ovariec- tomy. Following a month of recovery after surgery, either 60 μg/kg 17β-estradiol (Sigma-Aldrich) or 20 mg/kg progesterone (Sigma-Aldrich), both dissolved in 10% ethanol and olive oil, were given intraperitoneally (ip) every day for 3 wk until euthanasia. The same procedures were performed with males treated with 17β-estradiol. For PRL treatment, eight male rats were randomized and injected ip with 25 μg/kg body wt or vehicle as a control group every day for 2 wk. PRL (murine recombinant, Sigma-Aldrich) was dissolved in water.

Ex vivo perfused rat kidney. The right kidney of male Wistar rats was mounted in the Langerdoff system as previously described (7, 33) and perfused with vehicle or PRL (murine recombinant, Sigma-Aldrich) at a rate of 0, 10, and 40 ng/ml, which had no effect on the perfusion pressure. After 30 min of perfusion, the kidney was manually separated into the cortex and medulla, and the corresponding fragments were frozen in liquid nitrogen.

Statistical analyses. Statistical significance was defined as two-tailed (P < 0.05), and results are presented as means ± SE. Statistical significance between male and female or intact and gonadectomized animals for two groups was determined by a nonpaired Student’s t-test and for three or more groups by one-way ANOVA with Dunnett correction.

RESULTS

NCC expression/phosphorylation is higher in females than in males. We compared NCC expression and phosphorylation in female and male rats. As shown in Fig. 1A, NCC expression was similar between female and male rats, but NCC phosphorylation at T58 was significantly higher in females. Thus the pNCC/NCC ratio was higher in female rats. This is consistent with our observations and similar to that of Fanestil and coworkers (8), who found that the diuretic and natriuretic response to a single dose of BFZT was higher in females than in males (Fig. 1B). Because thiazides inhibit the activity of NCC, these data suggest that the greater activity of NCC in female rats is due to greater phosphorylation of the cotransporter. We also observed differences in NCC expression/phosphorylation in mice and humans. In mice, in addition to the stimulation of NCC phosphorylation, NCC abundance was higher in females. Thus the pNCC/NCC ratio was similar (Fig. 1C).

Using urinary exosome analysis by Western blotting, we
also documented higher NCC expression and phosphorylation in humans (Fig. 1D).

Ovariectomy eliminates the NCC difference between female and male rats. To assess the role of the gonads in the observed NCC difference between female and male rats, we analyzed the effect of a gonadectomy on the NCC expression/phosphorylation and thiazide response. Our results show that ovariectomy reduced NCC phosphorylation in rats, whereas orchiectomy had no effect (Fig. 2, A and B). The efficiency of the gonadectomy was confirmed by the measurement of the corresponding hormone level. Estradiol was decreased by 96 and 75%, progesterone by 84 and 66%, and prolactin by 28 and 56% in males and females, respectively (Table 1). In addition, ovariectomy reduced the thiazide response in females, whereas...
orchiectomy had no effect in males (Fig. 2A). These observations indicate that the sexual dimorphism observed for NCC is due to female hormones and is associated with the modulation of NCC expression/phosphorylation. STE-20 proline-alanine-rich kinase expression and phosphorylation is higher in females. The kinase responsible for NCC activation by phosphorylation is STE-20 proline-alanine-rich kinase (SPAK) (34, 43). Therefore, we quantified the level of expression and phosphorylation of SPAK in male and female mice and rats. As shown in Fig. 3, both the expression and phosphorylation of SPAK in the kidney were higher in females than in males in both rodent species. No gender differences for OSR1 expression were observed in either mice or rats. These observations suggest that the responsible hormone for the NCC dimorphism is probably acting, at least in part, through the SPAK pathway.

Estradiol and progesterone promote NCC phosphorylation. Ovariectomy in rats significantly reduced NCC phosphorylation, suggesting that ovarian hormones are involved. By immunogold electron microscopy, 17β-estradiol administration was shown to promote increases in NCC presence in the apical membrane (52), but the effect on NCC phosphorylation is not known. In addition, the role of progesterone has not been assessed. We thus analyzed the effect of 17β-estradiol or progesterone administration on NCC expression and phosphorylation in ovariectomized rats. Estradiol or progesterone was injected daily for 3 wk. Hormone levels at the end of the steroid treatment are shown in Table 2. Consistent with observations in Fig. 1A, the NCC expression level was similar in the treated and control groups (Fig. 4A). In contrast, administration of either steroid induced a significant increase in NCC phosphorylation (Fig. 4A). The effect of 17β-estradiol was stronger

![Figure 2](http://ajprenal.physiology.org/)

**Fig. 2.** Ovarian hormones regulate NCC phosphorylation. Western blot analysis of NCC expression and phosphorylation (T58) of control and ovariectomized (A) female rats and control and orchiectomized (B) male rats in proteins extracted from individual renal cortex; 2 different Western blots from different subjects are indicated by black outline. Also shown is densitometric analysis of total NCC is expressed as the fold of NCC over β-actin, and NCC phosphorylation as the fold of phosphorylated NCC over total NCC. **P < 0.01 control vs. ovariectomized rats; n = 6 rats/bar. C: change (Δ) in urinary volume, sodium, and chloride excretion 2 h before vs. 2 h after BFTZ injection in intact (open bars) and gonadectomized (filled bars) male or female rats as stated. *P < 0.05 vs. female rats; n = 6 rats/bar.
than the effect of progesterone. We confirmed the estradiol activation/phosphorylation of NCC in male rats treated for 3 wk with the same 17β-estradiol dose as females. Hormone levels at the end of treatment are also shown in Table 2. As shown in Fig. 4B, estradiol administration in males is associated with increased NCC phosphorylation.

Ovarian steroids also had an effect not only on SPAK phosphorylation but also on SPAK isoform expression (Fig. 4A). In ovariectomized rats treated with vehicle, the most prominent band observed for SPAK and phospho-SPANK was -45 kDa, corresponding to the SPAK2 isoform (29). The 17β-estradiol or progesterone treatment increased the abundance of the full-length SPAK (60 kDa) and a dramatic reduction in phospho-SPAK2. It is known that increased full-length SPAK together with a reduction in SPAK2 promotes NCC phosphorylation (29). Thus it is likely that ovarian steroids increased NCC phosphorylation, at least in part, through modulation of SPAK activity. We found no differences in aldosterone levels between any of the above groups: vehicle 424 ± 25 (pg/ml), ovariectomized + 17β-estradiol 386 ± 32 (pg/ml), ovariectomized + progesterone 466 ± 43 (pg/ml), and sham 408 ± 59 (pg/ml).

Because the positive effect of 17β-estradiol and progesterone on NCC activity in rats is associated predominantly with increased NCC phosphorylation, rather than increased expression, it is possible that nongenomic effects are involved. Several nongenomic membrane-associated effects of classic estrogen receptors have been reported (47). A particular 33-kDa isoform of estrogen receptor α (ERα33) is highly expressed along the entire nephron (25). In addition, an estrogen 7-spanning membrane receptor, known as the G-coupled estrogen receptor 1 (GPER-1), has recently been shown to be expressed in the proximal tubule and the thick ascending limb of Henle’s loop (9). Because the specific DCT localization was not shown in that study, we used the same antibody to assess the presence of GPER-1 in the basolateral membrane of DCT cells in the mammalian kidney. Figure 5 shows expression of GPER-1 at the basolateral membrane in a nephron segment that is also positive for NCC or parvalbumin, indicating that GPER-1 is expressed in DCT.

**PRL promotes NCC phosphorylation.** Several studies have suggested that PRL could also be involved in the regulation of NCC activity. We therefore quantified NCC expression and phosphorylation in female and male mice lacking the PRL
receptor (PRLR<sup>−/−</sup>) (3). Wild-type mice from the same colony were used as controls. The difference in NCC expression and phosphorylation between female and male mice persisted despite the presence or absence of the PRL receptors. In addition, the expression and phosphorylation of NCC were similar in PRLR<sup>−/−</sup> and PRLR<sup>+/+</sup> female mice (data not shown). We reasoned that the difference between female and male PRLR<sup>−/−</sup> mice persisted due to the presence of estrogens in females. The pNCC/NCC ratio tended to be lower in PRLR<sup>−/−</sup> male mice than in PRLR<sup>+/+</sup> males, but the difference did not reach significance (P = 0.06) (Fig. 6A), suggesting that prolactin may play a role in modulating NCC activity. However, the absence of the PRL effect was compensated, although not entirely. This hypothesis was tested using two different models. First, treatment of male rats with PRL for 2 wk resulted in a significant increase in the pNCC/NCC ratio (Fig. 6B), and second, PRL perfusion ex vivo (7, 33) resulted in a significant increase in the pNCC/NCC ratio, indicating that PRL is an activator of NCC (Fig. 6C).

**DISCUSSION**

It has been clearly demonstrated that NCC activation is associated with increased phosphorylation of certain threonine/serine residues of the amino-terminal domain, among which T58 (T60 in humans) is the key regulatory site (34, 43). The role of these sites has been corroborated in multiple in vitro and in vivo models (15). We thus analyzed the expression and phosphorylation status of NCC in female and male rats, mice, and humans. Our data show that phosphorylation of NCC was
higher in female rats and mice. In rats, we did not see a difference in expression between female and male animals, but we observed an increased phosphorylation in the former. However, one study showed higher expression of NCC in females compared with male lean and obese Zucker rats (42). The difference could be strain dependent. In contrast, we did observe a difference between female and male NCC expression levels in mice and humans. The lack of difference in NCC expression levels in rats contrasted with the observation by Verlander et al. (52). In our study, we analyzed several rats and consistently found no difference in NCC expression levels between females and males (Fig. 1) or between control and ovariectomized rats (Figs. 2 and 4). The different strains of rats used (Sprague-Dawley and Wistar rats) and the different antibodies could account for this difference. However, our data show that phosphorylation of NCC was higher in female rats and mice, suggesting increased NCC activity in females.

We used urinary exosomes to quantify these parameters in humans. The analysis of urinary exosomes by Western blotting is a noninvasive strategy that allows for “molecular renal biopsies” through the quantification of the level of expression of certain proteins in the human kidney. Following the methodology developed by Knepper and coworkers (21, 36, 57), it is possible to detect a variety of proteins in urinary exosomes (17), including the apical membrane transporters (11). NCC was absent in urinary exosomes from Gitelman patients (26), whereas its abundance was increased in exosomes from patients with primary aldosteronism (51). Here, we show by comparing exosomes obtained from four young female and male subjects (aged 22–28) that there is a clear and significant increase in NCC abundance and phosphorylation in women. However, it is difficult to define with this methodology whether the primary increase in humans is at the NCC expression or phosphorylation level because exosomes mostly contain membrane proteins, where phosphorylated NCC is exclusively located (15). Phosphorylation of NCC prevents ubiquitination and thus prevents NCC retrieval from the membrane (23). Nevertheless, our data show that sexual dimorphic regulation of NCC occurs in humans.

Gonadectomy is a generally accepted tool for assessing the effect of gonadal steroids on dimorphic regulation of physiological processes. Here, we show that NCC phosphorylation in female rats is dramatically reduced 4 wk after the ovariectomy. No effect was observed after testis removal. These observations indicate that female gonadal hormones modulate the level of NCC phosphorylation. We substituted estradiol or progesterone in ovariectomized rats and observed that NCC phosphorylation is increased by administration of either steroid. Perhaps the role of progesterone is more relevant during pregnancy, in which this steroid is secreted in great amounts by the placenta. The major isoform of the classic estrogen receptor expressed in the kidney is the truncated ERα33 variant, known to be associated with the membrane and translate nongenomic effects (25, 40, 55). In addition, we observed that the G protein membrane receptor for estrogens (9, 14, 37) is heavily expressed in the basolateral membrane of DCT cells. Thus the estrogen effect on NCC could be occurring either by the classic genomic pathway or through a nongenomic membrane receptor-type effect through either the ERα33 or the GPER1 receptors. Further studies will be necessary to clarify the pathways involved.

It is known that the kinase responsible for most NCC phosphorylation in the DCT is SPAK (39, 43, 56), which in turn is known to be modulated by WNKs (53). In the kidney, in addition to full-length SPAK, several short forms resulting from proteolytic cleavage are present (27), two of which are known as SPAK-2, truncated in the amino-terminal domain, and a kidney-specific variant known as KS-SPAK, which lacks most of the kinase domain (29). These shorter forms are more apparent in physiological conditions associated with decreased NCC activity, while their present form is decreased when NCC requires activation, for instance during a low-salt diet or angiotensin II infusion (18, 29, 44). Thus the shorter forms are believed to function as dominant negative SPAK forms. In this regard, we observed that expression and phosphorylation of SPAK were higher in females than in males in both rats and mice. The higher expression of SPAK in females is eliminated by ovariectomy. In addition, the predominant expressed and phosphorylated SPAK isoform in this situation is SPAK2, whereas estradiol or progesterone promotes the expression and phosphorylation of the full-length SPAK isoform. These observations suggest that the positive effect of female hormones on NCC expression/phosphorylation is mediated, at least in part, through SPAK. Supporting these observations, a recent study showed that estradiol upregulates SPAK in the developing hypothalamus, thereby stimulating the activity of the Na⁺/K⁺-2Cl⁻ cotransporter NKCC1 (31). The increased activity/expression of NCC in females might sound counterintuitive given that blood pressure is not higher in female than in males. However, it is well known that estrogens and female hormones possess a potent vasorelaxing effect; by preventing the increase in blood pressure usually associated with salt retention, these hormones preclude the occurrence of pressure-natriuresis (1, 2, 19, 55), which could potentially explain the frequent complaint that the volume

## Table 1. Sex hormone levels in control and gonadectomized female and male rats

<table>
<thead>
<tr>
<th></th>
<th>Male Control (n = 6)</th>
<th>Gnx Male (n = 6)</th>
<th>Female Control (n = 5)</th>
<th>Gnx Female (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone, ng/ml</td>
<td>2 ± 0.4</td>
<td>0.08 ± 0.01 *</td>
<td>0.02 ± 0.002 †</td>
<td>0.005 ± 0.001 †</td>
</tr>
<tr>
<td>Estradiol, pg/ml</td>
<td>25 ± 2</td>
<td>4 ± 0.7 *</td>
<td>50 ± 7</td>
<td>17 ± 1.8 *</td>
</tr>
<tr>
<td>Prolactin, ng/ml</td>
<td>5 ± 0.1</td>
<td>3.7 ± 0.31 ‡</td>
<td>16 ± 1</td>
<td>7 ± 0.8 *</td>
</tr>
</tbody>
</table>

Values are means ± SE. Gnx, gonadectomized. *P < 0.05 vs. same control same sex. †P < 0.01 vs. male control. ‡P < 0.05 vs. female control.

## Table 2. Plasma estradiol and progesterone concentration in ovariectomized rats and males treated with vehicle, 17-β-estradiol, and progesterone

<table>
<thead>
<tr>
<th></th>
<th>E₂, pg/ml</th>
<th>P₄, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovx+vehicle</td>
<td>28 ± 6</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Ovx+estradiol</td>
<td>142 ± 40***</td>
<td>10 ± 1.6</td>
</tr>
<tr>
<td>Ovx+progesterone</td>
<td>33 ± 8</td>
<td>91 ± 6***</td>
</tr>
<tr>
<td>Male+vehicle</td>
<td>30 ± 1</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>Male+estradiol</td>
<td>182 ± 19***</td>
<td>8 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE. Ove, ovariectomized. ***P < 0.001 vs. vehicle-treated rats; n = 6 rats. **P < 0.001 vs. vehicle-treated rats; n = 4 rats.
retention associated with female hormones is often accompanied by edema formation (35, 48, 54).

Studies in ovariectomized females, with or without estradiol administration, cannot rule out the participation of PRL in the regulation of NCC function or expression, because its secretion is stimulated by estradiol (24, 28, 45, 46). Female rats exhibit higher levels of PRL in the blood compared with those in male rats. Ovariectomy reduces both estrogens and PRL serum levels (Table 1) (28). It is also known that PRL is an osmoregulator in lower vertebrates (20). This hormone increases salt and water reabsorption in teleost urinary bladders in which NCC mediates reabsorption (16, 50). We thus analyzed NCC expression and phosphorylation of NCC in kidneys from wild-type and PRL receptor knockout mice to compare the effects of PRL without changing the pituitary-hypothalamus axis. Female and male PRLR KO mice exhibited the gender regulation of NCC, but the presence of estrogens could be responsible for this difference. However, the positive effect of PRL was corroborated in rats treated with PRL and by using an ex vivo kidney perfusion system. Thus, consistent with the proposed role of PRL in the osmoregulation in teleosts and as a salt-retaining hormone in mammals, this peptide hormone is a positive regulator of NCC phosphorylation. The use of the ex vivo kidney perfusion system excludes the possibility that PRL administration increased NCC phosphorylation due to activation of extrarenal signals.

Fig. 5. G protein estrogen membrane receptor 1 (GPER-1) is expressed in the distal convoluted tubule (DCT). Distribution pattern between GPER-1, NCC, and parvalbumin is shown. Paraffin-embedded mouse kidney sections were immunostained for GPER-1 and NCC, and parvalbumin as a marker of DCT. GPER-1 and NCC were visualized with goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 594, respectively. Note: GPER-1 is localized to the basolateral membrane of epithelial cells in which NCC is apical and parvalbumin is intracellular.

Fig. 6. NCC phosphorylation is modulated by prolactin (PRL). A: representative Western blot of NCC expression and phosphorylation in wild-type and PRL receptor knockout (PRLR−−) male mice. The results from densitometric analysis of total NCC are expressed as the fold of NCC over β-actin, and NCC phosphorylation as the fold of pNCC over total NCC. *P = 0.06 vs. male control mice; n = 4–5 mice/bar. B: Western blot of NCC expression and phosphorylation in proteins extracted from individual renal cortex of male rats treated with 25 µg/kg PRL for 2 wk. The results from densitometric analysis of total NCC are expressed as the fold of NCC over β-actin, and NCC phosphorylation as the fold of pNCC over total NCC. *P < 0.05 vs. vehicle-treated rats; n = 4 rats/bar. C: Western blot analysis of NCC expression and phosphorylation in kidneys from male rats perfused ex vivo with vehicle or with PRL at 10 or 40 ng/ml. The results from densitometric analysis of total NCC are expressed as the fold of NCC over β-actin, and NCC phosphorylation as the fold of pNCC over total NCC. **P < 0.01 vs. vehicle-treated rats; n = 4 rats/bar.
The present study shows that physiological and molecular sex dimorphic regulation of NCC activity and expression are due to female hormones such as estrogens, progesterone, and PRL. It is known that periods in female life in which secretion of these hormones is increased are associated with the requirement of volume retention and expansion. For instance, women at the end of pregnancy had gained several kilograms due to the weight of the fetus, the placenta, the amniotic fluid, and the increased circulating blood. All of these contain considerable amounts of salt and water. In lactation, women lose several milliliters of water a day in milk production that must be promoting a salt-retaining state (54). At the end of the luteal phase, after several days of increased secretion of estrogens and progesterone, women often report the feeling of edema. Further studies will be interesting to pursue to specifically assess the role of NCC activation in each of these unique situations to female physiology.

ACKNOWLEDGMENTS

We thank Norma Vazquez for technical help, the gift of anti-GPER-1 antibodies to Shi-Bin Cheng and Edward J. Filardo and NCC or SPAK antibodies from Dundee University to Dario Alessi.

Parts of the present work were presented during the 2013 annual meeting of the American Society of Nephrology in Atlanta, GA, the 2014 annual Experimental Biology meeting in San Diego, CA, and were published in abstract form in the J Am Soc Nephrol (2013) and FASEB J (2014).

GRANTS

The present study was supported in part by Grants 59992 and 165815 from the Mexican Council of Science and Technology (CONACYT) to G. Gamba and the collaborative international grant 188712 from CONACYT and ANR-12-ISYS1-0001-01 from the Agence Nationale pour la Recherche (ANR; France) to G. Gamba and J. Hachdouel. Rojas-Vega was supported by a scholarship from CONACYT-Mexico and is a graduate student in the biomedical Science Ph.D. program of the Universidad Nacional Autónoma de México.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES