PROTOCOLO CINVA- NMM-801-13/131

"REGULACIÓN DIMÓRFICA SEXUAL DE LA EXPRESIÓN/ FUNCIÓN DEL CONSTRANSPORTADOR RENAL Na: Cl (NCC) EN RATAS HEMBRAS OVARIECTOMIZADAS"

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DEPARTAMENTO: NEFROLOGÍA Y METABOLISMO MINERAL

Abril 24, 2016

Dra. Norma A. Bobadilla
CINVA

RE: Informe final y cierre de proyecto.

Estimada Dra. Bobadilla:

Por medio de la presente comunico que ha finalizado el proyecto NMM-801-13/13-1 y le anexo como informe final el artículo publicado directamente con los resultados del mismo. Además, le informo también que este proyecto fue parte de la tesis Doctoral con la cual se recibió la Dra. Lorena Rojas Vega como Doctora en Ciencias Biomédicas el pasado 27 de enero de 2016, con Mención Honorífica.

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Am J Physiol Renal Physiol 308: F799–F808, 2015.
First published January 13, 2015; doi:10.1152/ajprenal.00447.2014.

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Ovarian hormones and prolactin increase renal NaCl cotransporter phosphorylation

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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.

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distal convoluted tubule; salt transport; thiazide; WNK; hypertension

THE SEXUAL FEMALE CYCLE, PREGNANCY, and lactation are physiological states unique to women in which volume retention is required. This can be achieved, at least in part, by decreasing urinary salt and volume lost through activation of the thiazide-sensitive Na^+-Cl^- cotransporter (NCC), which is expressed in the distal convoluted tubule (DCT) and represents a key step for NaCl reabsorption (10). It is known that NCC is subjected to sexual dimorphic regulation. Chen et al. (8) showed that the urinary response to thiazides, as well as binding of the thiazide-like diuretic [^{3}H]metolazone to renal cortical homogenates, was higher in female than in male rats and that these differences were reduced by ovariectomy. Verlander et al. (52) observed by immunogold electron microscopy that ovariectomy reduced the expression of NCC in DCT cells and that administration of 17β-estradiol to ovariectomized female rats restored NCC expression.

However, ovariectomy results in a concomitant decrease in estrogens, progesterone, and prolactin (PRL) (28). Although estrogen receptors are highly expressed in the kidney, their presence in the DCT has not been reported (13). In addition, previous studies also indicate a role for the peptide hormone PRL in the stimulation of NCC. PRL is a well-known osmoregulator in lower vertebrates (38). In teleosts, PRL increases salt and water reabsorption in the urinary bladder (20), an organ in which sodium reabsorption is mediated by NCC (16, 41, 50). In zebrafish, NCC expression in the gills is modulated by PRL (4). In mammals, PRL increases renal salt reabsorption in the distal nephron by a vasopressin-independent mechanism (49) and activates the Na^+-K^+-ATPase activity in DCT in a dose-dependent fashion (6). The expression of the PRL receptor in rat DCT has been reported (12, 30). In humans, excessive PRL caused by a pituitary PRL-secreting tumor, can produce renal salt retention (22) and hyperprolactinemia in patients,

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 bendroflumethiazide (BFTZ) 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 BFTZ, 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 protein extracts 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 Forte Western HRP substrate (Merck Millipore).

The following antibodies were polyclonal and raised in sheep: anti-NCC [recognizing residues 906–925 of human NCC (CHTKRFEDMIAPFRLNDGFKD) S965B], anti-SPAK [residues 196–210 of mouse (QSLSVHSDQAQPNAN), S150C], anti-phosphorylated NCC at threonine 58 [residues 54–66 of human (RTFGYNT*IDVVPTRR), S995B], and anti-phosphorylated SPAK at serine 383 [residues (RRVPGS(P)SGRLHKT), S670B]. These antibodies were produced and validated at the Medical Research Council phosphorylation unit at Dundee University and given as a kind gift by Dr. Dario Alessi (39, 43).

Western blots in Figs. 1, *A* and *B*, 2*B*, 3*A*, and 4*A* 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, "CHTKRFEDMIAPFRLNDGFKD", S965B) (39), polyclonal anti-GPER-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 ovariectomy. 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. 1*A*, 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 BFTZ was higher in females than in males (Fig. 1*B*). 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. 1*C*). Using urinary exosome analysis by Western blotting, we

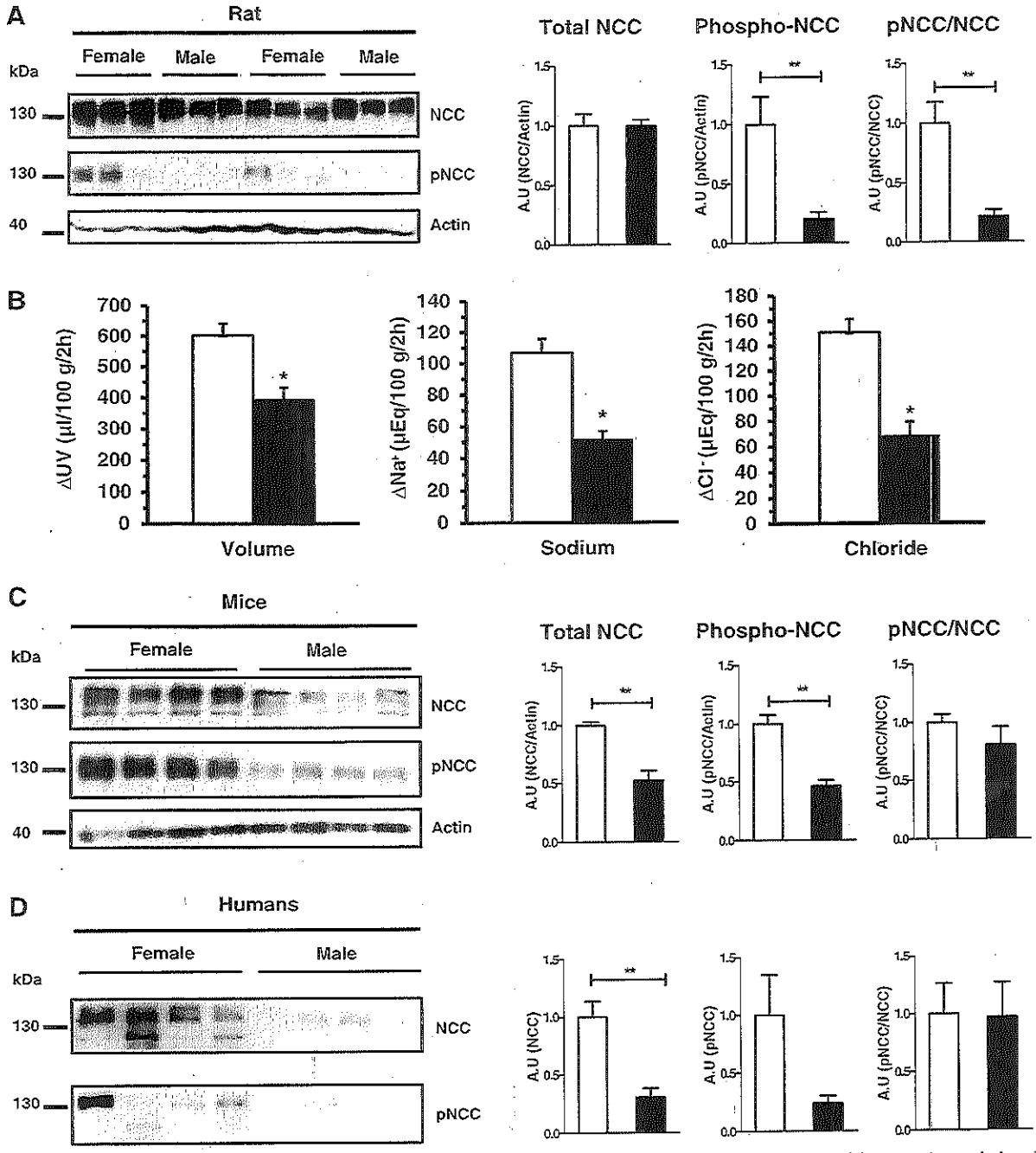

Fig. 1. Females have higher Na^+-Cl^- cotransporter (NCC) expression and phosphorylation. *A*: Western blot analysis of NCC expression and phosphorylation (T58) of female (open bars) and male (filled bars) rat proteins extracted from individual renal cortex. *B*: change in urinary volume, sodium, and chloride excretion 2 h before vs. 2 h after bendroflumethiazide (BFTZ) injection in female (open bars) and male (filled bars) rats. $*P < 0.05$ vs. female rats; $n = 12$ rats/bar. *C*: Western blot analysis of NCC expression and phosphorylation (T58) of female and male mice total kidneys. *D*: human urine exosomes of women and men normalized to urine creatinine. The result of densitometric analysis is expressed as the fold of NCC over β-actin, pNCC (T58) over β-actin, and NCC phosphorylation overexpression. $**P < 0.01$ in female rats; $n = 6$ (*A* and *B*). $**P < 0.01$ in female mice; $n = 4$ (*C*). $**P < 0.01$ in women; $n = 4$ (*D*).

also documented higher NCC expression and phosphorylation in humans (Fig. 1*D*).

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 orquiectomy 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

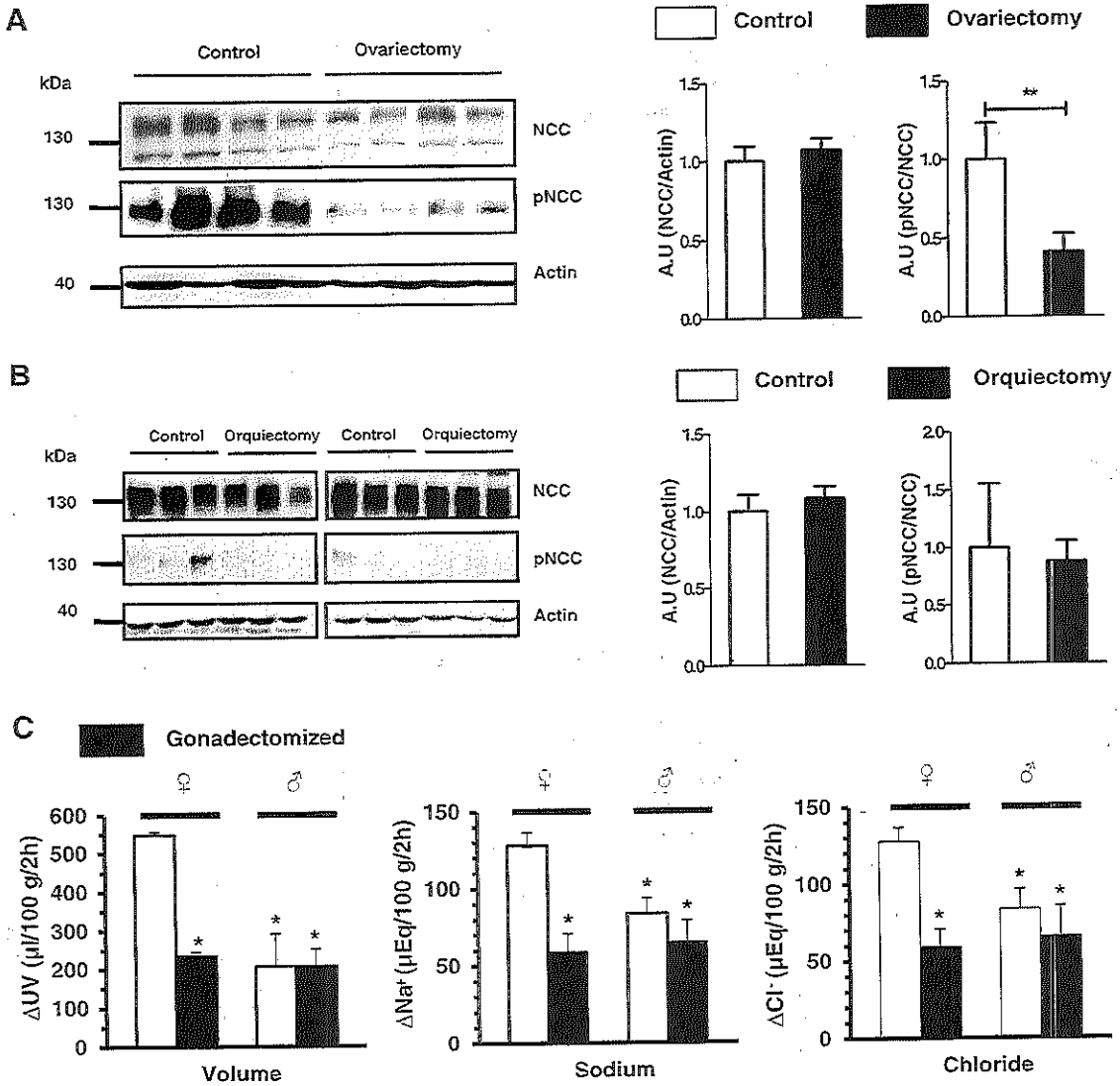

Fig. 2. Ovarian hormones regulate NCC phosphorylation. Western blot analysis of NCC expression and phosphorylation (T58) of control and ovariectomized (*A*) female rats and control and orquiectomized (*B*) male rats in proteins extracted from individual renal cortex; 2 different Western blots from different 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.

orquiectomy had no effect in males (Fig. 2*C*). 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. 1*A*, the NCC expression level was similar in the treated and control groups (Fig. 4*A*). In contrast, administration of either steroid induced a significant increase in NCC phosphorylation (Fig. 4*A*). The effect of 17β-estradiol was stronger

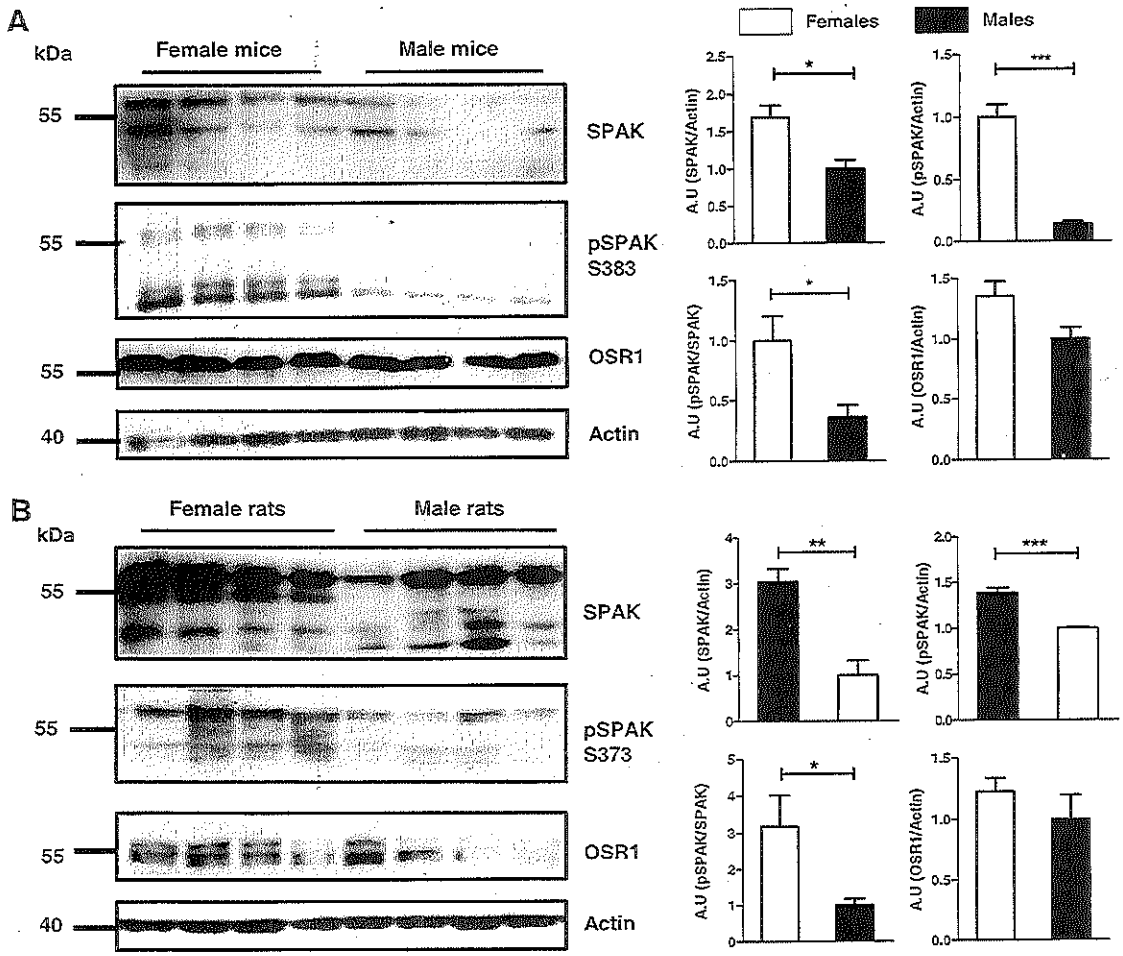

Fig. 3. Renal STE-20 proline-alanine-rich kinase (SPAK) is regulated by sexual hormones. Shown is Western blot analysis of SPAK expression and phosphorylation and OSR1 expression in control male and control female mice (*A*) and rats (*B*) in proteins extracted from whole kidney and cortex kidney, respectively. The results from densitometric analysis are expressed as the fold of total SPAK, phosphorylated SPAK, and phosphorylated SPAK over total SPAK. ***$P < 0.01$ vs. female mice. **$P < 0.01$ vs. female mice. *$P < 0.05$ vs. female mice; $n = 4$/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. 4*B*, 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. 4*A*). In ovariectomized rats treated with vehicle, the most prominent band observed for SPAK and phospho-SPAK 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

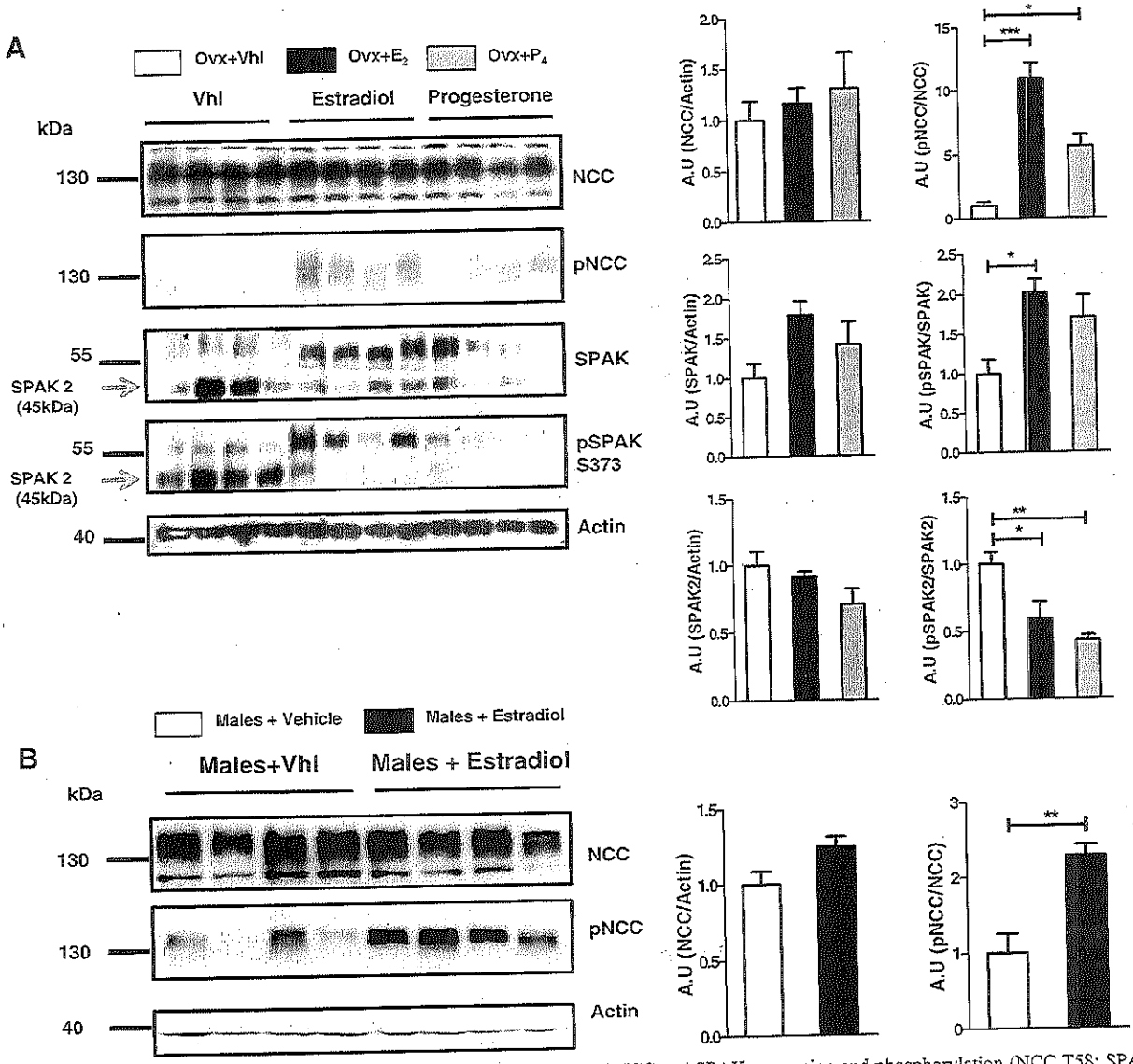

Fig. 4. Estrogens and progesterone phosphorylate NCC. *A*: Western blot analysis of NCC and SPAK expression and phosphorylation (NCC T58; SPAK S373) in ovariectomized rats treated with vehicle, 17-β-estradiol (60 μg/kg), or progesterone (100 mg/kg) for 3 wk. *B*: Western blot analysis of NCC expression and phosphorylation (NCC T58) in male rats treated with vehicle or 17-β-estradiol (60 μg/kg) for 3 wk. The results from densitometric analysis of total NCC are expressed as the fold of NCC and SPAK over β-actin and NCC and SPAK phosphorylation as the fold of phosphorylated NCC or SPAK over total NCC or SPAK, respectively. $^*P < 0.05$, $^{**}P < 0.01$. $^{***}P < 0.001$ vs. vehicle-treated rats: $n = 4$ rats per bar and $^{**}P < 0.01$ vs. vehicle rats; $N = 4$ rats per bar respectively.

receptor ($PRLR^{-/-}$) (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 $PRLR^{+/+}$ and $PRLR^{-/-}$ female mice (data not shown). We reasoned that the difference between female and male $PRLR^{-/-}$ mice persisted due to the presence of estrogens in females. The pNCC/NCC ratio tended to be lower in $PRLR^{-/-}$ male mice than in $PRLR^{+/+}$ males, but the difference did not reach significance ($P = 0.06$) (Fig. 6*A*), 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. 6*B*), 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. 6*C*).

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

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

	Male Control ($n = 6$)	Gnx Male ($n = 6$)	Female Control ($n = 5$)	Gnx Female ($n = 6$)
Body weight, g	443 ± 10	437 ± 12	292 ± 5	294 ± 1
Testosterone, ng/ml	2 ± 0.4	0.08 ± 0.01*	0.02 ± 0.002†	0.005 ± 0.001†
Estradiol, pg/ml	25 ± 2	4 ± 0.7*	50 ± 7	17 ± 1.8*
Prolactin, ng/ml	5 ± 0.1	3.7 ± 0.31‡	16 ± 1	7 ± 0.8*

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.

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

	E_2, pg/ml	P_4, ng/ml
Ovx+vehicle	28 ± 6	11 ± 3
Ovx+estradiol	142 ± 40***	10 ± 1.6
Ovx+progesterone	33 ± 8	91 ± 6***
Male+vehicle	30 ± 1	16 ± 6
Male+estradiol	182 ± 19***	8 ± 3

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.

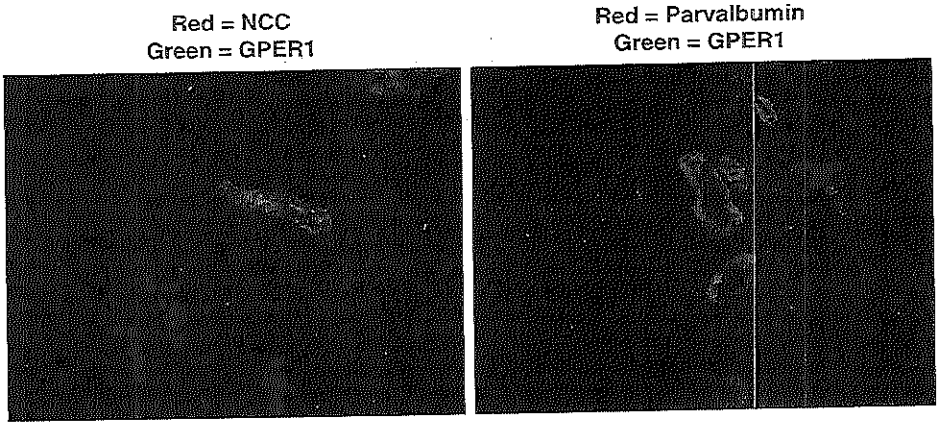

Fig. 5. G protein estrogen membrane receptor 1 (GPER-1) is expressed in the distal convoluted tubule (DCT). Distribution pattern between GPER-1, NCC, and parvalbumin is shown. Paraffin-embedded mouse kidney sections were immunostained for GPER-1 and NCC, and parvalbumin as a marker of DCT. GPER-1 and 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.

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.

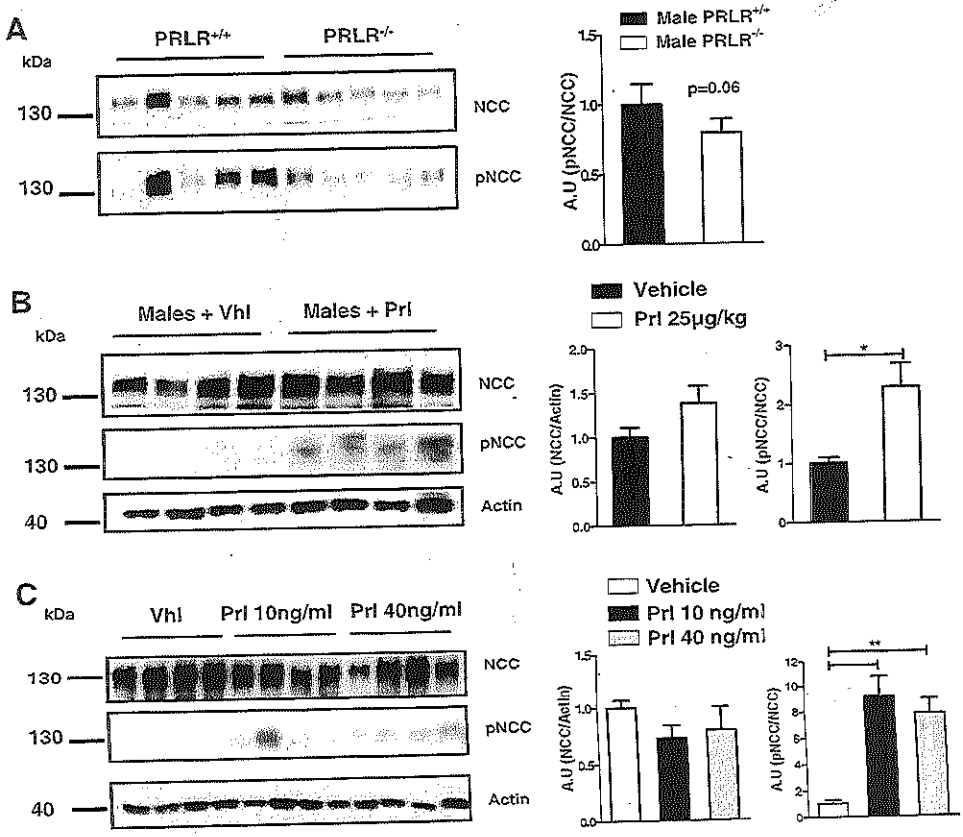

Fig. 6. NCC phosphorylation is modulated by prolactin (PRL). *A*: representative Western blot of NCC expression and phosphorylation in wild-type and PRL receptor knockout (*PRLR*$^{-/-}$) male mice. The results from densitometric analysis of total NCC are expressed as the fold of NCC over β-actin, and 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-ISVS1-0001-01 from the Agence Nationale pour la Recherche (ANR; France) to G. Gamba and J. Hadchouel. L. 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

Author contributions: L.R.-V., L.A.R.-C., V.R., R.B.-P., C.R., M.C.-B., P.M., P.D.I.H., I.A.-G., V.B., N.B., and J.H. performed experiments; L.R.-V., N.A.B., E.Z., and G.G. analyzed data; L.R.-V., N.A.B., E.Z., and G.G. interpreted results of experiments; L.R.-V. and G.G. prepared figures; L.R.-V. and G.G. drafted manuscript; L.R.-V., J.H., E.Z., and G.G. edited and revised manuscript; L.R.-V., N.A.B., and G.G. approved final version of manuscript; N.A.B. and G.G. provided conception and design of research.

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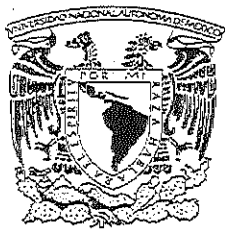

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DOCTORADO EN CIENCIAS BIOMÉDICAS
INSTITUTO DE INVESTIGACIONES BIOMEDICAS
FISIOLOGÍA RENAL

LA INVESTIGACIÓN BÁSICA Y TRASLACIONAL SOBRE EL COTRANSPORTADOR RENAL DE NACL. I. REGULACIÓN POR HORMONAS FEMENINAS. II. PAPEL EN LA HIPERTENSIÓN ARTERIAL INDUCIDA POR TACROLIMUS EN PACIENTES CON TRASPLANTE RENAL.

TESIS
QUE PARA OPTAR POR EL GRADO DE:
DOCTOR EN CIENCIAS

PRESENTA:

L.B. LORENA LEONOR ROJAS VEGA

TUTOR PRINCIPAL:

DR. GERARDO GAMBA AYALA

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que examinó a Lorena Jeanet Rojas Vega

para optar por el grado de Doctora

en Ciencias

hace constar que obtuvo la calificación de Aprobada

con mención honorífica

Ciudad Universitaria, a 27 de enero de 2016.

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"2015, Año del Generalísimo José María Morelos y Pavón"

México, D.F. a 23 de Junio del 2015

Dra. Norma Bobadilla Sandoval
Coordinadora de la CINVA
Presente

Estimada Dra. Bobadilla:

Por este conducto me permito solicitar el cierre del protocolo: " Regulación de la Fisiología de la Expresión/Función del Cotransportador Renal de Sodio en ratas hembra ovariectomizadas. " con registro **CINVA:** UMM-801-13/13-1 debido a que el protocolo ha concluido.

Sin otro particular por el momento, quedo de usted.

Atentamente,

Dr. Gerardo Gamba Ayala.
Nombre y Firma del (a) Investigador (a)

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"2015, Año del Generalísimo José María Morelos y Pavón"

México, D.F. a 23 de Junio del 2015

Dr. Gerardo Gamba Ayala
Depto. de Nefrología y Metabolismo
Minera.
Presente

Estimado Dr.Gamba.:

Por este conducto le informo que su proyecto: **"REGULACIÓN DIMÓRFICA SEXUAL DE LA EXPRESIÓN/FUNCIÓN DEL COTRANSPORTADOR RENAL NA:CL (NCC) EN RATAS HEMBRAS OVARIECTOMIZADAS."**, con registro **CINVA: NMM-801-13/13-1** finalizara el 8 de Julio de este año. Por lo que, le solicito de la manera más atenta me haga saber si el proyecto requerirá una prórroga. En caso afirmativo, favor de enviar a la **CINVA** el periodo de extensión que solicita y de requerir un mayor número de animales especificar y justificar como se utilizarán y los procedimientos experimentales que se llevarán a cabo con los mismos. En caso de no requerir una prórroga favor de llenar el formato de cierre del protocolo que se anexa a la presente.

Sin otro particular por el momento, quedo de usted.

Atentamente,

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la CINVA

INS. NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN" 29 JUN 2015 DIRECCIÓN DE INVESTIGACIÓN

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INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN SALVADOR ZUBIRAN INCMNSZ 26 JUN 2015 DEPARTAMENTO DE INVESTIGACIÓN EXPERIMENTAL Y BIOTERIO

c.c.p. Dr. Gerardo Gamba Ayala, Director de Investigación.
MVZ Mariela Contreras Escamilla, Jefa del Bioterio.

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"2014 Año de Octavio Paz"

INSTITUTO NACIONAL DE
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Y NUTRICIÓN
SALVADOR ZUBIRÁN

México, D.F., a 9 de Julio del 2014

DR. GERARDO GAMBA AYALA
Director de Investigación
P r e s e n t e .

Estimado Dr. Gamba:

Por este conducto me dirijo a usted para informarle que ha sido autorizada la prórroga solicitada hasta el 08 de Julio del 2015 para su proyecto intitulado " Regulación dimórfica sexual de la expresión/función del cotrasportador renal NaCl (NCC) en ratas hembras ovariectomizadas" con número de registro **CINVA 801, Clave NMM-801-13/131.**

Sin otro particular me despido de usted enviándole un cordial saludo.

Atentamente,

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la Comisión de Investigación en Animales

ccp: Dr. Rafael Hernández, Jefe del Bioterio

Leticia
9/07/14

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"2014, Año de Octavio Paz"

México, D.F., a 23 de mayo de 2014

DRA. NORMA BOBADILLA
COORDINADORA DE LA COMISIÓN DE INVESTIGACIÓN EN ANIMALES
DEPARTAMENTO DE NEFROLOGÍA Y METABOLISMO MINERAL

Estimada Dra. Bobadilla,

Por este conducto envío a usted la comunicación del Dr. Gerardo Gamba en su calidad de investigador en Ciencias Médicas "F" recibida el 21 de mayo del presente, referente a su solicitud de prórroga para el proyecto registrado con el título: "Regulación dimórfica sexual de la expresión/función del cotransportador renal NaCl (NCC) en ratas hembras ovariectomizadas" con clave de registro: NMM-801-13/131.

Lo anterior con el propósito de que la solicitud de prórroga pueda someterse a consideración de la CINVA en la próxima sesión a efectuarse el miércoles 4 de junio del presente.

Sin otro particular,

Atentamente

DR. RAFAEL HERNÁNDEZ GONZÁLEZ
JEFE DEL DEPARTAMENTO DE INVESTIGACIÓN EXPERIMENTAL Y BIOTERIO

C.c.p. Dr. Gerardo Gamba. Director de Investigación.

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICION "SALVADOR ZUBIRAN"
9:30
23 MAY 2014
DEPARTAMENTO DE NEFROLOGÍA Y METABOLISMO MINERAL

o de Quiroga No. 15
Colonia Sección XVI
Delegación Tlalpan
México, D. F. 14000
Tel. (52)54870900
www.incmnsz.mx

INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

Dra. Zobadilla

México, D.F. a 20 de Mayo de 2014

COMITÉ DE INVESTIGACIÓN EN ANIMALES
CINVA
Presente

El que suscribe Dr. Gerardo Gamba, investigador en ciencias médicas "F" del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Subiran. Se dirige a ustedes para solicitar una prórroga del proyecto registrado con el título: "Regulación dimórfica sexual de la expresión/función del cotransportador renal Na:Cl (NCC) en ratas hembras ovariectomizadas" con clave de registro: 'NMM-801-13/13-1', sometido y aprobado por el CINVA en este año. Dicha extensión se debe a la necesidad de repetir el protocolo en ratas macho y hembra para la verificación de los hallazgos experimentales encontrados en ratas hembras. La prórroga se solicita por un periodo de una año.

Dicho proyecto ha presentado sus avances en el congreso de: "Experimental Biology 2013 y 2014" con sedes en Boston y San Diego, California, respectivamente, para su exposición oral y en cartel.

ATENTAMENTE

Dr. Gerardo Gamba Ayala
Investigador en Ciencias Médicas "F"
Departamento de Nefrología y Metabolismo Mineral

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICION "SALVADOR ZUBIRAN"
9:30
23 MAY 2014
DEPARTAMENTO DE NEFROLOGÍA Y METABOLISMO MINERAL

Vasco de Quiroga No. 15
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Delegación Tlalpan
México, D. F. 14000
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INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICION
SALVADOR ZUBIRAN

"2014, Año de Octavio Paz"

México, D.F., a 22 de mayo de 2014

DR. GERARDO GAMBA
INVESTIGADOR EN CIENCIAS MÉDICAS "F"
DEPARTAMENTO DE NEFROLOGÍA Y METABOLISMO MINERAL

Estimado Dr. Gamba,

Por este conducto acuso recibo de su comunicación recibida el 21 de mayo del presente, que se refiere a la solicitud de prórroga para el proyecto registrado con el título: "Regulación dimórfica sexual de la expresión/función del cotransportador renal NaCl (NCC) en ratas hembras ovariectomizadas" con clave de registro: NMM-801-13/131.

Le comunico que he enviado su solicitud a la Dra. Norma Bobadilla, Coordinadora de la Comisión de Investigación en Animales para someter a su consideración de que se incluya para revisión en la próxima sesión de la CINVA a efectuarse el miércoles 4 de junio del presente.

Sin otro particular,

Atentamente

DR. RAFAEL HERNÁNDEZ GONZÁLEZ
JEFE DEL DEPARTAMENTO DE INVESTIGACIÓN EXPERIMENTAL Y BIOTERIO

C.c.p. Dra. Norma Bobadilla, Coordinadora de la CINVA
Gerardo Gamba. Director de Investigación.

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICION "SALVADOR ZUBIRAN"
9:30
23 MAY 2014
DEPARTAMENTO DE NEFROLOGÍA Y METABOLISMO MINERAL

Vasco de Quiroga No. 15
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INSTITUTO NACIONAL DE
CIENCIAS MEDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

Sep.20, 2013

Dr. Gerardo Gamba Ayala
Departamento de Nefrología y Metabolismo
Mineral
P r e s e n t e.

Por este medio me permito informar a usted que se aprueba su solicitud de prórroga al 18 de noviembre de 2013 del proyecto **"Regulación dimórfica sexual de la expresión/función del cotransportador renal Na:Cl (NCC) en ratas hembras ovariectomizadas"**con registro CINVA 801. Clave: NMM-801-13/13-1. Así mismo, le solicito enviar a esta Jefatura, información sobre la cantidad de animales de laboratorio que requerirá para el desarrollo del proyecto.

A t e n t a m e n t e

Dr. Rafael Hernández González
Coordinador de la Comisión de Investigación en Animales
Jefe del Departamento de Investigación Experimental y Bioterio

ccp. Dr. Rubén Lisker Y. Director de Investigación
MVZ., M.en C. Octavio Villanueva Sánchez. Secretario de la Comisión de Investigación en Animales
MVZ. Griselda Salmerón Estrada. CINVA
MVZ. M.en C. Ma. de la Luz Streber Jiménez. CINVA
Dr. Gonzalo M. Torres Villalobos. CINVA
Dra. Nimbe Torres y Torres. CINVA
Dr. Emiliano Tesoro Cruz. CINVA

Recibí Laura Rojas Vega
20/9/13

Vasco de Quiroga No. 15
Colonia Sección XVI
Delegación Tlalpan
México, D. F. 14000
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INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

México, D.F. a 18 de Septiembre de 2013

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN SALVADOR ZUBIRAN
INCMNSZ
18 SEP 2013
DEPARTAMENTO DE INVESTIGACIÓN EXPERIMENTAL Y BIOTERIO

COMITÉ DE INVESTIGACIÓN EN ANIMALES
CINVA
Presente

El que suscribe Dr. Gerardo Gamba, investigador en ciencias medicas "F" del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Subiran. Se dirige a ustedes para solicitar una prorroga del proyecto registrado con el título: "Regulación dimórfica sexual de la expresión/función del cotransportador renal Na:Cl (NCC) en ratas hembras ovariectomizadas" con clave de registro: 'NMM-801-13/13-1', sometido y aprobado por el CINVA en este año. Dicha extensión se debe a la necesidad de repetir el protocolo en ratas macho para la verificación de los hallazgos experimentales encontrados en ratas hembras. La prorroga se solicita por un periodo de dos meses.

Se autoriza prorroga por el periodo indicado

ATENTAMENTE

Dr. Gerardo Gamba Ayala
Departamento de Nefrología y Metabolismo Mineral

Vasco de Quiroga No. 15
Colonia Sección XVI
Delegación Tlalpan
México, D. F. 14000
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INNSZ

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

Enero 25,2013

DR. Gerardo Gamba Ayala
Investigador en Ciencias Médicas "F"
Departamento de Nefrología y Metabolismo Mineral
P r e s e n t e .

Con referencia al proyecto de investigación: "Regulación dimórfica sexual de la expresión/función del cotransportador renal Na:Cl (NCC) en ratas hembras ovariectomizadas"

Registro CINVA: 801

Clave: NMM-801-13/13-1

La Comisión de Investigación en Animales (CINVA), decidió **APROBARLO** para su desarrollo.

A t e n t a m e n t e

Dr. Rafael Hernández González
Coordinador de la Comisión de Investigación en Animales

ccp. Dr. Rubén Lisker Y.- Director de Investigación
MVZ., M.en C. Octavio Villanueva Sánchez .Secretario de la Comisión de Investigación en Animales
Dra. Nimbe Torres y Torres.- Comisión de Investigación en Animales

Recibí original
QFB Norma Vazquez
070213

Investigación
Tradición Servicio
Asistencia Docencia

• Vasco de Quiroga 15,
• Delegación Tlalpan
• C. P. 14000 México, D. F.
• Tel. 54-87-09-00

Instituto Nacional de Ciencias Médicas y Nutrición
Salvador Zubirán

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRAN
Dirección de Investigación
FORMA ÚNICA PARA REGISTRO DE PROYECTOS

FECHA DE RECEPCION: 15/11/2012 CLAVE: NMM-801-13/13-1
TITULO: Regulación dimórfica sexual de la expresión/función del cotransportador renal Na:Cl (NCC) en ratas hembras ovariectomizadas
INVESTIGADOR RESPONSABLE: Gamba Ayala Gerardo
DEPARTAMENTO O SERVICIO: DEPARTAMENTO DE NEFROLOGÍA Y METABOLISMO MINERAL
TIPO DE INVESTIGACION: Investigación Experimental
PATROCINADORES:

Patrocinador	Cantidad

VIGENCIA DEL PROYECTO: Del 21/01/2013 al 01/03/2013

Trimestre 1 Trimestre 2 Trimestre 3 Trimestre 4

COSTO TOTALES DE LA INVESTIGACION	
Personal	$ 0.00
(sueldos y sobresueldos al personal)	
Equipos	$ 0.00
(de laboratorio, cómputo, transporte, etc.)	
Materiales	$ 0.00
(reactivos, consumibles, desechables, etc.)	
Animales	$ 0.00
(adquisición, cuidado, procedimientos, etc.)	
Estudios	$ 0.00
(de laboratorio, gabinete,especiales,etc.)	
Viaticos	$ 0.00
(reuniones científicas y trabajo de campo)	
Publicaciones	$ 0.00
costo directos de publicación, sobregiro)	
Suscripciones	$ 0.00
libros, revistas, software, periódicos, etc)	
Varios	$ 0.00
(teléfono, fax, fotocopias, mensajería, etc)	
Fondo de apoyo	$ 0.00
(15% de la cantidad total de proyecto)	
Admon. Gastos pacientes	$ 0.00
Total :	$ 0.00

INSTITUCIONES PARTICIPANTES

FIRMAS

Investigador responsable Dr. Gerardo Gamba A.		Jefe de Departamento Dr. Ricardo Correa-Rotter
Comité de Investigación en Humanos		Comité de Investigación en Animales
Director de Investigación		Director General

Fecha de resolución
11-ABR-2014

Recibí Original
Omar Nuñez-O.
22/04/2014

INCMN SZ

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
"SALVADOR ZUBIRÁN"

México, D.F. a 20 de Noviembre de 2012

DECLARACIÓN DE LOS INVESTIGADORES

TÍTULO DEL PROYECTO: *Regulación dimórfica sexual de la expresión/función del cotransportador renal Na:Cl (NCC) en ratas hembras ovariectomizadas*
Clave de Registro: **'NMM-801-13/13-1'**

Los investigadores que participamos en el proyecto arriba mencionado sometemos voluntariamente a evaluación dicho proyecto ante la Comisión de Investigación en Animales y declaramos libremente:

- ***Que conocemos todos los aspectos del estudio y contamos con la capacidad de llevarlo a buen término.***
- ***Que la revisión minuciosa de los antecedentes científicos del proyecto justifican su realización y nos comprometemos a mantener un estándar científico elevado que permita obtener información útil para la sociedad.***
- ***Que conocemos los riesgos potenciales a los que exponemos al personal técnico, investigadores y los animales mismos involucrados en el proceso experimental, que se encuentran tanto en contacto directo como indirecto en el lugar donde se realiza la investigación. Por lo anterior, se establecen en el protocolo las medidas precautorias necesarias.***
- ***Que pondremos el bienestar de los animales sujetos de investigación y la seguridad del personal en contacto con ellos por encima de cualquier otro objetivo.***
- ***Que nos conduciremos de acuerdo con EL PRINCIPIO GENERAL DE LAS TRES R's descrito en el Manual del Usuario del SISTEMA ELECTRÓNICO DE REGISTRO DE PROTOCOLOS DE INVESTIGACIÓN: Comisión de Investigación en Animales, numeral 13 (http://132.247.8.50/latis invp/archivos/Manual.pdf). Asimismo, nos someteremos a los estándares de comportamiento ético y científico aceptados nacional e internacionalmente según lo establecido por la Ley General de Salud y el Reglamento en Materia de Investigación para la Salud de México, La NOM 062-ZOO-1999: "Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio" (publicada por SAGARPA en el Diario Oficial, 22 de agosto del 2001), y los lineamientos para el buen uso de los animales recopilados por el Consejo Internacional para La Ciencia de los Animales de Laboratorio en colaboración con la Organización Mundial de la Salud (ICLAS-WHO, http://www.iclas.org/harmonization.htm).***

Nombre del investigador	Firma
Dr. Gerardo Gamba Ayala	
Dra. Elena Zambrano	
EBC. Luis Antonio Reyes Castro	

Instituto Nacional de Ciencias Médicas y Nutrición
Salvador Zubirán

COMITÉ INSTITUCIONAL DE INVESTIGACIÓN BIOMÉDICA EN HUMANOS

FORMATO DE EVALUACIÓN DE PROYECTO DE INVESTIGACIÓN

No. de registro CIIBH: NMM-801-13/15-1

1. Título del proyecto

Regulación dimórfica sexual de la expresión/función del cotransportador renal Na:Cl (NCC) en ratas hembras ovariectomizadas

2. Investigadores

2a. Identificación

INVESTIGADOR	Posición institucional	Posición en el proyecto	Teléfono (ext.)	Correo-E
GAMBA AYALA GERARDO	INVESTIGADOR EN CIENCIAS MED F	Investigador responsable		gamba@quetzal.innsz.mx

2b. Pertinencia del grupo de investigadores con respecto del proyecto

3. Instituciones participantes

4. Patrocinio

4a. Organismos patrocinadores

4b. Especificar si los investigadores reciben pago (monetario o en especie) por su participación específica en la investigación.

5. Marco teórico

ANTECEDENTES:

El dimorfismo sexual evoluciono en los mamíferos superiores para asegurar el éxito reproductivo de los individuos, sin embargo los cambios se extienden mas allá de los órganos reproductores(15). Se cuenta con creciente evidencia de que la fisiología y fisiopatología renal se encuentran influenciadas por el genero o por las hormonas reproductivas. Por ejemplo existen transportadores que se expresan y/o regulan por andrógenos(1,6,29) la excreción renal de sodio puede verse reducida por la estimulación por estrógenos(13), existen diferencias renales sexuales en respuesta a la administración de angiotensina II(17)algunos compuestos son solo diuréticos en las ratas macho(10). Además de los marcados cambios ocurridos en la regulación de Na^+ durante el embarazo. Por otra parte es bien sabido que la progresión renal del daño renal crónico es mas rápido en hombres que en mujeres(20).

El contransportador de Na:Cl (NCC) es el principal mecanismo encargado de la reabsorción de Na^+ en el túbulo contorneado distal (DCT) en mamíferos, por lo que juega un papel importante en la determinación de lo niveles de presión arterial al estar fuera de la regulación de la macula densa y ser parte de la zona sensible a aldosterona

de la nefrona(4). Esta bien reportado que incrementos o decrementos en su función producen hipertensión o hipotensión respectivamente además existen fármacos, tiazidas, que inhiben de manera especifica al cotransportador, y que en la clínica se utilizan como diuréticos y como primer recurso como terapia para la hipertensión arterial(9).

Chen et al. fueron los primeros en mostrar evidencia de que la respuesta urinaria a tiazidas, así como la saturación de la unión del diuretico tipo tiazida [^{3}H]metolazona a los homogenados corticales renales son mas altos en hembras que en machos. Verlander et al. observaron por microscopia electrónica con anticuerpos marcados con oro contra NCC en el DCT de ratas ovariectomizadas que existe una reducción significativa, y que el administrar estrógenos restaura los niveles de NCC. Estos datos sugieren que NCC se expresa mas en hembras que en machos. Sin embargo la mayor respuesta a tiazidas en hembras puede deberse a la regulación dimorfica sexual del túbulo proximal o cambios compensatorios de zonas mas distales de la néfrona (14). Otra posibilidad es que el incremento en la expresión/función de NCC observados en hembras podría deberse al control hormonal de las hormonas sexuales femeninas: progesterona y estrógenos, o a la hormona hipofisiaria: prolactina, que en condiciones fisiológicas se encuentra mas elevada en mujeres. La progesterona y los estrógenos son atractivos candidatos pues existe evidencia de que existen incrementos en la reabsorción de Na^+ en forma de un decremento en la excreción renal de Na^+ asociados a la presencia de esta hormonas y disminución en ausencia de ellas, sin embargo existen incogruencias en los estudios realizados hasta la fecha (11,12,21-28). Por otro lado la prolactina parece un mejor candidato por la fuerte evidencia de que durante el embarazo y la lactancia fenómenos en donde los niveles de prolactina pueden elevarse diez veces (2) existe un incremento significativo en la reabsorción de Na^+ y que en vertebrados inferiores como los peces la prolactina funciona como una hormonas osmoreguladora (15)ADDIN CSL_CITATION{"mendeley": {"previouslyFormattedCitation": "(Manzon,2002)"}, "citationItems": [{"uris":["http://www.mendeley.com/documents/?uuid=10ae1685-77e0-4068-8cb6-fe3ffa60fb01"],"id": "ITEM-1", "itemData": {"DOI":"10.1006/gcen.2001.7746", "type": "article-journal","author": [{"given": "Lori A","family": "Manzon"}], "issued":{"date-parts": [["2002", "2", "1"]]},"abstract": "The protein hormone prolactin (PRL) was firstdiscovered as an anterior pituitary factor capable of stimulating milkproduction in mammals. We now know that PRL has over 300 different functions invertebrates. In fish, PRL plays an important role in freshwater osmoregulationby preventing both the loss of ions and the uptake of water. This paper willreview what is currently known about the structure and evolution of fish PRLand its mechanisms of action in relation to the maintenance of hydromineralbalance. Historically, functional studies of fish PRL were carried out usingheterologous PRLs and the results varied greatly between experiments andspecies. In some cases this variability was due to the ability of these PRLs tobind to both growth hormone and PRL receptors. In fact, a recurring theme inthe literature is that the actions of PRL cannot be generalized to all fish dueto marked differences between species. Many of the effects of PRL onhydromineral balance are specific to euryhaline fish, which is appropriategiven that they frequently experience sudden changes in environmental salinity.Much of the recent work has focused on the isolation and characterization offish PRLs and their receptors. These studies have provided the necessary toolsto obtain a better understanding of the evolution of PRL and its role inosmoregulation.", "title": "The role of prolactin in fishosmoregulation: a review.", "page": "291-310",

"volume":"125", "container-title": "General and comparativeendocrinology", "issue": "2", "id":"ITEM-1"}}], "properties": {"noteIndex": 0},"schema":"https://github.com/citation-style-language/schema/raw/master/csl-citation.json"} en donde NCC funciona como principal proteína de osmoregulación (29). En los humanos la PRL puede producir la retención renal de volumen urinario y la excreción de solutos se ve significativamente reducida en pacientes hiperprolactinemicos debido a tumores hipofisiarios secretores de prolactina (8). Se sabe que la prolactina es regulada por los niveles de estrógenos y que la ovariectomización disminuye la secreción de prolactina de los lactotrofos (3).

DEFINICION DE PROBLEMAS :

Todo lo anterior conlleva a la pregunta de si los efectos observados en el incremento en la reabsorción de Na^+ en la hembras en comparación a los machos se deben a la progesterona, a los estrógenos o a la prolactina, lo que a su vez genera la necesidad de utilizar un modelo en donde se puede aislar los efectos de cada una de ellas, para ello se puede utilizar el modelo de rata hembra ovariectomizada para poder suprimir los niveles de estrógenos y progesterona generados por los ovarios y el cuerpo luteo y por lo consecuente de prolactina que es estimulada por los picos hormonales estrales, y de esta manera poder disecar los efectos de cada una de estas hormonas y observar los efectos sobre NCC. Actualmente en el laboratorio se cuenta con la capacidad de no solo poder ver la expresión total de la proteína de NCC a través de anticuerpos, sino de poder cuantificar si determinados estímulos tienen la capacidad de activar a NCC utilizando fosfo-anticuerpos específicos contra residuos que se fosoforilan por estímulos que favorecen la función del contransportador, como la depleción de cloro(19), o por la activación por angiotensina II(5).

JUSTIFICACION :

El presente trabajo dilucidara la regulación hormonal de las hormonas sexuales femeninas sobre la expresión/función de NCC en ratas hembras gonadolectomizadas administradas con progesterona, estrógenos o prolactina. Este estudio permitirá obtener un conocimiento más profundo sobre los mecanismos de regulación de la reabsorción de sal a nivel del túbulo contorneado distal de la néfrona, que se ha implicado en el mantenimiento de la presión arterial y de la homeostasis electrolítica.

6a. Hipótesis

Las hormonas sexuales femeninas incrementan la activación/fosforilación del contransportador Na:Cl (NCC).

6b. Objetivos.

Determinar si la ovarectomización decrementa la activación/fosforilación de NCC y se restaura en presencia de las hormonas sexuales femeninas.

7. Metodología: Diseño general.

Modelo animal: Se utilizaran 40 ratas(el número fue determinado en base a estudios previos similares 2,7,31) hembras de 240 ± 20 g y entre 10 y 12 semanas de edad de la cepa wistar. Los animales serán mantenidos en el bioterio del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubian bajo condiciones cotroladas de luz-obscuridad (de 7:00 a 19:00 h), así como de humedad y temperatura (22-23°) alimentadas con la dieta estandar Purina 5001 y agua ad libitum.

Las ratas se trabajaran en una sola entrega para ser divididas en seis grupos : 1) ratas sham n=5 2) ratas ovx n=7 3)ratas ovx+vehículo n=7, 4) ratas ovx+estrógenos n=7 5) ratas ovx+progesterona n=7 6) ratas ovx+prolactina n=7.

La ovariectomización se realizará bajo anestesia con pentobarbital sódico 30 mg/kg (Flecknell, 2009) y en prescencia de atropina 0.05 mg/Kg (CCAC, 1984) para despejar las vías aereas; se rasurara el área dorso-lumbar y se desinfectara el área con yodo. Se realizara una incisión en la piel de 2 cm a lo largo de la línea media dorsal (caudalmente a la ultima costilla) y a través de la musculatura abdominal. Utilizando pinzas se desplazara hacia los costados dependiendo de cual ovario se intervendrá primero, se hará una pequeña incisión utilizando tijeras pequeñas para poder separar los músculos dorso-laterales abriendo las tijeras, para causar el minímo daño posible, se localizará el ovarío se ligará de manera próxima a la trompa de falopio con una sutura absorbible de 5 ceros, dejando cabos cortos para impedir el rose con las paredes del abdomen y minimizar el trauma en las ratas. Después de ligar se cortará el ovario y se regresará con cuidado la trompa a la cavidad abdominal.Se suturara el corte de la línea media dorsal primero el músculo con una sutura absorbible del numero 3.0 y después la piel. La cirugía sham se hace lo mismo pero sin remover los ovarios. La cirugía dura 10 minutos por animal, los instrumentos son esterilizados entre animal y animal utilizando un esterilizador de perlas de cristal. Posterior a la cirugía recibirán por una semana 1.1–2.5 mg/kg de flunixin de manera SC durante la primera semana (Fleknell, 1992).

Cuatro semanas después de la cirugía se realizara un frotis vaginal para cuantificar que los niveles de estrógenos y progesterona se encuentren abatidas. Una vez confirmado se procedera a aplicar vehículo (aceite de maíz), estrogenos, progesterona y prolactina por inyección intraperitoneal durante 5 días tras lo cual se obtendrán los riñones a través de escisión quirúrgica, bajo anestesia, que posteriormente se llevara a cabo a través de una sobredosis de anestesia por vía intravenosa, los restos de los animales serán colocados

en las bolsas amarillas (asignadas para residuos peligros biológico infecciosos: RPBI), posteriormente se congelaran a -20°C (en el depósito de cadáveres de animales para necropsia que el Bioterio del Instituto tiene asignado para tal propósito). Una compañía privada contratada por el INCMNSZ se encargará de la posterior incineración de los animales, de acuerdo a la norma establecida: NOM-087-ECOL-1995.

Criterios para dar por terminado el experimento en caso de que los animales presenten signos de sufrimiento.

Los animales que en el transcurso del experimento presenten algún tipo de sufrimiento (no generado por el diseño experimental), serán sometidos a eutanasia. Los criterios son (www.ahc.umn.edu/rar/euthanasia.html):

1. Pérdida de peso mayor al 20-25%.
2. Pérdida del apetito: completa anorexia por 24 h o anorexia parcial (50% del la ingesta calórica) durante 3 días.
3. Debilidad o inhabilidad para obtener su alimento y agua.
4. Estado moribundo: signos de depresión o la falta de respuesta a estímulos.
5. Presencia de alguna infección.
6. Signos de disfunción severa de algún órgano o sistema
7. Presentación de alguna anormalidad física (tumores).

Para los siguientes procedimientos: maniobras conductuales, modificaciones ambientales, restricción física y ejercicio, inmunizaciones, inoculación de agentes biológicos, sustancias peligrosas, radiaciones, trauma, cirugía, NO APLICA.

Entrenamiento del personal que estará en contacto con los animales:

Las personas que estarán en contacto serán la Lic en Ciencias Biomedica Lorena Leonor Rojas Vega tesista de doctorado del Dr. Gerardo Gamba y el investigador asociado: EBC. Luis Antonio Reyes Castro del departamento de Biología de la Reproducción. Ambos personas con previo entrenamiento en el uso y manejo de especies de bioterio.

La ovariectomización así como el seguimiento, aplicación de las hormonas y eutanasia será realizada por EBC. Luis Antonio Reyes Castro con asistencia de la Lic. en Ciencias Biomédicas Lorena Rojas Vega.

Estudios Moleculares:

Mediante técnica bioquímicas (western blot y PCR en tiempo real) se analizará la expresión y fosforilación de proteínas de interés (WNK4, SPAK, NCC). También se estudiarán parámetros fisiológicos en condiciones basales como la presión arterial y la concentración plasmática de electrolitos.

Extracción de RNA: El RNA se extrajo de los tejidos almacenados a -80°C mediante homogenización con fenol y tiocianato de guanidina y ultracentrifugación con cloroformo. Para determinar la calidad del RNA se midió su concentración por espectrofotometría de UV (280 nm/260 nm).

RT-PCR (Transcripción Reversa): La transcripción reversa (RT) se llevó a cabo con

10 µg de RNA total del tejido. Primero se llevó el RNA a 65 °C por 10 min. La reacción se realizó utilizando 200 U de transcriptasa reversa del virus de la leucemia en el mono (Moloney murine leukemia virus reverse transcriptase, MMLV, Stratagene), 100 pmol de hexámeros al azar (random primers, Life Technologies), 0.5 mM de cada dNTP (una mezcla de dCTP, dATP, dGTP, dTTP, Sigma), y 1X de buffer de TR (75 mM KCl; 50 mM Tris-HCl; 3 mM MgCl2; 10 mM DTT, pH 8.3), se incubó a 37°C por 60 min y se llevó a un volumen final de 20 µl. Una vez transcurrido el tiempo de reacción, las muestras se llevaron a 95°C por 5 min para inactivar la transcriptasa reversa.

PCR en tiempo real: Se utilizaron sondas TaqMan específicas para amplificar fragmentos de DNAc de Applied Biosystems marcadas con FAM (6-carboxyfluoresceina) o VIC, para el análisis de marcadores de fibrosis y/o inflamación: IL-6, TNF-a y MCP-1 y 18S RNAr como amplificación control. FAM y VIC son antes fluorescentes utilizados para detectar la amplificación de productos. De esta forma la cantidad de FAM o VIC, liberada por la degradación de la sonda TaqMan por exonucleasa en la reacción de PCR, es medida en función del cada ciclo de amplificación por reacción PCR mediante el uso de un termociclador en tiempo real ABI 7000 Prism (Applied Biosystems). La expresión de cada gen se cuantificó en forma relativa usando el método comparativo de Ct.

Western Blott: Mediante una electroforesis en gel se separan las proteínas previamente obtenidas de tejido renal almacenado a -80°C atendiendo al criterio que se desee: peso molecular, estructura, hidrofobicidad, etc. Luego son transferidas a una membrana adsorbente (típicamente de nitrocelulosa o de PVDF) para poder buscar la proteína de interés con anticuerpos específicos contra ella, en este caso contra NCC y pNCC. Finalmente, se detecta la unión antígeno-anticuerpo por actividad enzimática, fluorescencia entre otros métodos. De esta forma se puede estudiar la presencia de la proteína en el extracto y analizar su cantidad relativa respecto a otras proteínas.

8. Metodología: Criterios de selección

No aplican pues se utilizaría el modelo animal: rata wistar hembra.

9. Metodología: Desenlaces y variables

Desenlaces esperados

Se espera que la ovariectomización disminuya la función de NCC en comparación con las ratas sham.

Se espera encontrar los efectos positivos sobre la función/expresión de cada una de las hormonas sexuales femeninas: estrógenos y progesterona y de la hormona hipofisiaria: prolactina sobre el contransportador de NaCl de ratas ovariectomizadas.

Frecuencia de las mediciones

Previo a la cirugía de ovariectomización se medirá el peso de las ratas, para poder tener un control

postquirúrgico. Así como de cada semana durante el mes postoperatorio.

Después de un mes se tomara un frotis vaginal para medir los niveles hormonales.

Criterios de éxito y falla

Unicamente se excluirán del estudio a los animales que por algún motivo fallezcan durante el estudio, los demás ánimales se tomaran como individuos experimentales dentro del estudio.

Estratégia de análisis estadístico

Al final del procesamiento descrito previamente en lel apartado de "Metodología: Diseño General"de muestras se analizaran los western blotts por densitometría y para determinar la significancia d elos grupos una prueba T-Student no empatada.

10. Riesgos y beneficios del estudio

BENEFICIO :

Este estudio permitirá obtener un conocimiento más profundo sobre los mecanismos de regulación de la reabsorción de sal a nivel del túbulo contorneado distal de la néfrona, que se ha implicado en el mantenimiento de la presión arterial y de la homeostasis electrolítica.

RIESGOS:

MOLESTIAS GENERADAS : No aplica
COMPLICACIONES DE PROCEDIMIENTO : No aplica
EFECTOS ADVERSOS : No aplica.
EFECTOS PSICOLOGICOS : No aplica.
METODOS DE SEGURIDAD : No aplica
PROCEDIMIENTOS : No aplica
OTRO TIPO DE RIESGO : No aplica

11. Costos

COSTOS TOTALES DE LA INVESTIGACIÓN	
Admon. Gastos pacientes	**$ 0.00**
Animales	**$ 0.00**
Equipos	**$ 0.00**
Estudios	**$ 0.00**
Fondo de apoyo	**$ 0.00**
Materiales	**$ 0.00**
Personal	**$ 0.00**
Publicaciones	**$ 0.00**
Suscripciones	**$ 0.00**
Varios	**$ 0.00**
Viaticos	**$ 0.00**

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