Interaction of Estrogen and Progesterone in the Regulation of Sodium Channels in Collecting Tubular Cells

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Background: Renal Na^+ handling differs between males and females. Further, within females Na^+ metabolism changes during the menstrual cycle and pregnancy. Estrogen and progesterone could regulate α, β and γ amiloride-sensitive epithelial sodium channel (ENaC) subunit mRNA levels in female rat kidney. The aim of our study is to clarify the role of the female gender steroids in the regulation of ENaC activity.

Methods: We conducted an *in vitro* study on cultured collecting tubular cells. The collecting tubular cells were cultured under different concentrations of estrogen and/or progesterone. We analyzed the mRNA expression and protein translation of ENaC by reverse transcription polymerase chain reaction (RT-PCR) and Western blotting. Sodium channel activity was measured by short circuit current.

Results: Estrogen and/or progesterone alone mildly increased ENaC activity. A low concentration of estrogen together with progesterone stimulated ENaC activity more than 2-fold. A high concentration of estrogen almost completely inhibited the stimulated ENaC activity.

Conclusion: Female gender steroid hormones affect RNA expression, protein translation and ENaC activity in renal collecting tubule cells. The effects might suggest that pre-menstruation edema is a result of subtle interaction between the female gender steroid hormones on ENaC in renal collecting tubule cells.
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Key words: female gender steroid, collecting tubular cell, sodium channel

Edema is defined as a palpable swelling produced by expansion of the interstitial fluid volume. Various clinical conditions are associated with the development of edema, including heart failure, cirrhosis, nephrotic syndrome and many other diseases. There are two basic steps involved in edema formation. An alteration in capillary hemodynamics favors the movement of fluid from the vascular space into the interstitium.[1,2] Further, the kidney retains dietary or intravenously administered sodium and water.[3]

The importance of the kidneys in the development of edema should not be underestimated. Edema does not become clinically apparent until the interstitial volume has increased by at least 2.5 to 3 liters.[4] Since the normal plasma volume is only about 3 liters, it is clear that patients would develop marked

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hemoconcentration and shock if the edema fluid were derived only from the plasma. These complications do not occur because movement of fluid from the vascular space into the interstitium reduces the plasma volume and consequently tissue perfusion. In response to these changes, the kidney retains sodium and water. Some of this fluid stays in the vascular space, returning the plasma volume toward normal. However, the alteration in capillary hemodynamics, due to the local transcapillary hydraulic pressure gradient exceeding the opposing colloid osmotic pressure gradient throughout the length of capillary bed, results in most of the retained fluid entering the interstitium and eventually becoming apparent as edema.[1,2] The net effect is a marked expansion of the total extracellular volume with maintenance of the plasma volume at closer to normal levels. Renal sodium and water retention plays a vital role in the development of edema formation.

The normal menstrual cycle is a tightly coordinated cycle of stimulatory and inhibitory effects that results in the release of a single mature oocyte from a pool of hundreds of thousands of primordial oocytes. Varieties of factors contribute to the regulation of this process including monthly hormone variation. The average adult menstrual cycle lasts 28 days, with approximately 14 days in the follicular phase and 14 days in the luteal phase (Fig. 1).[5,6] Gradually rising progesterone concentrations appear in the middle to late luteal phase. Estrogen is also maintained at a high level. The pre-menstruation phase corresponds to the late luteal phase, which is associated with the decline in estrogen and progesterone release from the resolving corpus luteum.[5,6] Pre-menstruation edema is one of the most common causes of generalized edema seen by the clinician. Some adult females have a generalized accumulation of extracellular fluid before menstruation. The edema subsides spontaneously at the onset of menstruation (Fig. 1).[7] However, there is little definite clinical evidence of a quantitative relationship between levels of progesterone/estrogen and edema formation in these patients compared with normal controls.[7]

Sodium homeostasis in humans is maintained mainly by the kidney. The cortical collecting duct (CCD) is the final nephron segment for the fine-tuning regulation of sodium transport.[8] Sodium enters the CCD cells via epithelial sodium channels (ENaC) in the apical membrane and leaves the basolateral

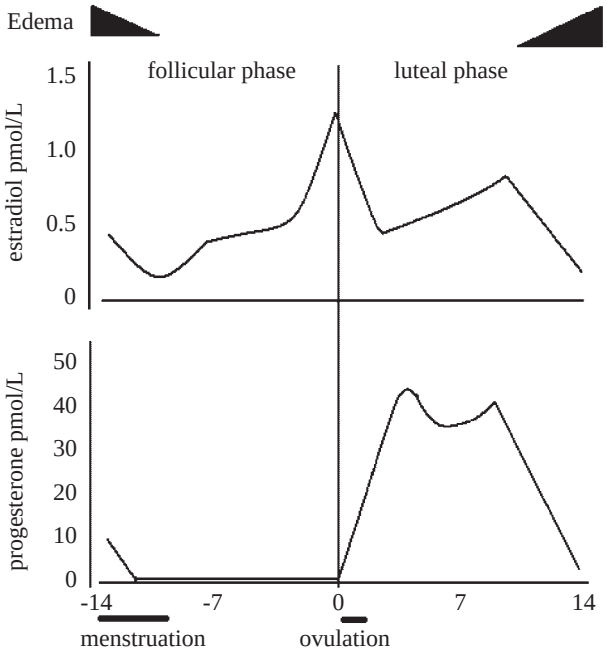

Fig. 1 Typical plasma progesterone and estrogen concentrations during a 28-day human menstrual cycle (modified from the figure in Ref. 6). Body weight gain and peripheral edema often develop during the luteal phase of the menstrual cycle. The edema subsides spontaneously at the onset of menstruation.

membrane via Na-K-ATPase. Reabsorption of sodium on the apical side of the CCD creates a lumen negative transepithelial potential. This negative potential drives potassium secretion to the lumen side of the CCD and enhances chloride passive transport via a paracellular route.[9] Hyperfunction of ENaC has been implicated as the pathogenic cause of salt-sensitive hypertension and sodium retention in glomerulonephritis.[10]

The ENaC is the key component of transepithelial sodium transport in epithelium of different tissues, such as airway, sweat gland or CCD etc. It is a heteromultimeric complex of three subunits, a, b and ğ ENaC, that is specifically inhibited by amiloride.[11] αENaC constitutes 90% of normal ENaC function. The rate of sodium transport across these epithelia is controlled to a large extent by ENaC synthesis and surface expression.

Renal Na^+ handling differs between males and females. Further, within females, Na^+ metabolism changes during the menstrual cycle and pregnancy.

Electrolyte homeostasis and extracellular volume are maintained primarily by regulated transport of Na^+ via the amiloride-sensitive Na^+ channel. Estrogen and progesterone could regulate α, β and γ amiloride-sensitive ENaC subunit mRNA levels in female rat kidney.[12] However, the regulation of mRNA expression provides inadequate information to describe the effects of estrogen and progesterone in the regulation of ENaC activity. Furthermore, the results of female hormone studies on ENaC activity are conflicting.[12,13]

With this background, it is interesting to ask about the role of the female gender steroids in the regulation of ENaC activity. We hypothesize that progesterone can enhance ENaC activity. High estrogen levels in the middle luteal phase prevent the stimulatory effect. The early decline of estrogen in the late luteal phase decreases the inhibitory effects and predisposes to pre-menstruation sodium retention via ENaC activation.

METHODS

Culture cells

To test the hypothesis, we conducted an *in vitro* study on cultured collecting tubular cells. We used the cultured mpkCCD cells derived from male C57BL/6 mice as previously described.[14] The mpkCCD cells are derived from isolated CCDs microdissected from the kidneys of SV-PK/Tag transgenic mice. Briefly, the kidneys of a 1-month-old SV-PK/Tag transgenic mouse were removed under sterile conditions, sliced and incubated in medium (Dulbecco's Modified Eagle's Medium (DMEM): Ham's F12 (HFM), 1:1 vol/vol) containing 0.1% (wt/vol) collagenase for 1 h at 37°C. The slices were rinsed in medium and CCD fragments were microdissected. Pools of 5-10 isolated CCDs were rinsed, transferred to collagen-coated 24-well trays and cultured in a modified DM medium (DMEM: HFM, 1:1 vol/vol; 60 nM sodium selenate; 5 μg/ml transferrin; 2 mM glutamine; 50 nM dexamethasone; 1 nM triiodothyronine; 10 ng/ml epidermal growth factor (EGF); 5 μg/ml insulin; 20 mM D-glucose; 2% fetal calf serum (FCS); 20 mM HEPES, pH 7.4) at 37°C in 5% CO2/95% air atmosphere. After several passages, the cells grew faster and were cultured in 25 cm^2 culture flasks (Corning Costar Corp., Cambridge, MA, USA). The mpkCCD cells were grown in the same modified DM medium described above. A line of mouse CCD cells can thus be subcultured for a long time (over 35 passages). The medium was changed every 2 days and all studies described in this study were performed on confluent cells taken between the 10th and 25th passages. Experiments were carried out 2 weeks after seeding using confluent cells that had developed high transepithelial electrical resistance (> 700 Ω cm^2).

Extraction of RNA and RT-PCR

Total RNA was extracted with Triazol reagent (Life Technologies Inc, Gaithersburg, MD, USA) from mpkCCD cells cultured in 6-well dishes or 24-well cell culture inserts (Corning Costar Corp., Cambridge, MA, USA) with or without incubation of estrogen and/or progesterone.[15] Reverse transcription (RT) was done with Superscript II (Life Technologies Inc, Gaithersburg, MD, USA), primed by oligo-dT, at 42°C for 45 minutes. The primers for mouse αENaC were as follows: αENaC sense 5-CTA ATC ATG CTG GAC CAC C-3, αENaC anti-sense 5-AAA GCG TCT GGA TCC-3,[16] β-actin sense 5- GCC AGG ATA GAG CCA CCA ATC- 3; and β-actin anti-sense 5-ACT GCC CTG GCT CCT AGC A-3 (nucleotide). 100 ng cDNA and non-RT RNA were amplified for 28 cycles in 100 μl total volume containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 40 μM dNTP, 1.5 mM $MgCl_2$, 1 unit Taq polymerase and 29 pmol αENaC primers. The thermal cycling programmes were as follows: 94°C for 30 s, 54°C for 30 s and 72°C for 60 s. Each RT-polymerase chain reaction (RT-PCR) product was electrophoresized on 2% agarose gel containing ethidium bromide.

Extraction of protein and Western blotting

The mpkCCD cells cultured in the same condition as RT-PCR studies were homogenized with Dounce homogenize for protein extraction. αENaC was detected by Western blotting using an immunopurified anti-rabbit ENaC antibody. Confluent cells were scraped off, collected in phosphate-buffered saline (PBS), centrifuged (150 g for 5 min) and homogenized in 50 μl lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, containing 0.5% (w/v) sodium dodecyl sulfate (SDS), 0.1 mM phenylmethyl-sulfonylfluoride (PMSF) and 100 μg/ml leupeptin). Protein content was determined by

the Bradford method using bovine serum albumin as standard.[17] Cell homogenates (50 μg) were sub-fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels. The proteins were transferred to polyvinylidene difluoride membranes (NEN), probed (4 h at 4°C) with the anti-ENaC (1:1000) (a kind gift from Dr. Mark Knepper of National Institute of Health) and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Dako, Trappes, France) for 60 min at room temperature. Negative controls were performed by omitting the primary antibody. Blots were also incubated with a β-tubulin monoclonal antibody. The antigen-antibody complexes were detected using the NBT-BCIP alkaline phosphatase substrate (Sigma Chemicals, St Louis, MO, USA).

Electrophysiological studies

Confluent mpkCCD cells were grown on 0.33-cm^2 Transwell filters (Corning Costar Corp., Cambridge, MA, USA) in DM medium until confluent (day 14) and then in DM containing no EGF, hormones, FCS or HEPES (HFM containing 29 mM $NaHCO_3$) for the final 2 h. The filters were mounted in a modified Ussing-type chamber (Diffusion Chamber System, Corning Costar Corp., Cambridge, MA, USA) connected to a voltage clamp apparatus via glass barrel micro-reference Ag/AgCl electrodes. Cell layers were bathed on both sides (0.2 ml for the apical side and 0.6 ml for the basal side) with HFM warmed to 37°C and continuously gassed with 95% O2/5% CO_2 to keep the pH at 7.4. Short circuit current (Isc, $\mu A/cm^2$) was measured by clamping the open-circuit transepithelial voltage (VT) to 0 mV for 1 s.[18] By convention, a positive Isc value corresponds to a flow of positive charges from the apical to the basal solution.

RESULTS

Cultured mpkCCD cells

Cells from CCD fragments in glucose-enriched medium multiplied rapidly after the first passage. The confluent mpkCCD cells grown on plastic support were all cuboid in shape and formed domes (Fig. 2). The mpkCCD cells grew rapidly (doubling time 20 to 30 h) and had a long life span (more than 45 passages to date). As described in the previous study, mpkCCD cells contained a typical network of

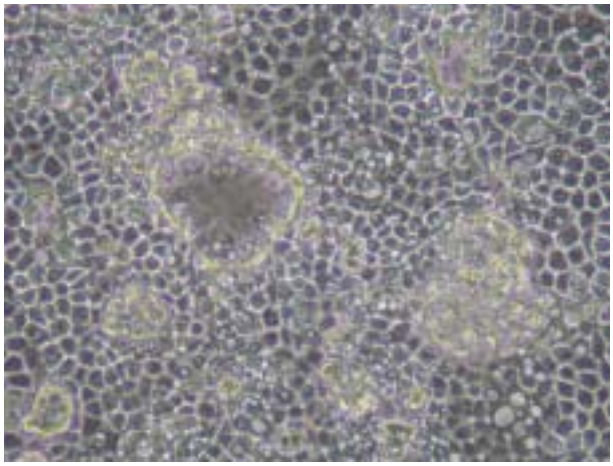

Fig. 2 Properties of cultured mouse mpkCCD cells. Confluent mpkCCD cells grown on Petri dishes form layers of cuboid-shaped cells and small domes, representing active fluid transport (magnification x 250).

cytokeratins K_8-K_{18} and the tight junction-associated protein ZO-1 at the cell peripheries.[18] The mpkCCD cells also had a basolaterally located Na^+-K^+ ATPase and apical ENaC. The cells maintained their structural polarity when grown on porous filters, and formed monolayers of epithelial cells closely apposed and sealed by typical junctional complexes.[18]

RT-PCR

Menstruation cycle is associated with various hormone changes. In the early and middle luteal phase, progesterone increases gradually with a background of high estrogen levels. In the late luteal phase, a gradual decrease in luteinizing hormone (LH) secretion results in a gradual fall in estrogen production by the corpus luteum in the absence of a fertilized oocyte. We investigated the effects of different concentrations of estrogen and/or progesterone on ENaC expression in mpkCCD cells. Fig. 3 illustrates the levels of αENaC mRNA expression within untreated and hormone-treated cells compared to the expression of β-actin, used as an internal standard. Incubating for 24 h with 25 nM estrogen induced a 2.2 fold increase in αENaC mRNA (Fig. 3). Increasing the concentration of estrogen to 50 nM increased the expression of αENaC up to 3.5-fold. Progesterone (10^{-6} M) also increased the expression of αENaC up to 3.0-fold. Concomitant appearance of low concentration estrogen (25 nM) and proges-

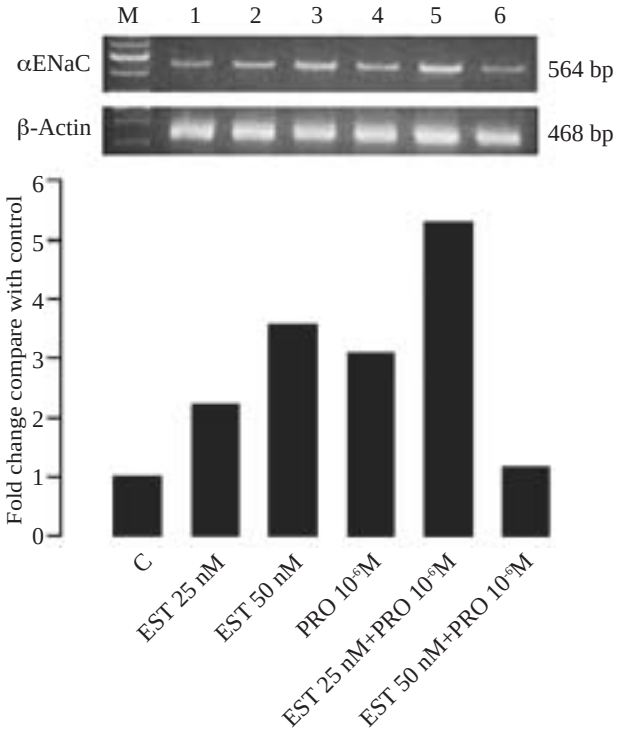

Fig. 3 Illustration of an ethidium bromide-stained 4% agarose gel showing the amplified products of expected size (564 bp) obtained with the *αENaC* primers in control mpkCCD cells (lane 1), cultured mpkCCD cells preincubated with estrogen 25 nM (lane 2), estrogen 50 nM (lane 3), progesterone 10^{-6} M (lane 4), estrogen 25 nM plus progesterone 10^{-6} M (lane 5) and estrogen 50 nM plus progesterone 10^{-6} M (lane 6). The expression of *β-actin* is also shown as an internal control (468 bp). Molecular weight standards (M) were the 1 kb ladder from GIBCO-BRL (Invitrogen, Carlsbad, CA, USA).

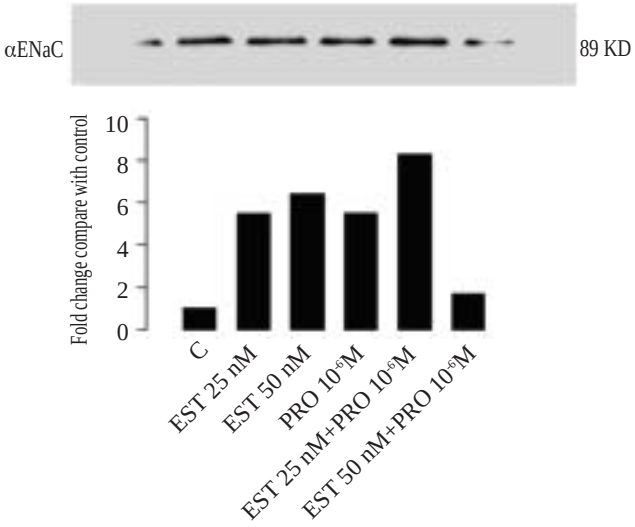

Fig. 4 Immunoblot of cultured mpkCCD cell membrane-enriched fraction probed with rat anti-*αENaC* antibody. A 89 kD band is detected in control mpkCCD cells (lane 1), cultured mpkCCD cells preincubated with estrogen 25 nM (lane 2), estrogen 50 nM (lane 3), progesterone 10^{-6} M (lane 4), estrogen 25 nM plus progesterone 10^{-6} M (lane 5) and estrogen 50 nM plus progesterone 10^{-6} M (lane 6).

terone (10^{-6} M) induced a 5.3-fold increase of αENaC mRNA expression. High concentration estrogen (50 nM) combined with progesterone (10^{-6} M) prevented the stimulatory effects (Fig. 3).

Western blotting

The Western blotting analysis to detect αENaC was performed on cell membrane-enriched preparation from confluent mpkCCD cells using an anti-αENaC antibody (Fig. 4). This antibody detected a protein band of about 89 kD representing the targeted αENaC protein in the membrane-enriched preparation. In parallel with mRNA changes, incubating for 24 h with 25 nM estrogen induced a 5.5-fold increase in αENaC protein translation (Fig. 4). Increasing the concentration of estrogen to 50 nM increased the translation of αENaC protein up to 6.4-fold. Progesterone (10^{-6} M) also increased the translation of αENaC protein up to 5.5-fold. Concomitant appearance of low concentration estrogen (25 nM) and progesterone (10^{-6} M) induced an 8.3-fold increase of αENaC protein translation. High concentration estrogen (50 nM) combined with progesterone (10^{-6} M) prevented the stimulatory effects (Fig. 4). The results indicated that the effects and interaction of the female gender steroid hormones were not limited to mRNA expression but also affected parallel changes of protein.

Short circuit current

It is very interesting to see that the changes in protein translation could be also observed in the sodium channel activity. We used the short circuit channel to measure sodium channel activity alteration in the presence of different concentrations of female gender steroid hormones. The short circuit current analysis to detect ENaC activity was performed on mpkCCD cells grown on permeable filters. In parallel with mRNA and protein changes,

incubating for 24 h with 50 nM estrogen induced a mild increase in ENaC activity (21%) compared with the control group. Progesterone (10^{-6} M) mildly increased the ENaC activity up to 16.2%. Concomitant appearance of low concentration estrogen (25 nM) and progesterone (10^{-6} M) induced a 137% increase in ENaC activity. High concentration estrogen (50 nM) combined with progesterone (10^{-6} M) induced a 14.7% increase in ENaC activity. Compared with low concentration estrogen and progesterone, high concentration estrogen and progesterone prevented the stimulatory effects of ENaC (Fig. 5). The results indicated that the effects and interaction of the female gender steroid hormones were not limited to mRNA expression and protein translation but also affected parallel changes in ENaC activity.

DISCUSSION

It has been suggested that woman gain weight and develop peripheral edema during the luteal phase of the menstrual cycle because they tend to retain sodium and water. Clinical edema is always associated with renal sodium retention.[4] It has been reported that the renal response to salt is characterized by a marked salt escape from the distal nephron in the luteal phase.[19] ENaC activity in collecting tubules plays an important role in the regulation of renal sodium reabsorption.[20] The ENaC activity is regulated by several factors, including the female gender hormones.[13] Pre-menstruation edema occurs in many females and can affect daily life. The exact mechanism is still unclear. Female gender steroid hormones were thought to play an important role in the development of pre-menstruation edema. The mid-luteal phase is characterized by elevated progesterone and high estrogen levels. Estrogen and progesterone levels decline rapidly during the late luteal phase corresponding to clinical pre-menstruation edema.

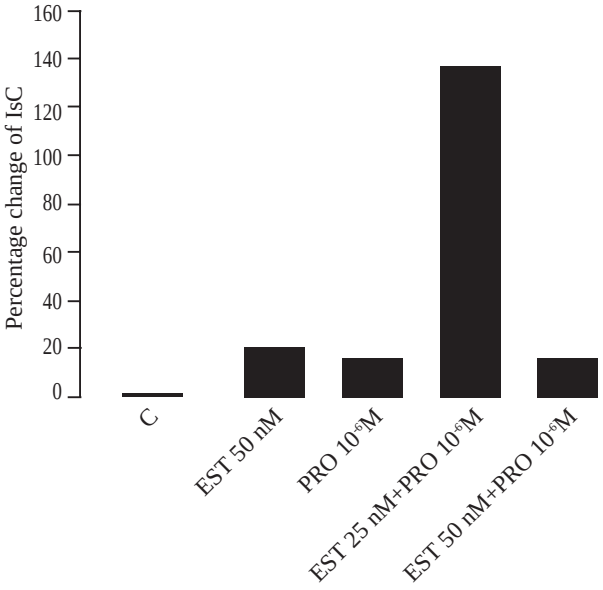

Fig. 5 Effect of estrogen and/or progesterone on short circuit current. Alternations in the short circuit current measurements are demonstrated in % change after incubation without (Control) or with different concentrations of hormones.

Taken together, it is interesting to know the effects of female gender steroids on the ENaC in collecting tubules. In the previous *in vivo* study in rats, treatment of ovariectomized rats with estrogen increased αENaC mRNA abundance in the kidney and progesterone inhibited the effect of estrogen on αENaC mRNA.[12] However, a conflicting result that showed a combination of progesterone and estrogen increased the ENaC transcription in lung epithelium of rats has been reported.[13] We used a model of well-characterized CCD cultured cells for this study. The effects of progesterone with various concentrations of estrogen were investigated. We analyzed mRNA expression, protein translation and sodium channel activity. Our results suggested that estrogen and/or progesterone alone mildly increased ENaC activity. Low concentration estrogen together with progesterone stimulated ENaC activity more than 2-fold. A high concentration of estrogen almost completely inhibited stimulated ENaC activity. The observed change correlated to the hormone change during the luteal phase. The elevation of estrogen and progesterone during the mid-luteal phase inhibited ENaC activity. Decline of estrogen production during the late luteal phase prevented the inhibitory effect. ENaC activity was enhanced and might explain observed pre-menstruation edema.

It is still unknown how these hormones interact within the collecting tubular cells to affect ENaC activity. Glucocorticoid-regulated kinase 1 (Sgk1) is the common pathway to regulate the activity of ENaC.[9] Sgk1 is mainly regulated by glucocorticoid and mineralocorticoid hormone.[8] It is likely that female gender steroids compete with these hormones via intracellular receptors.[21] The exact intracellular

mechanisms deserve further study.

We conclude that female gender steroid hormones affect RNA expression, protein translation and ENaC activity in renal collecting tubule cells. The effects might suggest that pre-menstruation edema is a result of subtle interaction between the female gender steroid hormones on ENaC in renal collecting tubule cells.

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雌性素與黃體素在集尿小管細胞鈉通道活性調控上的交互作用

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背 景：女性與男性的腎臟鈉離子處理有差異存在，此外女性鈉離子代謝亦會隨著月經週期及孕程變化。在母老鼠的實驗，雌性素與黃體素可以調控腎臟鈉通道訊息核糖核苷酸的表現。本實驗的目的是嘗試回答女性激素固醇在調控腎臟鈉通道活性上的角色。

方 法：我們利用細胞培養的方法將腎小管細胞在不同濃度的雌性素與黃體素下培養，再以反轉錄聚合酶鏈反應及西方點墨法分析鈉通道訊息核糖核苷酸表現及蛋白質轉譯，並以短回路電流分析鈉通道活性。

結 果：單獨的雌性素或黃體素可使腎小管細胞鈉通道活性略爲增加。低濃度的雌性素加上黃體素一起作用可使鈉通道活性增加約兩倍。高濃度的雌性素幾乎可以抑制被刺激的納通道活性。

結 論：女性激素固醇可以影響腎小管細胞鈉通道訊息核糖核苷酸表現，蛋白質轉譯及活性。女性激素固醇在調控腎小管細胞鈉通道之交互作用可能與女性經期前水腫有關。

(長庚醫誌 2007;30:305-12)

關鍵詞：女性激素固醇，集尿小管細胞，鈉通道

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