Original Paper

Experimental Nephrology

Exp Nephrol 2002;10:355–364
DOI: 10.1159/000065300

Received: February 22, 2001
Accepted: August 31, 2001

Estradiol-17β Stimulates Phosphate Uptake and Is Mitogenic for Primary Rabbit Renal Proximal Tubule Cells

Ho Jae Han[a] Yeune Hee Lee[a] Kwon Moo Park[a] Mary Taub[b]

[a]Department of Veterinary Physiology, College of Veterinary Medicine, Hormone Research Center, Chonnam National University, Kwangju, Korea and [b]Department of Biochemistry, School of Medicine, State University of New York at Buffalo, N.Y., USA

Key Words
Estradiol-17β · Kidney · Phosphate · Na/P_i cotransport

Abstract
The direct effects of estradiol-17β (E_2) on phosphate (P_i) uptake and on DNA synthesis in the primary rabbit kidney proximal tubule cells (PTCs) have been investigated. In the present study, E_2 (>10^{-9} *M*, over 9 days) causes an increase both in [^{3}H]thymidine incorporation and the number of PTCs. The anti-estrogen tamoxifen completely prevented the E_2-induced increase in [^{3}H]thymidine incorporation, and ameliorated the stimulatory effect of E_2 on growth. E_2 (>10^{-9} *M*, over 5 days) also stimulated the P_i uptake and its effect was due to the V_{max} values but not to the K_m value for P_i uptake. Estriol and estrone also exerted significant stimulatory effects on P_i uptake. Progesterone, tamoxifen, actinomycin D and cycloheximide prevented the E_2-induced stimulation of P_i uptake. In conclusion, estrogens at physiological concentrations stimulate P_i uptake and DNA synthesis in the renal proximal tubule cells, and these effects are estrogen receptor mediated.

Introduction

Estrogens are most noted for their ability to stimulate cell proliferation in the uterus, vagina and mammary gland [1]. However, in addition, estrogens also have physiological effects on other target tissues, including the kidney [2, 3]. Included amongst the evidence that the kidney is an estrogen-sensitive tissue is the presence of the estrogen receptor, which increases in abundance after estrogen treatment, as well as the formation of tumors in the renal cortex of the hamster after prolonged estrogen treatment [4, 5]. Of particular interest in these regards, the renal cell carcinoma, an estrogen-dependent tumor, is of renal proximal tubule origin. Moreover, the studies of Bojar et al [6, 7] indicate the presence of estrogen receptors in this type of human tumor. However, the role of estrogen receptors in regulating normal physiologic processes in the renal proximal tubule is poorly understood. In addition to the regulation of growth, estrogens may be involved in other renal processes including the regulation of mineral reabsorption.

The transport processes involved in the reabsorption of phosphate (P_i) by the renal proximal tubule have been extensively studied. Transport studies with intact cells and with membrane vesicles have clearly shown that the

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1018–7782/02/0106–0355$18.50/0

Accessible online at:
www.karger.com/journals/exn

Dr. Ho Jae Han
Department of Veterinary Physiology, College of Veterinary Medicine
Hormone Research Center, Chonnam National University
Kwangju 500-757 (Korea)
Tel. +82 62 5302831, Fax +82 62 5302809, E-Mail hjhan@chonnam.ac.kr

reabsorption of P_i by the renal proximal tubule is mediated largely by a Na^+-dependent phosphate (Na/P_i)-cotransport system in the brush border membrane [8]. Recently, two different classes of brush border membrane Na/P_i cotransporters have been 'cloned' (types I and II) [9]. However, only the type II Na/P_i cotransporter has characteristics typical of the 'major' apical membrane Na/P_i cotransporter. Included amongst these properties are regulation by parathyroid hormone (PTH) and the dietary P_i level [10–13]. Thus, type II Na/P_i cotransporters are hypothesized to play an important role in the maintenance of P_i homeostasis in the kidney.

Despite these advances at the molecular level, the entire spectrum of hormones and effector molecules which regulate P_i transport in the renal proximal tubule have not yet been defined, in part due to the complexity of the in vivo environment. Of particular interest in this report is the regulation of estrogens, which may ultimately affect mineral deposition in bone. Several lines of evidence reported that decreasing circulating levels of P_i are correlated with estrogen treatment [14, 15]. These reports suggest the possibility that estrogens have an effect on P_i transport in kidney, since renal proximal tubular reabsorption of P_i is a major determinant of the circulating level of P_i and contributes to the maintenance of P_i homeostasis.

A convenient means to evaluate the effects of estradiol-17β (E_2) on renal proximal tubule epithelial cells is by means of in vitro studies with differentiated cell cultures. In this report, primary rabbit kidney proximal tubule cells (PTCs) are examined in these regards. Such a primary culture system permits a comparison with previous transport studies conducted with renal tissue. The primary rabbit renal PTCs retain a number of differentiated functions distinctive of the renal proximal tubule, including a sodium-dependent sugar transport system [16], a sodium-dependent phosphate transport system [17], γ-glutamyl transpeptidase activity [16], probenecid-sensitive *p*-aminohippurate transport [18] and gluconeogenic capacity [19].

The observed set of responses of the primary PTCs to hormones also indicates that this cell culture system consists of a population of cells which is highly enriched with cells originating from the renal proximal tubule. Typical of the renal proximal tubule, PTH stimulates cyclic AMP production, unlike the case with arginine vasopressin and calcitonin [16]. Suggestive of the presence of insulin receptors, another proximal tubule marker, primary PTCs respond to physiologic levels of insulin with an inhibition of the cells' phosphoenolpyruvate carboxykinase (PEPCK) activity [20]. In a more recent report, we show that yet another physiologically significant effector molecule, angiotensin II (Ang II), modulates the rate of Na^+ uptake into the PTCs by the Na^+/H^+ antiport system [21].

Of particular interest to this report are our previous studies concerning the effects of steroid hormones on the primary PTCs [22]. We have found that estrogens and testosterone cause an increase in the number of cells in primary PTC cultures similar in extent to the growth-stimulatory effect of glucocorticoids [22]. The stimulatory effect of E_2 in particular was found to occur within a physiologically significant range, and was associated with the presence of an estrogen receptor. Although stimulatory effects of E_2 on the activity of several apical membrane proteins, γ-glutamyl transpeptidase and alkaline phosphatase were reported, apical membrane transport systems were not examined with regard to possible estrogen effects.

In this report, we further examine the effects of E_2 on primary PTC growth as well as on apical membrane transport. We present evidence indicating that E_2 stimulates P_i uptake, as well as growth as a consequence of the specific interactions of E_2 with the estrogen receptor.

Methods

Materials

E_2, estrone, estriol, progesterone, cycloheximide, actinomycin D, insulin, apo-transferrin and ouabain were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). $^{22}Na^+$, $^{32}P_i$ and ^{14}C-α-methyl-*D*-glucopyranoside were purchased from Dupont/NEN. Fetal bovine serum was purchased from HyClone (Logan, Utah, USA). Phenol red free (PRF) D-MEM/F12, class IV collagenase, and soybean trypsin inhibitor were purchased from Life Technologies (Grand Island, N.Y., USA). The basal medium, PRF D-MEM/F12, consisted of a 50:50 mixture of Dulbecco's modified Eagle's medium, and Ham's nutrient mixture F-12, further supplemented with 15 m*M* Hepes buffer (pH 7.4), and 20 m*M* sodium bicarbonate, but lacking phenol red. The culture medium (Medium RK-2) consisted of the basal medium further supplemented with two additional growth supplements, 5 μg/ml insulin, and 5 μg/ml transferrin, added immediately prior to use. The iron oxide used in culture preparations was made by the method of Cook and Pickering [23]. Stock solutions of iron oxide in 0.9% NaCl were sterilized using an autoclave and diluted with phosphate-buffered saline (PBS) prior to use [24].

Preparation of Primary Cell Cultures

Kidneys obtained from male New Zealand White rabbits (1.5–2.0 kg) were used to prepare primary rabbit kidney PTC cultures, by a modification of the method of Chung et al. [16]. To summarize, kidneys were perfused via the renal artery, first with PBS, and subsequently with PRF-D-MEM/F-12 containing 0.5% iron oxide (w/v), until the kidney turned gray-black in color. Renal cortical slices pre-

pared from the kidney were homogenized with 4 strokes of a sterile glass homogenizer. The homogenate was poured first through a 253-μm, and then through an 83-μm mesh filter. Tubules and glomeruli on the 83-μm filter were transferred into a tube containing sterile PRF-D-MEM/F-12 and a magnetic stir bar. Glomeruli (containing iron oxide) were removed with the magnetic stir bar. The remaining proximal tubules were incubated 2 min at 23°C in PRF-D-MEM/F-12 containing 60 μg/ml collagenase (class IV) and 0.025% soybean trypsin inhibitor. The dissociated tubules were washed by centrifugation, resuspended in Medium RK-2, and transferred into 35-mm tissue culture dishes. The cultures were then maintained at 37°C, in a 5% CO_2-95% air, humidified environment. Medium was changed 1 day after plating, and every 3 days thereafter.

Cell Growth Studies

To determine the effect of hormones on growth, purified rabbit renal proximal tubules were incubated in 35-mm plastic dishes containing factors to be tested experimentally in Medium RK-2. Periodically, cells from representative dishes were detached utilizing 0.05% trypsin/0.5 m*M* EDTA. The cell number was then determined utilizing a Coulter Model ZF particle counter. All determinations were in triplicate.

[^{3}H]thymidine Incorporation

[^{3}H]thymidine incorporation experiments were conducted as described by Brett et al. [25] using primary PTCs (70–80% confluent). To summarize, immediately prior to the study, the medium was changed either to Medium RK-2 further supplemented with E_2, or to Medium RK-2 with no further supplements. 1 μCi of [methyl-^{3}H]thymidine was added to each of the cultures. The incubation with [^{3}H]thymidine was continued for 24 h at 37°C. The PTCs were then washed twice with PBS, fixed in 10% trichloroacetic acid (TCA) at 23°C for 15 min, and then washed twice with 5% TCA. The acid-insoluble material was dissolved in 2 *N* NaOH for 12 h at 23°C. Aliquots were removed for the determination of radioactivity using a scintillation counter. All values are averages (±SE) of triplicate determinations. Values were converted from absolute counts to the percentage of the control ($-E_2$) in order to allow for comparison between experiments.

P_i Uptake Studies

Monolayers (80–90% confluent) were maintained in Medium RK-2 either in the presence or in the absence of 10^{-9} *M* E_2 over a 5-day period. Subsequently, P_i uptake experiments were conducted as described by Rabito [26]. To summarize, immediately prior to the uptake study, the culture medium was removed by aspiration. The monolayers were gently washed twice with P_i uptake buffer (150 m*M* NaCl, 1.2 m*M* $MgSO_4$, 0.1 m*M* $CaCl_2$, and 10 m*M* MES/Tris, pH 7.4) and then incubated at 37°C for 30 min in uptake buffer containing 1.5 μCi/ml ^{32}P-phosphate, and 1 m*M* unlabeled phosphate. At the end of the uptake period, the monolayers were washed 3 times with ice-cold uptake buffer. The cells in each dish were solubilized in 1 ml 0.1% SDS. To determine the $^{32}P_i$ incorporated intracellularly, a portion of the solubilized material was removed, and counted in a liquid scintillation counter (LS6500; Beckman Instruments, Fullerton, Calif., USA). To determine protein concentration, the Bradford method [27] was employed with the remaining material. The radioactive counts in each sample were normalized with respect to protein, and corrected for zero-time uptake. All uptake values are averages of triplicate determinations. α-Methyl-*D*-glucopyranoside (α-MG) uptake and Na^+ uptake experiments were conducted as described by Sakrani et al. [28] and Rindler et al. [29], respectively.

Statistical Analysis

Results were expressed as means ± SE. Statistical significance was estimated by ANOVA and by unpaired t test as appropriate. The difference was considered statistically significant when $p < 0.05$.

Results

Effect of E_2 on Cell Growth

When PTCs were grown in the presence of increasing concentrations of E_2, the number of primary PTCs increased significantly (fig. 1a), ranging from 162 ± 8% of the control in the presence of 10^{-9} *M* E_2, to 143 ± 9% of the control in the presence of 10^{-6} *M* E_2. In order to determine whether the observed increase in cell number in E_2-treated cultures could be explained by increased DNA synthesis, the effect of 10^{-9} *M* E_2 on the ability of the PTCs to incorporate [^{3}H]thymidine into TCA precipitable material was examined. Figure 1b also demonstrates that E_2 (>10^{-9} *M*) increased [^{3}H]thymidine incorporation. Figure 2a shows a significant increase in cell number in cultures treated with 10^{-9} *M* E_2, both at day 9 (145 ± 13% of control) and at day 13 (156 ± 13% of control). A similar increase in [^{3}H]thymidine incorporation was also observed at days 9 and 13 in E_2-treated primary cultures (fig. 2b). A significant effect of E_2 on cell growth and [^{3}H]thymidine incorporation was not observed at an earlier time interval (5 days), consistent with our previous report [22]. These results suggest that at least after prolonged incubations, the stimulatory effect of E_2 on cell number can be explained at least in part by increased DNA synthesis.

Previously, we have shown that the estrogen receptor is present in the PTCs [22]. If the growth-stimulatory effect of E_2 is indeed mediated by such an estrogen receptor, then other estrogen receptor antagonists, such as the anti-estrogen tamoxifen, may very likely prevent the growth-stimulatory effect of E_2 by preventing the binding of E_2 to the estrogen receptor. In order to examine this possibility, the ability of 10^{-6} *M* tamoxifen to block the stimulatory effect of E_2 on [^{3}H]thymidine incorporation was examined. Figure 3a shows that an E_2-induced stimulation of [^{3}H]thymidine incorporation was completely prevented by tamoxifen. Tamoxifen alone at 10^{-6} *M* had no significant affect on [^{3}H]thymidine incorporation. Similarly, figure 3a shows the stimulatory effect of 10^{-9} *M* E_2 on cell number was significantly reduced in the presence of 10^{-6} *M* tamoxifen. Tamoxifen alone at 10^{-6} *M* was not

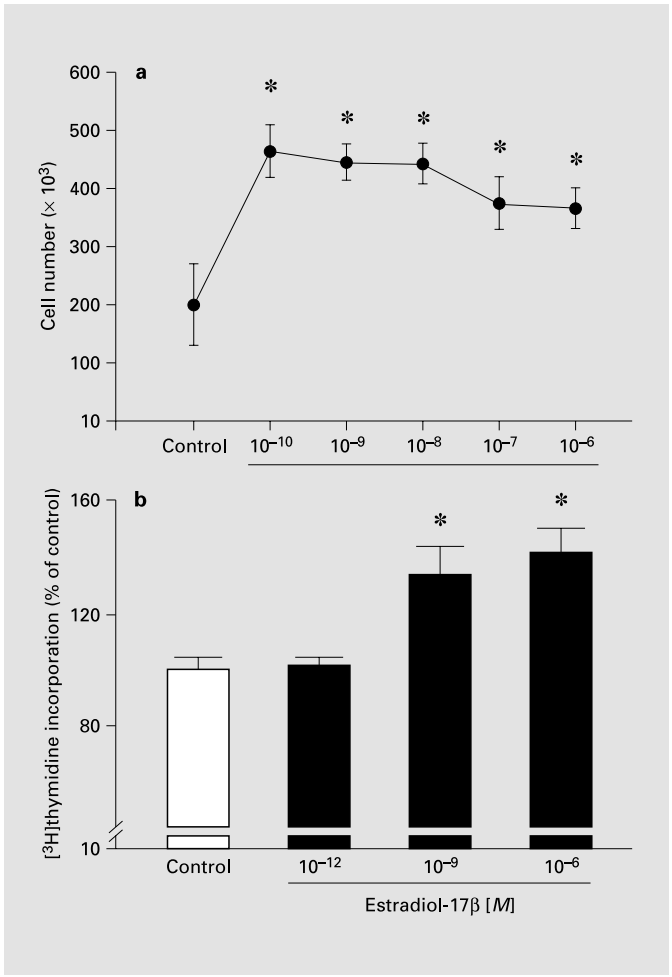


Fig. 1. Effect of estrogens on cell growth. a Dose-response effect of estradiol-17β (E_2): Proximal tubules from a rabbit kidney were cultured for 13 days in phenol red free hormonally defined medium. The medium contained E_2 at various concentrations (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} or 10^{-10} *M*) in a separate dish and no added hormone. * $p < 0.05$ vs. control. b Dose-dependent effect of E_2 on [^{3}H]thymidine incorporation into the PTCs. Cells were incubated for 24 h in the absence or presence of 10^{-12}, 10^{-9} or 10^{-6} *M* E_2, and pulsed with 1 μCi of [^{3}H]thymidine for 24 h. Values are means ± SE of three independent experiments with triplicate dishes. * $p < 0.05$ vs. control.

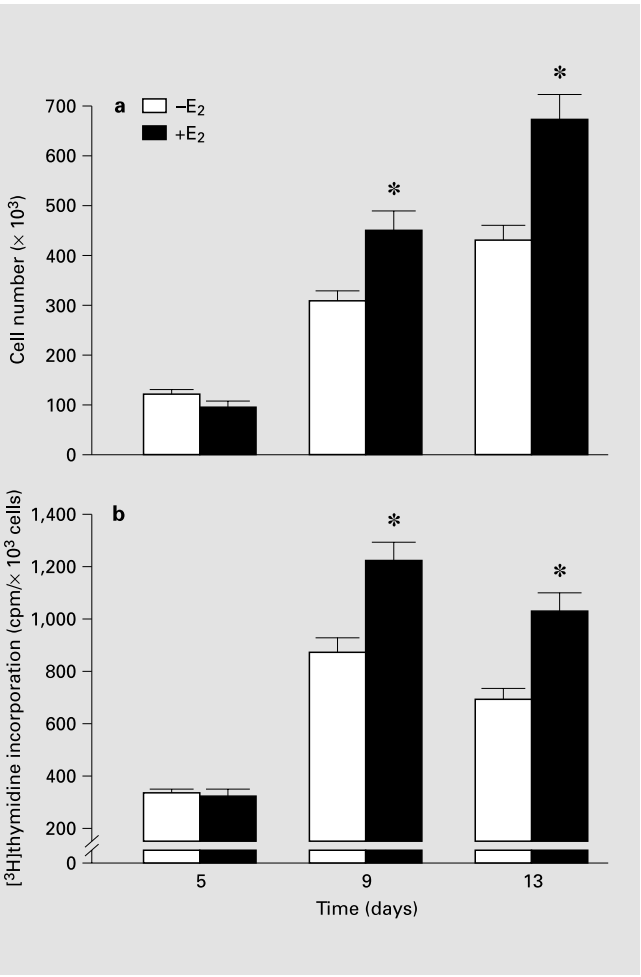


Fig. 2. Effect of E_2 on cell growth (a) and [^{3}H]thymidine incorporation (b) into the PTCs on cell stage. Cells were incubated each day in the absence or presence of 10^{-9} *M* E_2 and pulsed with 1 μCi of [^{3}H]thymidine for 24 h. Experiments were performed from days in culture to 5, 9 and 13 days respectively. Values are means ± SE of three independent experiments with triplicate dishes. * $p < 0.05$ vs. each control.

growth stimulatory (fig. 3b), unlike our previously published results with 10^{-9} *M* tamoxifen [22].

Effect of E_2 on P_i Uptake

Estrogens play a major role in mineral homeostasis, affecting the two major mineral components of bone, calcium (Ca^{2+}) and P_i. Not only is bone resorption affected, but the urinary excretion of P_i is reduced following estrogen treatment. In order to evaluate whether the inhibitory effects of estrogens on urinary P_i excretion may be attributed in part to increased tubular reabsorption of P_i, the effect of E_2 on P_i uptake by primary PTCs was examined.

Primary PTCs were incubated for 5 days with E_2 at either 10^{-12}, 10^{-9} or 10^{-6} *M*. Figure 4a shows a significant stimulatory effect of E_2 on P_i uptake at 10^{-9} *M* as well as 10^{-6} *M*, whereas at 10^{-12} *M* E_2 had no significant effect. The stimulatory effect of 10^{-9} *M* E_2 on P_i uptake was examined as a function of incubation time. Both short-term (0.5–5 h) as well as long-term (up to 10 days) incubation times were employed. Figure 4b shows the lack of a

significant effect of E_2 during the short-term incubations, as well as a lack of an effect of E_2 after a more extended 3-day incubation period with this steroid. However, a significant stimulatory effect of E_2 on P_i uptake was observed after the incubation period was extended to 5 days (27%). This stimulatory effect of E_2 on P_i uptake was retained until the end of the 10-day incubation period.

E_2 may stimulate P_i uptake by affecting either the affinity of P_i for the uptake process (K_m) or the maximal velocity (V_{max}). In order to evaluate this possibility, P_i

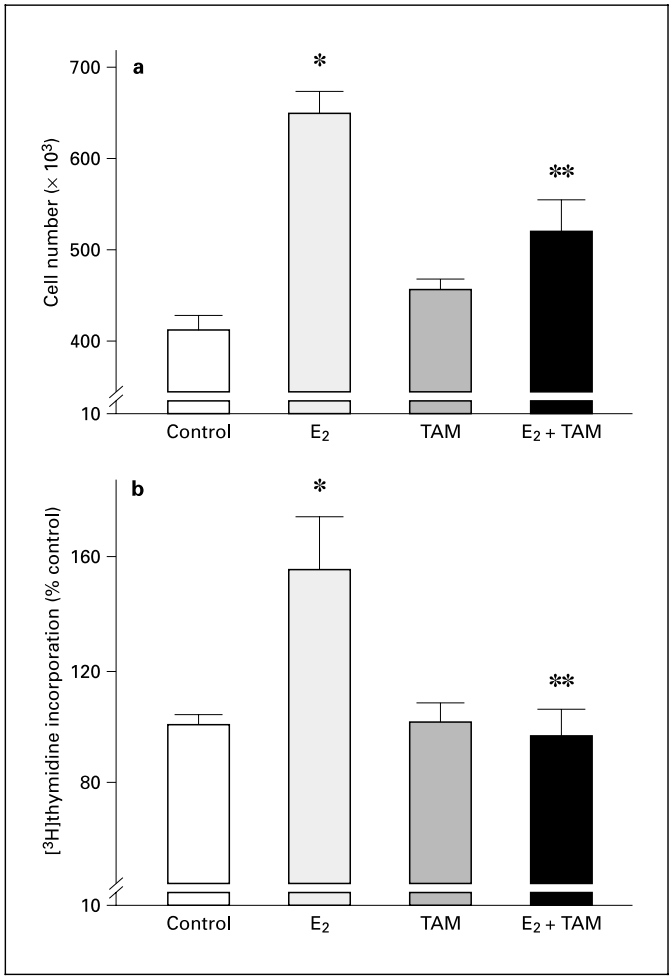
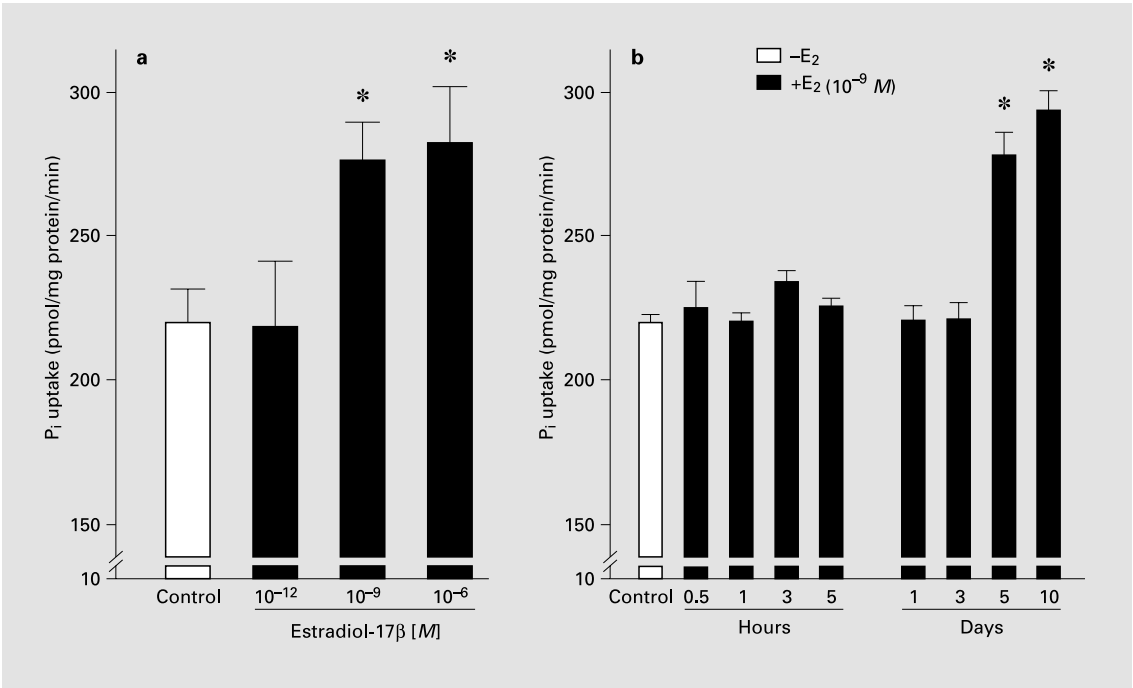


Fig. 3. a Effect of tamoxifen (TAM) on E_2-induced stimulation of cell growth and [^{3}H]thymidine incorporation. PTCs were incubated with TAM (10^{-6} *M*) and E_2 (10^{-9} *M*) or alone for 13 days. Cell counts were performed on 13 days using a Coulter Model ZF particle counter. Each point represents means ± SE of five independent experiments with triplicate dishes. * $p < 0.05$ vs. control, ** $p < 0.05$ vs. E_2. b Effects of E_2 antagonists on E_2-induced increase of [^{3}H]thymidine incorporation into the PTCs. Cells were incubated for 24 h in the presence 10^{-6} *M* tamoxifen; TAM and 10^{-9} *M* E_2 or alone and pulsed with 1 μCi of [^{3}H]thymidine. Values are means ± SE of four independent experiments with triplicate dishes. * $p < 0.05$ vs. control, ** $p < 0.05$ vs. E_2.

Fig. 4. Effects of E_2 on P_i uptake. a Dose-dependent effect of E_2. E_2 was treated to PTCs at concentrations of 10^{-12}, 10^{-9} and 10^{-6} *M* for 5 days. b Time course effect of 10^{-9} *M* E_2 on P_i uptake. PTCs were incubated with E_2 or with vehicle for various periods of time (30 min to 10 days) prior to the P_i uptake study. Values are means ± SE of six (a) and four (b) independent experiments with triplicate dishes. * $p < 0.05$ vs. control.



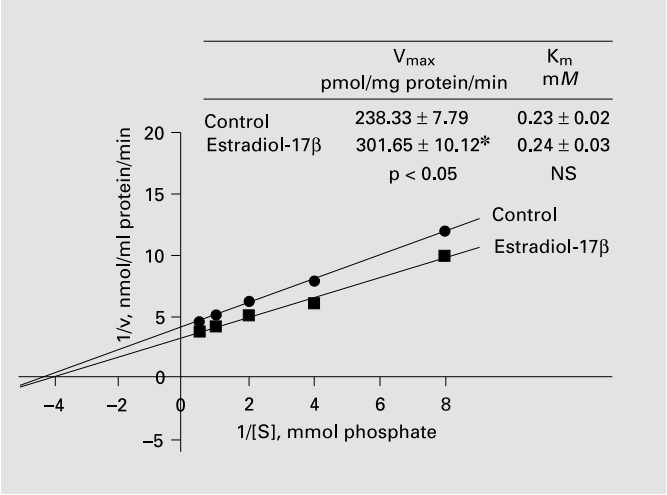


Fig. 5. Effect of E_2 on the kinetic parameters of P_i uptake. PTCs were incubated for 5 days in the presence of 10^{-9} *M* E_2 or vehicle. Cells were then incubated 30 min with the indicated concentrations of phosphate. P_i uptake was measured in a buffer containing $^{32}P_i$ (1.5 μCi/ml) in the presence of 1, 0.5, 0.25, 0.125 or 0.0625 m*M* unlabeled phosphate. Values are means ± SE of three independent experiments with triplicate dishes. * $p < 0.05$ vs. control.

Table 1. Effects of E_2 on Na/P_i cotransport, Na/glucose cotransport and Na^+/H^+ antiport activity

	$^{32}P_i$ pmol/mg protein/min	[^{14}C]-α-MG pmol/mg protein/min	$^{22}Na^+$ nmol/mg protein/min
Control	240 ± 1	610 ± 13	14 ± 0.4
E_2 (10^{-9} *M*)	302 ± 2*	474 ± 13*	12.0 ± 0.6

Primary cultured renal proximal tubular cells were grown in D-MEM/F-12 supplemented with insulin (5 μg/ml) and transferrin (5 μg/ml). Cells were treated for 5 days with E_2 (10^{-9} *M*). E_2-treated cells were then incubated with $^{32}P_i$ 1.5 μCi/ml, [^{14}C]-α-MG 0.5 μCi/ml and $^{22}Na^+$ 0.25 μCi/ml for 30 min at 37°C. Uptake experiments were performed as described in Methods. Values are means ± SE of five independent experiments with triplicate dish. * $p < 0.05$ vs. control.

uptake was examined as a function of the extracellular P_i concentration, both in control and in E_2-treated cultures. Figure 5 shows that P_i uptake was saturable as a function of increasing P_i concentrations. A kinetic analysis of the uptake data indicated a significant 23% increase in the V_{max} for P_i uptake in E_2-treated cultures, while the apparent K_m value was unaffected.

Possibly, the increase in the V_{max} for P_i uptake may reflect a generalized increase in the apical membrane transport capacity. In order to examine this possibility, the initial rate of α-MG uptake and of Na^+ uptake was examined. Table 1 shows, however, that the initial rate of α-MG uptake was significantly reduced in E_2-treated cultures, while the Na^+ uptake rate remained unchanged.

Effects of Other Estrogens and Estrogen Antagonists, and RNA and Protein Synthesis Inhibitor on P_i Uptake

In order to evaluate the specificity of the estrogen effect on P_i uptake, we examined the effects of several different types of estrogens (estriol and estrone) on P_i uptake following a 5-day incubation. In addition, the effects of testosterone and several estrogen antagonists (including progesterone (P_4) and tamoxifen) on P_i uptake were also examined. Figure 6 (insert) shows that both 10^{-9} *M* estriol and 10^{-9} *M* estrone were stimulatory to P_i uptake. However, progesterone and testosterone did not affect P_i uptake [Han et al., unpubl. data], suggesting that amongst the sex steroids, the effect is estrogen-specific. While 10^{-9} *M* P_4 and 10^{-6} *M* tamoxifen individually had no affect on P_i uptake (fig. 6), when added in combination with 10^{-9} *M* E_2, both 10^{-9} *M* P_4 and 10^{-6} *M* tamoxifen significantly reduced the E_2-induced stimulation of P_i uptake.

In order to examine whether the E_2-induced stimulation of P_i uptake is dependent upon transcription and/or protein synthesis, the effects of actinomycin D (10^{-8} *M*) and cycloheximide (4×10^{-7} *M*) were examined. As illustrated in figure 6, the P_i uptake rate in the absence of E_2 was not significantly affected by actinomycin D and cycloheximide. However, in the presence of E_2, actinomycin D significantly reduced the E_2-induced stimulation of P_i uptake, suggesting a requirement for transcription. In the presence of cycloheximide, E_2 had no significant effect on P_i uptake, suggesting a requirement for the synthesis of new proteins.

Discussion

The cellular effects of estrogens are tissue- and organ-specific. Not only do estrogens have proliferative effects on bone and the uterus, but also the kidney [30–33]. Of particular interest in these regards is the renal proximal tubule. That the renal proximal tubule is indeed a target of estrogen action is shown quite clearly by the estrogen-induced ultrastructural changes localized to the cells in this nephron segment. In the hamster, prolonged estrogen

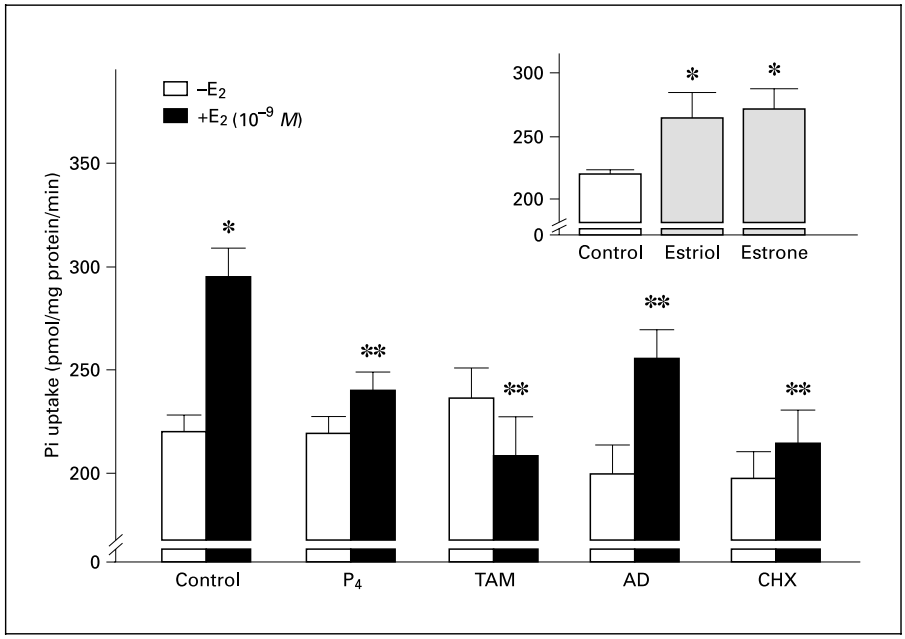


Fig. 6. Effects of progesterone (P_4), tamoxifen (TAM), actinomycin D (AD) and cycloheximide (CHX) on E_2-induced stimulation of P_i uptake. PTCs were incubated with E_2 antagonists (10^{-9} *M* P_4, 10^{-6} *M* TAM), AD (10^{-8} *M*), or CHX (4×10^{-7} *M*) for 3 h prior to incubation with 10^{-9} *M* E_2 or its vehicle. P_i uptake was performed 5 days later. Insert shows that the effect of estriol (10^{-9} *M*) and estrone (10^{-9} *M*) on P_i uptake. Values are means ± SE of four or five independent experiments with triplicate dishes. * $p < 0.05$ vs. control, ** $p < 0.05$ vs. E_2.

treatment often results in the formation of renal cortical tumors. The direct involvement of estrogens in such processes is supported by the presence of estrogen receptors in this locale. Our results lend further support to the overall contention that the kidney is an estrogen-responsive organ, which is a site at which important changes in mineral metabolism and growth occur as a consequence of changes in estrogen status.

Previously, we demonstrated that E_2 and α-estradiol increased the number of primary PTCs in hormonally defined serum-free medium [22]. In this report we show that E_2 also stimulates cell growth and the incorporation of [^{3}H]thymidine into acid-precipitable material. These results indicate that the E_2 effect on cell number can be attributed at least in part to an increase in the rate of DNA synthesis, rather than simply to improved cell adhesion (for example).

The involvement of the estrogen receptor in mediating the stimulatory effect of E_2 on growth and P_i uptake is substantiated by our observation that physiologic E_2 concentrations (10^{-9} *M*) cause an increase both in the number of primary PTC cells, in [^{3}H]thymidine incorporation and in P_i uptake. Our observation that such stimulatory effects of E_2 on [^{3}H]thymidine incorporation, growth and P_i uptake are ameliorated by the anti-estrogen tamoxifen also substantiates the involvement of estrogen receptors in mediating these effects of E_2. Presumably, tamoxifen at 10^{-6} *M* is preventing the stimulatory effects of 10^{-9} *M* E_2 by competing with E_2 for binding to the estrogen receptor. Admittedly, tamoxifen, like E_2, elicits a growth-stimulatory effect at 10^{-9} *M*, although this effect is no longer observed at higher tamoxifen concentrations. In contrast, in the case of E_2, a growth-stimulatory effect is still observed as the concentration is raised to 10^{-6} *M*, albeit to a lower extent than at 10^{-9} *M*. Thus, the ability of 10^{-6} *M* tamoxifen to block the growth-stimulatory effect of 10^{-6} *M* E_2 can be explained by the ability of 10^{-6} *M* tamoxifen to compete with E_2 for estrogen receptor binding. In addition, the reduced growth-stimulatory effect of E_2 in the presence of tamoxifen may be due to a secondary set of effects initiated through the binding of tamoxifen to the estrogen receptor. Our previous immunoprecipitation studies do indeed indicate that the PTCs possess estrogen receptors, and substantiate our contention that the growth-stimulatory effects of estrogens are very likely by means of an estrogen-receptor-mediated signal transduction pathway(s) [22].

The involvement of the estrogen receptor in mediating the effects of E_2 on P_i uptake is further substantiated by the stimulatory effects of other types of estrogens (estriol and estrone) on P_i uptake. Finally, the observation that progesterone, a physiologic estrogen antagonist, also prevented (at least in part) the stimulatory effect of E_2 on P_i uptake, substantiates the involvement of the estrogen receptor. Progesterone alone (like testosterone alone) did not stimulate P_i uptake.

Presumably, the phosphate transport system responsible for the P_i uptake in these studies is analogous to the Na^+/P_i cotransport system localized in renal brush border membrane vesicles [8]. Our previous studies indicate that such a Na^+-dependent carrier-mediated process is present in the PTCs [17]. In this report, kinetic parameters for P_i uptake were determined. The K_m value, 0.23 m*M* P_i, was somewhat higher than K_m values previously determined from studies with rabbit renal brush border membrane vesicles (0.060 m*M* [34] and 0.160 m*M* [35]). Such differences in the K_m values for P_i may be due to differences in the intracellular as well as the extracellular milieu of the cells and/or the membrane vesicles.

Of the three types of cDNAs for Na/P_i cotransporters which have been isolated by expression cloning techniques [8], only the type II Na/P_i cotransporter has characteristics consistent with being the 'main' brush border membrane Na/P_i cotransporter [8]. A K_m value of 0.114 m*M* P_i was obtained in Sf9 insect cells which expressed rat renal NaP_i-2, in a range similar to that previously obtained with the PTCs [36]. These K_m values, obtained for P_i uptake by NaP_i-2, are significantly higher than the K_m values for type III P_i transporters (found in a variety of nonrenal tissue) which are in the 20-μ*M* range.

In the rabbit kidney cortex, mRNA for a type I as well as a type II Na/P_i cotransporter has been identified. By immunohistochemical techniques, homologous proteins have been identified in renal proximal tubule brush border membranes [8]. However, the type I cotransporter does not exhibit a pH dependence, unlike the type II transporter. However, such a pH dependence for P_i uptake is a property shared by our primary PTC cells [37].

Incubation with E_2 caused a significant increase in the V_{max} for P_i uptake, without significantly affecting the K_m. The increase in the V_{max} for P_i uptake in E_2-treated PTCs may be due to an increase in the number of Na/P_i cotransporters in the plasma membrane, which in turn may result from an increase in the level of expression of the gene for the type II Na/P_i cotransporter. Indeed, the stimulatory effect of E_2 on P_i uptake requires a 5-day incubation with E_2, compatible with the common time frame of steroid hormones acting via regulation at the gene and protein level. The ability of actinomycin D and cycloheximide to block the stimulatory effect of E_2 on P_i uptake suggests a requirement for mRNA and protein synthesis, and is also consistent with transcriptional regulation.

However, hormonal regulation of the level of Na/P_i cotransporters in the apical membrane is not necessarily the result of transcriptional regulation. Indeed the inhibition of P_i uptake by PTH has been attributed to PTH-induced membrane retrieval and lysosomal degradation of the Na/P_i cotransporter, rather than to specific affects at the gene level [8]. However, the possibility of generalized effects on cellular protein synthesis is unlikely, as indicated by our previously published protein synthesis studies [22]. The possibility that there are effects of estrogens on the metabolism of P_i into nucleotide and deoxynucleotide pools, which affect DNA synthesis [38, 39], is also unlikely, as only a small fraction of the $^{32}P_i$ (<4%) was incorporated into such labeled nucleotide pools during our ^{32}P-labeled uptake [17].

Plasma P_i is maintained within a narrow range through the actions of hormones, and other effector molecules at a number of target sites, including the kidney. The Na/P_i cotransport system localized in the apical membrane of the renal proximal tubule plays an important part in this control. The effects of a number of physiologic changes on brush border Na/P_i cotransport activity have been clearly defined. Renal brush border Na/P_i cotransport activity increases following decreases in dietary P_i, as do type II Na/P_i cotransporter mRNA levels [8, 13, 40]. In contrast, decreased Na/P_i cotransport activity occurs following a P_i overload, the secretion of PTH (under conditions of low blood Ca^{2+}), as well as in response to renal dopamine and glucocorticoids [41, 42]. A decrease in type II Na/P_i cotransporter mRNA in response to dexamethasone has been reported [43]. Our previous report of inhibitory effects of glucocorticoids on P_i uptake by primary PTCs is consistent with the results published with intact tissue [42, 43].

Unlike the examples of regulatory effects given above, previous reports concerning the effects of estrogens on the renal handling of P_i are conflicting. In humans, estrogen administration causes a decrease in the urinary excretion of P_i [41]. This observation could be explained in part by increased renal proximal tubule reabsorption of P_i. Indeed, Uremura et al. [14] have reported that estrogens do have a stimulatory effect on renal P_i reabsorption in humans. However, others have found a normal, or a slightly increased urinary P_i excretion rate following prolonged estrogen treatment.

Beers et al. [44] reported an inhibitory effect of E_2 administration on Na/P_i cotransport activity in brush border membrane vesicles obtained from kidneys of thyroparathyroidectomized and ovariectomized female rats. Such differences in experimental results between the present report and this previous report very likely relate to differences in the species used (rabbit vs. rat vs. human), and in experimental technique. Indeed, in rats, estrogen administration has a phosphaturic effect, which differs

from humans, for example. In addition, in the intact animal, E_2 stimulates the synthesis of dopamine, which has well-documented inhibitory effects on the rate of Na/P_i cotransport across the brush border membrane [44].

A major advantage of our in vitro studies with a primary renal cell culture system as compared with studies with in vivo material, is that hormonally defined culture conditions are utilized. Not only is phenol red, which has structural similarity to estrogens [45, 46], not included in the medium, but in addition, hydrocortisone is deleted. Thus, when using our in vitro cell culture model system in hormonally defined culture conditions, we were able to minimize such concerns about indirect effects of other regulatory factors on growth and on membrane transport, and thus were able to define estrogen-specific effects on these processes.

Acknowledgements

This study was supported by grants from the Korea Research Foundation (KRF 2001-015-DP0524) and the Korea Science and Engineering Foundation (HRC 2001 G0301).

References

1 Zheng J, Johnson ML, Redmer DA, Reynolds LP: Estrogen and progesterone in the ovine uterus during early pregnancy. Endocrinology 1996;137:340–348.
2 DeVries JR, Ludes JH, Fanestil D: Estradiol renal receptor molecules and estradiol-dependent antinatriuresis. Kidney Int 1972;2:95–100.
3 Winter ML, Liehr JG: Possible mechanism of induction of benign prostatic hyperplasia by estradiol and dihydrotestosterone in dogs. Toxicol Appl Pharmacol 1996;136:211–219.
4 Li JJ, Talley DJ, Li SA, Vallee CA: An estrogen binding protein in the renal cytosol of the intact castrated and estrogenized golden hamster. Endocrinology 1974;96:1106–1113.
5 Li JJ, Li SA, Oberley TD, Parsons J: Carcinogenic activities of various steroidal and nonsteroidal estrogens in the hamster kidney: Relation to hormonal activity and cell proliferation. Cancer Res 1995;55:4347–4351.
6 Bojar H, Blazer K, Dreyfurst R, Staib W: Identification and partial characterization of specific oestrogen-binding components in human kidney. J Clin Chem Clin Biochem 1976;14:515–520.
7 Bojar R, Dreyfurst R, Maar K, Staib W: Distribution of estradiol receptor molecules in human kidney. J Clin Chem Clin Biochem 1977;15:319–321.
8 Murer H, Forster I, Hilfiker H, Pfister M, Kaissling B, Lotscher M, Biber J: Cellular/molecular control of renal Na/P_i-cotransport. Kidney Int Suppl 1998;65:S2–S10.
9 Murer H, Biber J: Molecular mechanisms of renal apical Na/phosphate cotransport. Annu Rev Physiol 1996;58:607–618.
10 Dousa TP: Modulation of renal Na-P_i cotransport by hormones acting via genomic mechanism and by metabolic factors. Kidney Int 1996;49:997–1004.
11 Pfister MF, Ruf I, Stange G, Ziegler U, Lederer E, Biber J, Murer H: Parathyroid hormone leads to the lysosomal degradation of the renal type II Na/P_i-cotransporter. Proc Natl Acad Sci USA 1998;95:1909–1914.
12 Verri T, Markovich D, Perego C, Norbis F, Stange G, Sorribas V, Biber J, Murer H: Cloning of a rabbit renal Na-P_i cotransporter, which is regulated by dietary phosphate. Am J Physiol 1995;268:F626–F633.
13 Murer H, Biber J: Control of proximal tubular apical Na/P_i cotransport. Exp Nephrol 1996;4:201–204.
14 Uemura H, Irahara M, Yoneda N, Yasui T, Genjida K, Miyamoto KI, Aono T, Takeda E: Close correlation between estrogen treatment and renal phosphate reabsorption capacity. J Clin Endocrinol Metab 2000;85:1215–1219.
15 Castelo-Branco C, Martinez de Osaba MJ, Pons F, Gonzalez-Merlo J: The effect of hormone replacement therapy on postmenopausal bone loss. Eur J Obstet Gynecol Reprod Biol 1992;44:131–136.
16 Chung SD, Alavi N, Livingston D, Hiller S, Taub M: Characterization of primary rabbit kidney cultures that express proximal tubule functions in hormonally defined medium. J Cell Biol 1982;95:118–126.
17 Waqar MA, Seto J, Chung SD, Hiller-Grohol S, Taub M: Phosphate uptake by primary renal proximal tubule cell cultures grown in hormonally defined medium. J Cell Physiol 1985;124:411–423.
18 Yang HS, Goldinger JM, Hong SK, Taub M: Preparation of basolateral membranes that transport *p*-aminohippurate from primary cultures of rabbit kidney proximal tubule cells. J Cell Physiol 1988;135:481–487.
19 Jung JC, Lee SM, Kadakia N, Taub M: Growth and function of primary rabbit kidney proximal tubule cells in glucose-free serum-free medium. J Cell Physiol 1992;150:243–250.
20 Wang Y, Taub M: Insulin and other regulatory factors modulate the growth and the phosphoenolpyruvate carboxykinase (PEPCK) activity of primary rabbit kidney proximal tubule cells in serum-free medium. J Cell Physiol 1991;147:374–382.
21 Han HJ, Park SH, Koh HJ, Taub M: Mechanism of regulation of Na^+ transport by angiotensin II in primary renal cells. Kidney Int 2000;57:2457–2467.
22 Han HJ, Jung JC, Taub M: Response of primary rabbit kidney proximal tubule cells to estrogens. J Cell Physiol 1999;178:35–43.
23 Cook WF, Pickering GW: A rapid method for separating glomeruli from rabbit kidney. Nature 1958;182:1103–1104.
24 Han HJ, Kang JW, Park KM, Lee JH, Yang IS: Functional characterization of primary culture cells grown in hormonally defined, serum-free medium and serum-supplemented medium. Kor J Vet Res1996;36:551–563.
25 Brett M, Emerman G, Emerman JT: Effects of estrogen, epidermal growth factor, and transforming growth factor-β on the growth of human breast epithelial cells in primary culture. Exp Cell Res 1992;201:113–118.
26 Rabito CA: Phosphate uptake by a kidney cell line (LLC-PK1). Am J Physiol 1983;245:F22–F31.
27 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
28 Sakhrani LM, Behnam BD, Walter T, Nabil M, Andrew GL, Leon GF: Transport and metabolism of glucose by renal proximal tubular cells in primary culture. Am J Physiol 1984;246:F757–F764.
29 Rindler MJ, Taub M, Saier MH Jr: Uptake of $^{22}Na^+$ by cultured dog kidney cells (MDCK). J Biol Chem 1979;254:11431–11439.
30 Ernst M, Schmid C, Froesch ER: Enhanced osteoblast proliferation and collagen gene expression by estradiol. Proc Natl Acad Sci USA 1988;85:2307–2310.
31 Weisz A, Bresciani F: Estrogen induces the expression of c-fos and c-myc protooncogenes in rat uterus. Mol Endocrinol 1988;2:816–824.
32 Dick IM, John AST, Heal S, Prince RL: The effect of estrogen deficiency on bone mineral density, renal calcium and phosphorus handling and calcitropic hormones in the rat. Calcif Tissue Int 1996;59:174–178.
33 Li JJ, Li SA: Estrogen carcinogenesis in the hamster kidney: A hormone-driven multistep process. Prog Clin Biol Res 1996;394:255–267.

34 Verri T, Markovich D, Perego C, Norbis F, Stange G, Sorribas V, Biber J, Murer H: Cloning of a rabbit renal Na-P_i cotransporter, which is regulated by dietary phosphate. Am J Physiol 1995;268:F626–F633.
35 Park K, Kim KR, Kim JY, Park YS: Effect of cadmium on Na-P_i cotransport kinetics in rabbit renal brush-border membrane vesicles. Toxicol Appl Pharmacol 1997;145:255–259.
36 Fucentese M, Winterhalter K, Murer H, Biber J: Functional expression of rat renal Na/P_i-cotransport (NaP_i-2) in Sf9 cells by the baculovirus system. J Membr Biol 1995;144:43–48.
37 Han HJ, Kim DH, Park SH: Regulatory mechanisms of Na/P_i cotransporter by glucocorticoids in renal proximal tubule cells: Involvement of cAMP and PKC. Kidney Blood Press Res 2000;23:1–9.
38 Plagemann PG: Nucleotide pools in Novikoff rat hepatoma cells growing in suspension culture. 3. Effects of nucleosides in medium on levels of nucleotides in separate nucleotide pools for nuclear and cytoplasmic RNA synthesis. J Cell Biol 1972;52:131–146.
39 Edwards DP, Munthy SR, McGuire WL: Effects of estrogen and anti-estrogen on DNA polymerase in human breast cancer. Cancer Res 1980;40:1722–1726.
40 Murer H, Biber J: A molecular view of proximal tubular inorganic phosphate (P_i) reabsorption and of its regulation. Pflügers Arch 1997; 433:379–389.
41 Ritz E, Kreusser W, Bommer J: Effects of hormones other than parathyroid hormones on renal handling of phosphate; in Massry SG, Fleisch H (eds): Renal Handling of Phosphate. New York, Plenum, 1980, pp 137–195.
42 Park SH, Taub M, Han HJ: Regulation of phosphate uptake in primary cultured rabbit renal proximal tubule cells by glucocorticoids: Evidence for nongenomic as well as genomic mechanisms. Endocrinology 2001;242:710–712.
43 Jehle AW, Forgo J, Biber J, Lederer E, Krapf R, Murer H: Acid-induced stimulation of Na-P_i cotransport in OK cells: Molecular characterization and effect of dexamethasone. Am J Physiol 1997;273:F396–F403.
44 Beers KW, Thompson MA, Chini EN, Dousa TP: β-Estradiol inhibits Na^+-P_i cotransport across renal brush border membrane from ovariectomized rats. Biochem Biophys Res Commun 1996;221:442–445.
45 Berthois Y, Katzenenellbogen JA, Katzenenellbogen BS: Phenol red in tissue culture media is a weak estrogen: Implication concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 1986;83:2496–2500.
46 Walsh-Reitz MM, Toback FG: Phenol red inhibits growth of renal epithelial cells. Am J Physiol 1992;262:F687–F691.