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Interaction of 17β-Estradiol with EGF and IGF-I on Proliferation and P_i Uptake in Primary Cultured Rabbit Renal Proximal Tubular Cells

Ho Jae Han and Yeun Hee Lee

Department of Veterinary Physiology, College of Veterinary Medicine, Hormone Research Center, Chonnam National University, Kwangju 500–757, Korea

The most significant direct role of estrogen *in vivo* is its ability to elicit receptor-mediated cellular proliferation in mammalian target tissues. However, the mechanism by which exogenously added estrogen causes the neoplastic transformation of renal cortical cells is yet to be uncovered. The present study was designed to evaluate interaction of 17β-estradiol (E_2) with epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) on proliferation and P_i uptake in primary cultured rabbit renal proximal tubular cells in phenol red-free, hormonally defined-medium. [^{3}H]-thymidine incorporation increased markedly by about 133% and 141% more in the presence of 10^{-9} and 10^{-6} M E_2, respectively, than that of control. Cell count was 162% and 143% greater in the presence of 10^{-9} and 10^{-6} M E_2 , respectively, compared with control. Among all time points examined, there was an increase in [^{3}H]-thymidine incorporation in the presence of 10^{-9} M E_2 at day 9 or 13, respectively. However, E_2 (10^{-9} M) significantly drove up cell count to 160% of that of control at day 13, while it had a slight but statistically insignificant effect at day 9. E_2-induced stimulation of [^{3}H]-thymidine incorporation was completely reversed by E_2 antagonists (progesterone or tamoxifen). E_2 (10^{-9} M) or EGF (10^{-8} M) significantly stimulated [^{3}H]-thymidine incorporation by 144% and 154% of control. E_2 plus EGF was synergistic on [^{3}H]-thymidine incorporation (204% of control), while E_2 plus IGF-I showed a slight but no significant synergistic effect. Cell number also displayed similar pattern. E_2 (10^{-9} M) significantly stimulated P_i uptake to 134% of control. E_2-induced stimulation of P_i uptake was partially reversed by E_2 antagonists. EGF or IGF-I (10^{-8} M) significantly also increased P_i uptake to 132% or 129% of control. E_2 plus EGF had synergistic effect on P_i uptake, while E_2 plus IGF-I did not. In conclusion, E_2 may act not only directly interaction with its receptors but also indirectly as a modulator of EGF in proliferation and P_i uptake of primary cultured rabbit renal proximal tubular cells.

Key Words: Estrogen, Kidney, EGF, IGF-I, Na^+/P_i cotransporter

INTRODUCTION

The most significant direct role of estrogen action *in vivo* is its ability to elicit receptor-mediated cellular proliferation in mammalian target tissues such as the uterus, vagina, and mammary gland (Zheng et al, 1996). However, estrogens also have physiological effects in a number of other target tissues, including kidney (DeVries et al, 1972; Winter et al, 1996). The hamster renal cortex is clearly an estrogen-sensitive target tissue, as indicated by an increase of a specific estrogen and a 13-fold induction of progesterone receptor in this organ after estrogen treatment (Li et al, 1974; Li et al, 1995). In the hamster, prolonged estrogen treatment caused the formation of tumors in the renal cortex (Li et al, 1995). However, the mechanisms by which exog-

Corresponding to: Ho Jae Han, Department of Veterinary Physiology, College of Veterinary Medicine, Chonnam National University, Kwangju 500-757, Korea. (Tel) 062-530-2831 (Fax) 062-530-2809, E-mail: hjhan@chonnam.chonnam.ac.kr

enously added estrogen cause the neoplastic transformation of renal cortical cells are yet to be determined, and a little has been known regarding the role of estrogens in regulating the function of normal renal cortical cells.

It has been reported that estrogens trigger the production of growth factors which are mitogenic and stimulate growth. Among the growth factors known to elicit mitogenic response in estrogen-dependent cell lines, epidermal growth factor (EGF), transforming growth factor-α or -β (TGF-α or -β), insulin-like growth factor-I or -II (IGF-I or -II), fibroblast growth factor (FGF), and amphiregulin have received considerable attention as a mediator (Beleh et al, 1993; Wattiez et al, 1996). It has been well documented that estrogen proliferates the human breast cancer by inducing EGF-related peptide (Dickson et al, 1986) and the TGF-α (Bates et al, 1988) autocrine system. In particular, 17β-estradiol (E_2) induced mRNA and protein for EGF (Heut-Hudson et al, 1990; Ignar-Trowbridge et al, 1992). EGF has a strong stimulatory effect on the proliferation and growth of a variety of nontumor cells (Nowak & Schnellmann, 1995). EGF-specific antibody administered prior to E_2 partially blocks estrogen-induced uterine epithelial cell proliferation (Nelson et al, 1991). This result suggests that the production of EGF may be necessary for estrogen-induced response. EGF also accelerates Na^+/phosphate cotransport, glycolysis, and glucose and amino acid uptake in a variety of cultured epithelial cells (Boerner et al, 1985; Goodyer et al, 1988; Nehar et al, 1993). Beer et al (1996) have reported that E_2 decreased P_i uptake in brush border membrane vesicles of kidneys from ovarectomized and thyroparathyroidectomized rats, whereas Na^+-cotransports of SO_4, L-proline, and D-glucose showed no change. However, there is no report about the interaction between E_2 and growth factors on the growth and Na^+/phosphate cotransporter activity of renal proximal tubular cells (PTCs).

Tissue culture cell *in vitro* is the easiest way to study the mechanisms by which estrogens regulate the growth and functions of differentiated cells in target tissue *in vivo*. Thus, primary cultured rabbit renal proximal tubular cells in phenol red-free, hormonally-defined, serum-free medium would be an efficient tool for studying the action mechanisms of E_2 (Han et al, 1996). The present study was designed to evaluate the interaction of E_2 with EGF and IGF-I on proliferation and P_i uptake of primary cultured rabbit renal proximal tubular cells in phenol red-free, hormonally defined-medium.

METHODS

Materials

Male New Zealand White rabbits (1.5~2.0 kg) were used in this study. Dulbecco's Modified Eagle's Medium (phenol red-free), F-12 Nutrient Mixture, Class IV collagenase, and soybean trypsin inhibitor were purchased from Life Technologies (Grand Island, NY). Estradiol-17β (E_2), estriol, estrone, insulin, transferrin, tamoxifen (TAM), progesterone (P_4), epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), and trichloroacetic acid (TCA) were obtained from Sigma Chemical Corp (St. Louis, Mo). $^{22}Na^+$ and [methyl-^{3}H]-thymidine was purchased from Dupont/NEN. All the other reagents were of the highest purity commercially available. Liquiscint was obtained from National Diagnostics (Parsippany, NY). Iron oxide was prepared by the method of Cook and Pickering (1958). Stock solutions of iron oxide in 0.9% NaCl were sterilized using an autoclave and diluted with phosphate buffered saline (PBS) prior to use.

Methods

Preparation of culture medium and primary cell culture: This study used male New Zealand White rabbits (1.5~2.0 kg). Rabbit renal proximal tubules were prepared by a modification of the method of Chung et al (1982). The PTCs were grown in 50 : 50 mixture of Dulbecco's Modified Eagle's Medium (D-MEM) and Ham's nutrient mixture F-12 with 15 mM HEPES buffer (pH 7.4) and 20 mM sodium bicarbonate, which lacks phenol red. Immediately prior to the use of the medium, two growth supplements (5 μg/ml insulin, 5 μg/ml transferrin) were added. Kidneys were perfused via the renal artery, first with PBS, and subsequently with D-MEM/F-12 containing 0.5% iron oxide (wt/vol) until the kidney turned gray-black in color. Renal cortical slices were prepared by cutting the renal cortex and then homogenized with 4 strokes of a sterile glass homogenizer. The homogenate was poured first through a 253 μm mesh filter and then 83 μm one. Tubules and glomeruli on the top of 83 μm filter were transferred

into sterile medium containing a magnetic stirring bar. Glomeruli (containing iron oxide) were removed with a magnetic stirring bar. The remaining proximal tubules were briefly incubated in medium containing 60 μg/ml collagenase (Class IV) and 0.025% soybean trypsin inhibitor. The dissociated tubules were then washed by centrifugation, resuspended in medium containing the two supplements, and transferred into tissue culture dishes. PTCs were maintained at 37°C, in a 5% CO_2-humidified environment in phenol red-free medium containing the two supplements. Medium was changed one day after plating and every three days thereafter.

[^{3}H]-Thymidine incorporation: Medium was changed every 3 days, and cultures were observed daily. When the fastest growing cultures become 70 ~80% confluent, a final media change was done. [^{3}H]-thymidine incorporation experiments were conducted as described by Brett et al (1992). Cells were incubated in medium in the absence or presence of various concentrations of E_2 for 24 hr and were pulsed with 1 μCi of [methyl-^{3}H]-thymidine for 24 hr at 37°C. The cells were then washed twice with PBS and were fixed in 10% TCA at room temperature for 15 min and then washed twice in 5% TCA. The acid-insoluble material was dissolved in 2 N NaOH at room temperature for 12 hr. Aliquots from each dish were measured for [methyl-^{3}H]-thymidine incorporation into DNA by scintillation counting. All experiments were made in triplicate. Values were converted from absolute counts to the percentage of control to allow for comparison between experiments.

Cell growth studies: To determine the effect of steroids and growth factors on the growth of PTCs, purified rabbit kidney proximal tubules were inoculated into 35 mm plastic dishes containing phenol red free D-MEM/F-12 supplemented other specified factors which are required by the experimental protocol. Periodically, during growth period, representative culture dishes were removed at an indicated day. The cells were detached from the culture dishes utilizing 0.05% trypsin/0.5 mM EDTA solution, and the proteolytic action was then inhibited by soybean trypsin inhibitor (0.05 mg/ml). The cell suspensions were diluted with PBS and the cell number was determined utilizing a Coulter Model ZF Particle counter. All experiments were made in triplicate.

P_i uptake studies: To study the effect of E_2 on P_i uptake, the 80~90% confluent monolayers were incubated with 10^{-9} M E_2 for 5 days before P_i uptake experiments. Uptake experiments were conducted as described by Rabito (1983). After culture medium was removed by aspiration, the monolayers were gently washed twice with the uptake buffer [150 mM NaCl, 1.2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 10 mM MES/Tris, pH 7.4]. After the washing procedure, the monolayers were incubated at 37°C for 30 min in an uptake buffer that contained 1.5 μCi/ml ^{32}P-phosphate and 1 mM unlabeled phosphate. At the end of the incubation period, the monolayers were again washed three times with ice-cold uptake buffer, and the cells were dissolved in 1 ml 0.1% SDS. To determine the $^{32}P_i$ incorporation intracellularly, 900 μl of each sample was collected and counted in a liquid scintillation counter (Beckmann Co). The remainder of each sample was used for protein determination (Bradford, 1976). The radioactive counts in each sample were then normalized with respect to protein and were corrected for zero-time uptake per mg protein. All uptake measurement was made in triplicate.

Statistical analysis: Results were expressed in mean ± standard errors (S.E.). Data were analyzed by the ANOVA procedure using the Statistical Analysis System. The difference was considered statistically significant when $P<0.05$.

RESULTS

Effect of E_2 on cell proliferation

To determine the dose-response of the effect of E_2 on DNA synthesis and cell growth, PTCs were grown in phenol red-free, hormonally defined medium in the absence or presence of various concentrations of E_2. [^{3}H]-thymidine incorporation markedly increased in the presence of each 10^{-9} and 10^{-6} M E_2, growing about 133±10% and 141±8% more, respectively, than that of control. Cell number also was 162±8% and 143±9% greater in the presence of 10^{-9} and 10^{-6} M E_2, respectively, compared with control (Fig. 1).

PTCs grew in different degrees depending on whether E_2 at 10^{-9} M, an optimal concentration level found to effect maximum tubular cell proliferation, is present or not. Among all time points examined (day 5, 9, and 13), there was 152±18% and 143±6% increase in [^{3}H]-thymidine incorporation when the medium contained E_2 compared to cultures not to exposure to E_2 at day 9 and 13, respectively.

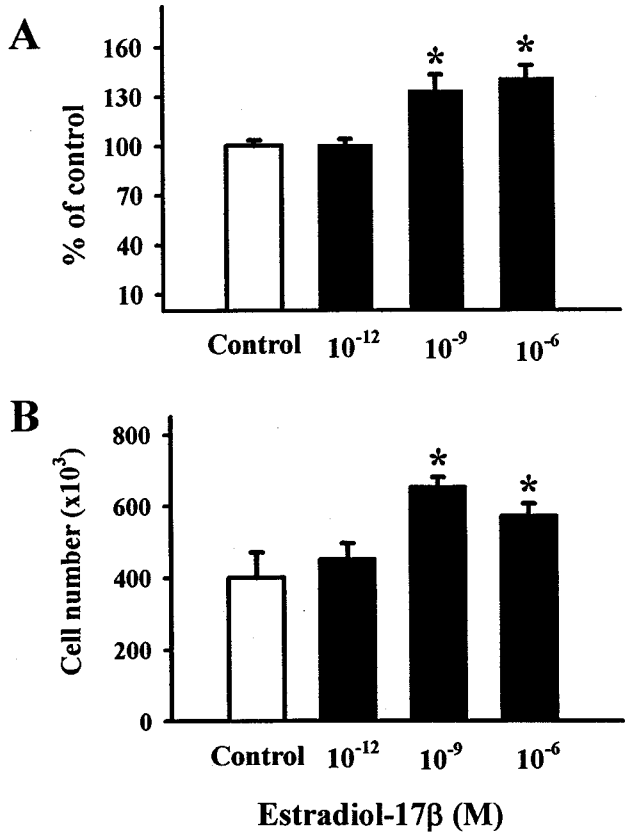

Fig. 1. A: Dose-dependent effect of E_2 on [^{3}H]-thymidine incorporation. Cells were incubated for 24 hr in the absence or presence of 10^{-12}, 10^{-9}, or 10^{-6} M E_2, and pulsed with 1 μCi of [^{3}H]-thymidine for 24 hr. Values are means±S.E. of three independent experiments with triplicate dishes. *P<0.05 vs. control. B: Dose-dependent effect of E_2 on cell growth. Proximal tubules were cultured in medium containing E_2 at 10^{-12}, 10^{-9}, 10^{-6} M, or no add hormone, respectively. Cell counts were performed on 13 day. Values are means±S.E. of six independent experiments with triplicate dishes. *P<0.05 vs. control.

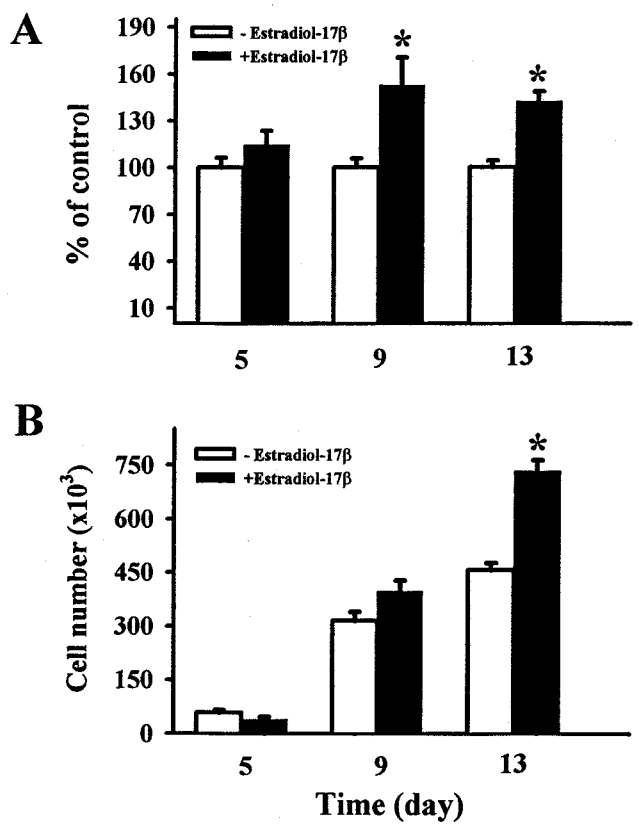

Fig. 2. A: Cell stage dependent effect of E_2 on [^{3}H]-thymidine incorporation. Cells were incubated for 24 hr in the absence or presence of 10^{-9} M E_2 and pulsed with 1 μCi of [^{3}H]-thymidine for 24 hr. Experiments were performed from days in culture to 5, 9 and 13 day respectively. Each point represents means±S.E. of three independent experiments with triplicate dishes. *P<0.05 vs. each control. B : Time dependent effect of E_2 on cell growth. Proximal tubules were cultured in medium containing 10^{-9} M E_2. Cell counts were performed on 5, 9 and 13 day. Each point represents means±S.E. of six independent experiments with triplicate dishes. *P<0.05 vs. control.

However E_2 (10^{-9} M) significantly push up the cell count to 160±7% of control at day 13, while at day 9, it showed only a slight effect without significance (Fig. 2). These data showed that E_2 promotes DNA synthesis and cell growth in dose-, cell stage-dependent manners *in vitro*.

To assess the specificity and potency of this proliferative response by E_2 on PTCs, exposure of culture to E_2 metabolites was examined. Estriol (10^{-9} M) significantly stimulated [^{3}H]-thymidine incorporation to 133±15% of control and cell number to 132 ±8% of control (P<0.05), both of which were less than with E_2. However, estrone at 10^{-9} M did not affect any significant change in [^{3}H]-thymidine incorporation and cell number (Fig. 3).

Effects of E_2 antagonists (progesterone and tamoxifen) on E_2-induced cell proliferation

Another set of experiments was then designed to test the receptor dependence of the E_2 effect on cell proliferation. Antiestrogens such as progesterone and tamoxifen prevent the growth of many E_2-dependent tumors by competing with E_2 for binding to the E_2 receptor and thereby preventing E_2-mediated effects

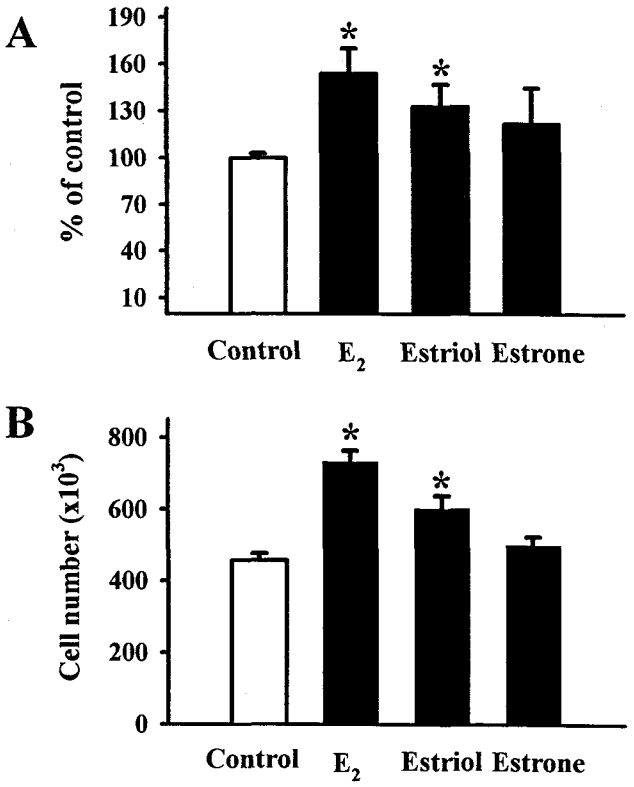

Fig. 3. A: Effects of estrogen metabolites on [^{3}H]-thymidine incorporation. Cells were incubated for 24 hr in the presence of estrogen metabolites (10^{-9} M estriol or estrone) or no add hormone and pulsed with 1 μCi of [^{3}H]-thymidine for 24 hr. Values are means±S.E. of five independent experiments with triplicate dishes. *P< 0.05 vs. control. B: Effects of estrogen metabolites on cell growth. Proximal tubules were cultured in medium containing estrogen metabolites (10^{-9} M estriol or estrone). Cell counts were performed on 13 day. Values are means±S.E. of six independent experiments with triplicate dishes. *P<0.05 vs. control.

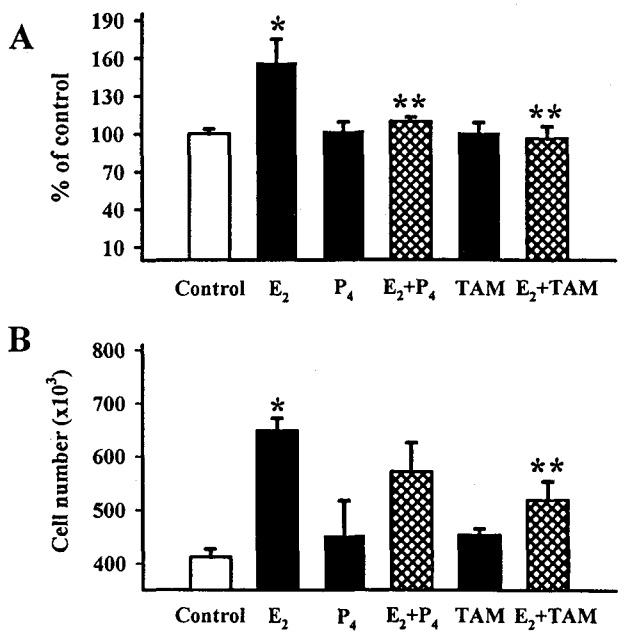

Fig. 4. A: Effects of E_2 antagonists on E_2-induced increase of [^{3}H]-thymidine incorporation. Cells were incubated for 24 hr in the presence of E_2 antagonists (10^{-9} M progesterone; P_4 or 10^{-6} M tamoxifen; TAM) and 10^{-9} M E_2 or alone and pulsed with 1 μCi of [^{3}H]-thymidine for 24 hr. Values are means±S.E. of four 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 stimulation of cell growth. Proximal tubules were cultured in medium containing E_2 antagonists (10^{-9} M P_4, 10^{-6} M TAM) and 10^{-9} M E_2 or alone. Cell counts were performed on 13 day. Values are means± S.E. of four independent experiments with triplicate dishes. *P<0.05 vs. control, **P<0.05 vs. E_2.

(Rochefort & Borgna, 1981). E_2-induced stimulation of [^{3}H]-thymidine incorporation was completely reversed by progesterone (10^{-9} M) or tamoxifen (10^{-6} M). Also, cell number was decreased by tamoxifen. However, progesterone or tamoxifen alone at this concentration did not affect [^{3}H]-thymidine incorporation and cell growth of PTCs (Fig. 4).

Interaction of E_2 with EGF and IGF-I on cell proliferation

E_2-induced cell proliferation may be mediated by the secretion of various growth factors. We examined the proliferation effects of E_2 (10^{-9} M), EGF (10^{-8} M) or IGF-I (10^{-8} M) alone and in combination. E_2 or EGF was added to culture to examine their effects on proliferation of PTCs. E_2 and EGF significantly stimulated [^{3}H]-thymidine incorporation by 144± 14% and 154±14% of control (P<0.05), respectively. A combination of E_2 and EGF resulted in a synergistic effect (204±18% of control). The effect of E_2 and IGF-I on [^{3}H]-thymidine incorporation was also examined. In this experiment, the degree of stimulation by IGF-I equaled to or was lower than that of EGF and E_2. The effect of E_2 and IGF-I combined had a slight but no significant synergistic effect (Fig. 5). The results of cell growth were also similar pattern.

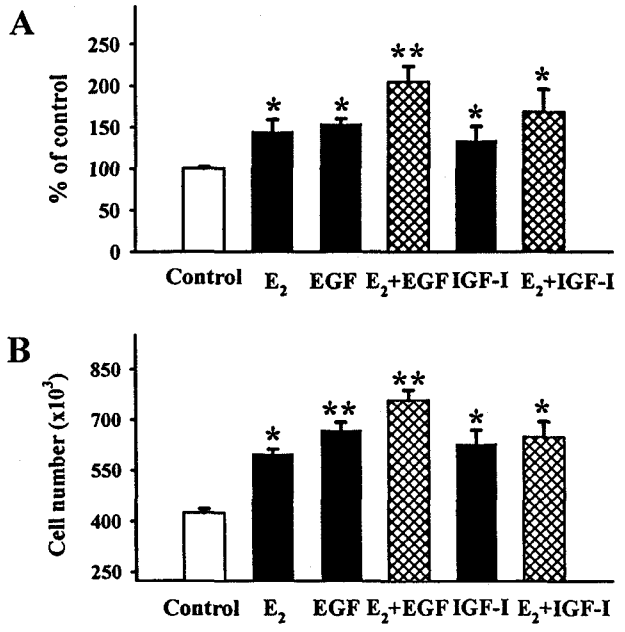

Fig. 5. A: Effects of EGF and IGF-I on E_2-induced increase of [^{3}H]-thymidine incorporation. Cells were incubated for 24 hr in the presence of growth factors (10^{-8} M EGF or IGF-I) and 10^{-9} M E_2 or alone and pulsed with 1 μCi of [^{3}H]-thymidine for 24 hr. Values are means $\pm$S.E. of five independent experiments with triplicate dishes. *$P<0.05$ vs. control, **$P<0.05$ vs. E_2 and EGF. B : Effects of growth factors on E_2-induced stimulation of cell growth. Proximal tubules were cultured in medium containing growth factors (10^{-8} M EGF, IGF-I) and 10^{-9} M E_2 or alone. Cell counts were performed on 13 day. Values are means$\pm$S.E. of four independent experiments with triplicate dishes. *$P<0.05$ vs. control, **$P<0.05$ vs. E_2.

Interaction of E_2 with EGF and IGF-I on P_i uptake

A major portion of filtered phosphate is reabsorbed by Na^+/phosphate cotransport in renal proximal tubule. The effect of E_2 on P_i uptake was evaluated. E_2 (10^{-9} M) significantly stimulated P_i uptake to 134 $\pm$5% of control ($P<0.05$). E_2-induced stimulation of P_i uptake was partially reversed by progesterone (10^{-9} M) or tamoxifen (10^{-6} M). However, progesterone or tamoxifen alone at this concentration did not affect P_i uptake of PTCs (Fig. 6). We also examined the effects of each E_2, EGF and IGF-I on P_i uptake. EGF and IGF-I (10^{-8} M) significantly increased P_i uptake to 132$\pm$12% and 129$\pm$6% of control, respectively ($P<0.05$). In these experiments, E_2 plus EGF had a synergistic effect, while E_2 plus IGF-I was not synergistic (Fig. 7).

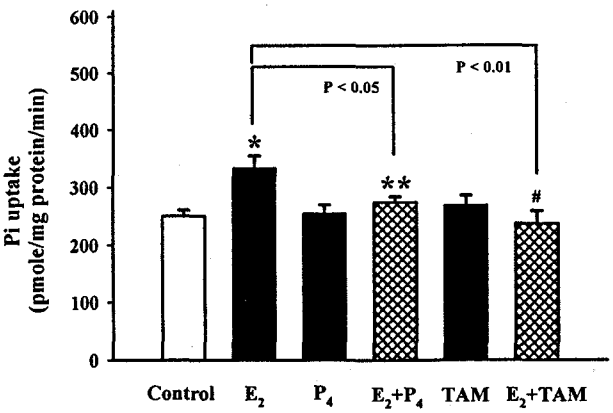

Fig. 6. Effects of E_2 and E_2 antagonists on P_i uptake. Proximal tubular cells were incubated for 5 days with 10^{-9} M E_2 and E_2 antagonists (10^{-9} M P_4, 10^{-6} M TAM) or alone and then P_i uptake was determined. Values are means$\pm$S.E. of four independent experiments with triplicate dishes. *$P<0.05$ vs. control, **$P<0.05$ vs. E_2, $^{\#}P<0.01$ vs. E_2.

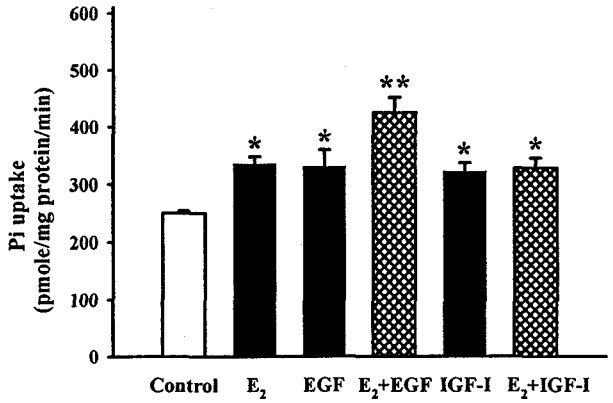

Fig. 7. Effects of EGF and IGF-I on E_2-induced stimulation of P_i uptake. Proximal tubular cells were incubated for 5 days with 10^{-9} M E_2 and growth factors (10^{-8} M EGF, IGF-I) or alone and then P_i uptake was performed. Values are means$\pm$S.E. of four independent experiments with triplicate dishes. *$P<0.05$ vs. control, **$P<0.05$ vs. E_2 and EGF.

DISCUSSION

These investigations were conducted in phenol red-free, hormonally defined, serum-free medium. Phenol red bears some structural resemblance to certain nonsteroidal estrogens, and binds to estrogen receptors with an affinity which is only 0.001% of that of E_2 (Berthois et al, 1986). Nevertheless, phenol red significantly stimulates cell growth and specific

protein synthesis in some estrogen responsive cells, including the human breast cancer cell line MCF-7. For this reason, phenol red has been reported to interfere with the observation of growth stimulatory effects of estrogens in such a cell culture system. The use of this medium has alleviated effects of steroids and other factors in the serum and estrogenic effects of phenol red (Van der Burg et al, 1992; Walsh-Reitz & Toback, 1992).

Estrogen receptor has been detected in whole kidney, kidney sections, and kidney tubules of various animal species (Davidoff et al, 1980; Hangenfeldt & Eriksson, 1988). In addition, we have reported that PTCs from male New Zealand White rabbits contain receptor for E_2 (Han, 1993). The cellular effect of E_2 is tissue and organ specific. E_2 has been found to be mainly proliferative in osteoblasts (Ernst et al, 1988), female reproductive organ (Weisz & Bresciani, 1988), and renal proximal tubular epithelium from hamsters, but not those from rats or mice (Oberley et al, 1989). These effects seem to depending not only on cell or tissue type but also on the specific culture and incubation conditions of the individual experiments performed. In this study, 10^{-9} M and 10^{-6} M of E_2 increased cellular proliferation, but at a lower dose (10^{-12} M), E_2 did not affect proliferation. Also, 10^{-9} M of E_2 significantly increased cellular proliferation at middle and late stages of PTCs but slightly suppressed proliferation at an early stage of PTCs. We also found that 10^{-9} M of E_2 stimulated DNA synthesis of PTCs, as evaluated by [^{3}H]-thymidine incorporation into the cell. These results suggest that the interaction of progesterone or tamoxifen with the E_2 receptor may have caused suppression of E_2-induced stimulation of DNA synthesis and cell growth. In addition, E_2 seemed to interact with specific cytoplasmic or nuclear receptor proteins, which then become associated with chromatin, causing gene expression and synthesis of specific proteins leading to cell division.

These estrogen-specific effects are of particular significance in that it occurs at physiologic hormone concentrations and under serum-free hormone defined culture condition. It is of note that several laboratories have been unable to demonstrate an effect of estrogens on proliferation of estrogen-responsive tissues in culture (Cooke et al, 1986; Tomooka et al, 1986). When H-301 (estrogen dependent kidney tumor cell line) cell growth was examined in culture, an addition of estrogens or other steroid hormones to the medium over the range of 0.1 to 10 ng/ml did not affect cells (Sirbasku & Kirkland, 1976). These observations suggest that estrogen involvement in the cell growth of kidney tumor may be mediated indirectly *in vivo*.

Accumulated lines of evidence have shown that estrogen or other steroid hormones regulate the production of some growth factors or their cognate receptors, and in turn, certain growth factors also down- or up- regulate steroid receptors (Sirbasku, 1978; Bulter et al, 1983). Estrogen modulates the biosynthesis or secretion of several growth factors such as IGF-I, EGF, TGF-α. Thus, E_2 may similarly stimulate PTCs growth indirectly by stimulating the production of such autocrine growth factors. E_2 plus EGF was synergistic on DNA synthesis and cell growth, while E_2 plus IGF-I was not. This fact contradicts the results that IGF-I is involved in E_2-induced cell proliferation (Narayan & Roy, 1993). These inconsistencies may result from the differences in the species, organs, or methods employed. Our results suggested that EGF, not IGF-I, may be involved in E_2-induced PTCs growth stimulation. On the other hand, P_i uptake was markedly stimulated after 5 days of E_2 treatment. These effects were partially blocked by two different E_2 antagonists, progesterone and tamoxifen. Our findings with regard to P_i uptake provide further evidence of estrogen responsiveness in PTCs. In contrast, Beer et al (1996) reported that the E_2 decreased Na^+/P_i cotransport activity in brush border membrane vesicles from kidneys of thyroparathyroidectomized and ovarectomized female rats. The differences in results probably relate to experimental model used in the measurement of P_i uptake. E_2 plus EGF had also a synergistic effect on P_i uptake, while E_2 plus IGF-I did not. These results give support to the idea that E_2 may be involved not only directly in the interaction with its receptors as a mitogen but also indirectly as a modulator of some growth factors including EGF in proliferation and P_i uptake of PTCs. Further investigations are required to determine the proposed direct and indirect functions of E_2.

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