Kor J Physiol (1995) 29(2), 191-202

Effects of Insulin and IGFs on Growth and Functional Differentiation in Primary Cultured Rabbit Kidney Proximal Tubule Cells

-Growth and membrane transport-

Ho-Jae Han and Kwon-Moo Park

Department of Veterinary Physiology, College of Veterinary Medicine, Chonnam National University, Kwangju, Korea. 500-757

=ABSTRACT=

The purpose of this study was to compare effects of insulin and IGFs on growth, apical membrane enzyme activities and membrane transport systems of primary cultured rabbit kidney proximal tubule cells. Results were as follows:

1. Insulin and IGF-I produced significant growth stimulatory effects at 5×10^{-10} M. IGF-II(5×10^{-10} M) did not stimulate significant cell growth.

2. Insulin stimulated the phosphorylation of a 97 KD protein. It was difficult to determine whether this band represents insulin and/or the IGF-I receptor.

3. The activities of apical membrane enzymes (alkaline phosphatase, leucine aminopeptidase, and γ -glutamyl transpeptidase) were observed to be diminished after the cells were placed in the culture environment.

4. The uptake of α-MG, Pi and Na was significantly increased in cells incubated with insulin or IGF-I. IGF-II had no effect on the uptake of these substrates.

5. Na-pump activity, as assayed by Rb uptake, was significantly increased in cells treated with insulin or IGFs.

In conclusion, insulin and IGF-I exert stimulatory effects on growth and membrane transporter(glucose, Na, Pi, and Na-pump) activities in primary cultured rabbit kidney proximal tubule cells. IGF-II had no effect on cell growth and membrane transporter(glucose, Na and Pi) activities.

Key Words: Kidney, Insulin, IGF, Transporter

INTRODUCTION

The compensatory hypertrophic response of nephrons surviving after renal injury appears to be ultimately injurious to nephrons, and may underlie the progressive nature of kidney disease (Cross & Dexter, 1991). However, the neurohumoral mechanisms involved in mediating this response are poorly understood. In diabetic renal disease, increase in kidney size and associated microalbuminuria appear to precede the development of diabetic nephropathy and end stage renal disease (Mogensen, 1987). In-

This study was supported by grants awarded to Dr. H. J. Han from NON DIRECTED RESEARCH FUND, Korea Research Foundation (1994) and Korea Science and Engineering Foundation through Hormone Research Center (HRC-95-0404)

creased intake of dietary protein is known to be a potent stimulus of renal hypertrophy and has also been shown to accelerate the progression of experimental renal disease in the rat (Brenner, 1985). Studies of the processes involved in renal hypertrophy have focused on the structural changes involved and on the role of renotrophic and other factors which might be responsible for the functional adaptations. Although less is known about the renotrophic factors that are responsible for the functional adaptations (increased RBF and GFR) in this model, IGF-I has been suggested to have an important role in the kidney during the process of hypertrophy (Fine & Norman, 1989). Developing an understanding of the mechanisms involved in renal hypertrophy, therefore, is important in developing future therapeutic strategies to slow the progression of established renal diseases.

Insulin and IGFs are commonly required for animal cell growth and differentiation in serum free medium (Czech, 1985). These peptide hormones and growth factors act by distinct mechanisms to modulate a number of different functions in different tissues. The physiological actions of insulin, IGF-I and IGF-II are initiated by the binding of hormone to receptors located on the plasma membrane of target cells (Rechler & Nissley, 1985). Insulin and IGFs are known to affect on glycogen synthesis, protein synthesis and RNA synthesis (Kahn, 1985). Both insulin and IGFs have been reported to influence developmental processes (Sara & Hall, 1990; Kahn, 1985). Not only have IGFs been shown to stimulate the differentiation of myoblasts, osteoblasts and adipocytes, but also to affect ovarian and testicular cell function (Sara & Hall, 1990). Whereas insulin is produced by pancreatic beta cells, a wide variety of cells synthesize IGFs (Sara & Hall, 1990). It is becoming increasingly clear that insulin and IGFs play important roles in modulating renal proximal tubule cell growth and function in vivo. The regulatory role of insulin in altered renal proximal tubule cells has long been known, largely due to function and cellular hypertrophy which occur in the renal proximal tubule in diabetes (Hammerman, 1985).

The proximal tubular cell in primary culture is an important model system with which one can examine mechanisms of hypertrophy and hyperplasia and the interrelationships between membrane transport processes and the regulation of growth by specific growth factors (Ham, 1982; Taub, 1990). One of the significant advantages of working with cells in culture is the ability to manipulate the milieu over prolonged periods. Since the bulk of the increase in kidney size observed during the process of compensatory hypertrophy is due to an increase in proximal mass, the primary proximal tubule culture has been used to gain insight into the process of renal growth. The purpose of this study was to compare effects of insulin, IGF-I and IGF-II on growth, apical membrane enzyme activities and membrane transport systems of primary cultured rabbit kidney proximal tubule cells.

MATERIALS AND METHODS

Materials

Male New Zealand white rabbits (1.5~2.0 kg) were used for these experiments. Dulbecco's Modified Eagle's Medium (D-MEM), F-12 nutrient mixture and soybean trypsin inhibitor were obtained from Life Technologies (Grand Island, NY). Insulin, IGF-I, IGF-II, enzyme assay substrates (p-nitrophenylphosphate, L-leucine p-nitroanilide, γ-glutamyl-p-nitroanilide), and other chemicals were purchased from Sigma Chemical Corp. (St. Louis, MO). Class IV collagenase was supplied by Worthington (Freehold, NJ). Radionuclides, [^{14}C]Methyl-α-D-glucopyranoside (α-MG), Rubidium-86 (^{86}Rb), Phosphate(^{32}P), and Sodium(^{22}Na) were purchased from Dupont/NEN. Liquiscint was obtained from National Diagnostics (Parsippany, NY). All other reagents were of the highest purity commercially available. 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

Isolation of rabbit kidney proximal tubules and culture conditions

Primary rabbit kidney proximal tubule cell cultures were prepared by a modification of the method of Chung et al (1982). The basal medium, D-MEM/F12, pH 7.4, was a 50 : 50 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12. The basal medium was further supplemented with 15 mM HEPES buffer (pH 7.4) and 20 mM sodium bicarbonate. Immediately prior to the use of the medium, two growth supplements (transferrin and hydrocortisone) were added. Water utilized in medium preparation was purified by means of a MilliQ deionization system. Kidneys of a male New Zealand white rabbit were perfused via the renal artery, first with PBS, and subsequently with D-MEM/F12 containing 0.5% iron oxide (wt/vol) until the kidney turned grey-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 and then a 83 μm mesh filter. Tubules and glomeruli on top of the 83 μm filter were transferred into sterile D-MEM/F12 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 D-MEM/F12 containing 80 μg/ml collagenase (Class IV) and 0.025% soybean trypsin inhibitor. The dissociated tubules were then washed by centrifugation, resuspended in D-MEM/F12 containing the three supplements, and transfered into tissue culture dishes. Primary rabbit kidney proximal tubule cells were maintained at 37 ℃, in a 95% air/5% CO_2-humidified environment in D-MEM/F12 medium, supplemented with 5 μg/ml transferrin and 5×10^{-8} M hydrocortisone only (control) or, in addition, with either insulin (5×10^{-10} M), IGF-I (5×10^{-10} M), or IGF-II (5×10^{-10} M) (Chung et al, 1982). Medium was changed one day after plating and every two days thereafter.

Cell growth studies

To determine effects of insulin and IGFs, primary proximal tubule cells in culture were initiated in 35mm dishes for cell growth studies. Briefly, tubules were inoculated at one-fourth the normal inoculum (the normal inoculum was 0.3 mg protein/ dish measured by the method of Bradford, 1976; Chung et al, 1982). Cell growth was monitered on day 3, 5, 9, 13, and 19 in culture. Cells were maintained in a 37℃, 95% air/5% CO_2 humidified environment in D-MEM/ F12 medium supplemented with 5 μg/ml transferrin and 5×10^{-8} M hydrocortisone only(control) or, in addition, with either insulin (5×10^{-10} M), IGF-I (5×10^{-10} M), or IGF-II (5×10^{-10} M) (Chung et al, 1982). During this time, cell counts were determined on day 3, 5, 9, 13, and 19 from triplicate culture plate using a Coulter Model ZF particle counter. The cells were dislodged by incubation with phosphate buffered saline (PBS) containing 0.05% trypsin and 0.5 mM EDTA. The proteolytic action was then inhibited by soybean trypsin inhibitors (0.05 mg/ml). The cell suspensions were diluted with PBS. Values are the mean ± S.E. of five independent determinations.

Identification of purified insulin and/or IGFs receptors

Wheat germ agglutinin(WGA) agarose chromatography was employed to evaluate whether insulin and/ or IGF-I receptors are present in the primary rabbit kidney proximal tubule cells. Briefly, primary rabbit kidney cells were grown to confluency in 100 mm dishes in antibiotic free D-MEM/F12 supplemented with transferrin and hydrocortisone as

described above. The monolayers were labelled with ^{32}Pi and incubated in the medium supplemented with 20 ng/ml of insulin or vehicle. Primary proximal tubule cell extracts were prepared and applied to WGA agarose columns. The insulin receptor and IGF-I receptor were eluted with buffer containing N-acetylglucosamine. The labelled eluates were pooled, aliquoted and stored at -70℃ prior to use. Samples of the WGA eluate were run on reducing SDS gels (7.5% acrylamide). The gels were dried and subjected to autoradiography.

Marker enzymes assay

Alkaline phosphatase activity was assayed using p-nitrophenylphosphate as a substrate (Linhardt & Walter, 1963). The culture medium was removed and the cultures were washed three times with PBS (pH 8.0). The cultures were incubated with PBS containing 2 mg p-nitrophenyl phosphate/ml. Absorbance of p-nitrophenol released was assayed at 420 nm(E 420 for p-nitrophenol = 18,300) after 15 minutes of incubation. The samples were solubilized with 0.1 N NaOH. Leucine aminopeptidase activity was assayed using L-leucine p-nitroanilide as a substrate. The culture medium was removed and the cultures were washed three times with PBS. The cultures were incubated with PBS containing 0.5 mg/2 ml L-leucine p-nitroanilide for 15 minutes at room temperature. Absorbance of p-nitroanilide was measured at 405 nm (E 405 for p-nitroanilide = 9,600). γ-glutamyl transpeptidase activity was determined by using γ-glutamyl-p-nitroanilide as substrate and glycylglycine as the amino acid acceptor (Tate & Meister, 1974). Reagents were added to a Eppendorf tube as follows: 0.2 mg of L-γ-glutamyl-p-nitroanilide (final concentration, 1 mM), 0.2 ml of glycylglycine (final concentration, 20 mM) and 0.6 ml of 0.1 M Tris-HCl buffer, pH 8.0. The solution was brought to 37℃ in a water bath. Cells in culture were washed in ice cold PBS (pH 7.3) and then lysed by a 50 mM imidazole-HCl buffer (pH 7.2 at 25℃) containing 1%(V/V) Triton X-100. The reaction was initiated by adding a suitable amount of cell lysate and the rate of release of p-nitroanilide was measured at 410nm (E 410 or p-nitroanilide = 9,600) after a 10 minute incubation. Each measurement was made using triplicate dishes and was standardized with respect to protein. Protein determination was performed by the methods of Bradford (1976) using bovine serum albumin as a standard. Values quoted are the mean ± S.E. of three independent measurements.

Membrane transport studies

Primary rabbit kidney proximal tubule cell cultures were grown to confluence in 35 mm dishes containing serum free D-MEM/F12 medium supplemented with 5 μg/ml transferrin and 5×10^{-8} M hydrocortisone only(control) or, in addition, either insulin (5×10^{-10} M), IGF-I (5×10^{-10} M), or IGF-II (5×10^{-10} M). Intact monolayers were then used for uptake studies.

α-methyl-D-glucoside uptake studies: The nonmetabolisable sugar, α-methyl-D-glucoside (α-MG), was used in glucose uptake studies. Uptake experiments were conducted as described by Sakhrani et al (1984). Before the uptake period, the monolayers were washed twice with uptake buffer containing (in mM): NaCl, 136; KCl, 5.4; $CaCl_2$, 1.3; $MgSO_4$, 0.41; $MgCl_2$, 0.49; Na_2HPO_4, 0.44; KH_2PO_4, 0.44; HEPES, 5; glutamine, 2 and bovine serum albumin, 0.5 μg/ml. 1.5 milliliters of this buffer containing 0.5 mM α-MG and [^{14}C] α-MG (0.5 μCi/ml) were then added to the dishes. Other compounds as indicated were incubated in the uptake assay medium. The plates were incubated at 37℃ for 30 minutes. At the end of the uptake period, the monolayers were rapidly washed three times with ice-cold isotonic mannitol buffer. The cells were solubilized in 1 ml 0.1% SDS. To determine the [^{14}C] α-MG incorporated intracellularly, nine-tenth of each sample was removed and counted in a scintillation counter (Packard Co.). The remainder of each sample was used for protein

determination. The radioactive counts in each sample were then normalized with respect to protein and were corrected for zero-time uptake per mg protein (i.e., label not removed by washing procedure). All uptake measurements were made in triplicate.

Na^+ uptake studies: For ^{22}Na uptake studies, the medium was removed by aspiration. The monolayers were washed twice with 100 mM Tris-HCl buffer, pH 7.3. Na influx then proceeded at 37℃ for 30 minutes in an uptake buffer (10mM Tris buffer, pH 7.3, made isotonic to 110mM NaCl with sacarose) containing 0.25 μCi/ml $^{22}Na^+$ and 1×10^{-5} M ouabain (pH 7.4). At the end of this period, the monolayers were gently washed three times with ice cold 100 mM Tris-HCl buffer, pH 7.3. After the final wash, the cells were solubilized. The next steps were conducted as described in α-MG uptake study.

Phosphate uptake studies: To study phosphate uptake, the culture medium was first removed by aspiration. The monolayers were gently washed twice with the uptake buffer. The uptake buffer contained 150 mM NaCl, 1.2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 10 mM MES/Tris, pH 6.5. After the washing procedure, the monolayers were incubated at 37℃ for 30 minutes in an uptake buffer that contained 0.5 μCi/ml [^{32}P]-phosphate and 1 mM phosphate. At the end of this incubation period, the monolayers were again washed three times with ice cold uptake buffer. After the final wash, the cells were solubilized. The next steps were conducted as described in α-MG uptake study.

Rb^+ uptake studies: To examine Rb^+ uptake, confluent monolayers on 35 mm dishes were washed twice with 2 ml of uptake buffer (10 mM Tris-HCl, pH 7.4, and 140 mM NaCl). The cells were then submitted to a 30 minute uptake period at 37℃ in uptake buffer containing 1 mM RbCl, 1 μCi $^{86}Rb^+$/ml, and 1 mM ouabain. At the end of the uptake period, the reaction mixture was removed by aspiration, and the monolayer was rapidly washed three times with ice-cold wash buffer (10 mM Tris-HCl, pH 7.4, and 100 mM $MgCl_2$). The cell cultures were solubilized. The next steps were conducted as described in α-MG uptake study.

Statistical analysis: Results were expressed as means ± standard errors(S.E). The difference between two mean values was analysed by Student's t-test. The difference was considered statistically significant when $p < 0.05$.

RESULTS

Growth studies

Insulin and IGFs have been implicated in modulating renal proximal tubule cell growth and function in vivo. In the first series of experiments, we compared the growth responsiveness of primary rabbit kidney proximal tubule cells to insulin (5×10^{-10} M), IGF-I (5×10^{-10} M) and IGF-II (5×10^{-10} M). Fig. 1

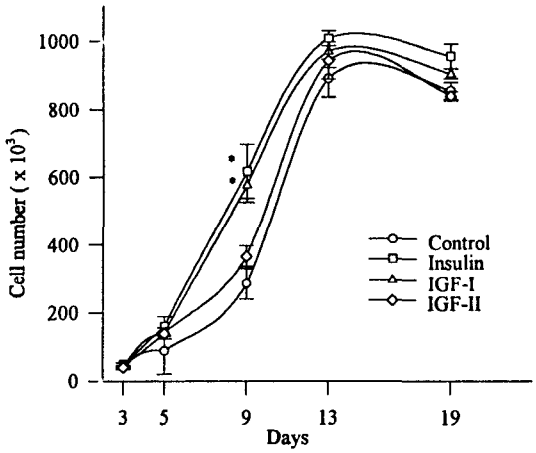

*Fig. 1. Effect of insulin, IGF-I, and IGF-II on renal proximal tubule cell growth. Primary proximal tubule cells were grown in DME/F12 supplemented with transferrin (5 μg/ml) and hydrocortisone only (5×10^{-8} M) (control) or in addition, with either insulin (5×10^{-10} M), IGF-I (5×10^{-10} M), or IGF-II (5×10^{-10} M). The medium was changed every 3 days. During this time, cell counts were determined on days 3, 5, 9, 13 and 19 from triplicate culture plates using a Coulter Model ZF particle counter. Values are the means ± S.E. from five independent determinations (n=15). *, $p<0.05$ vs. control and IGF-II-treated cells.*

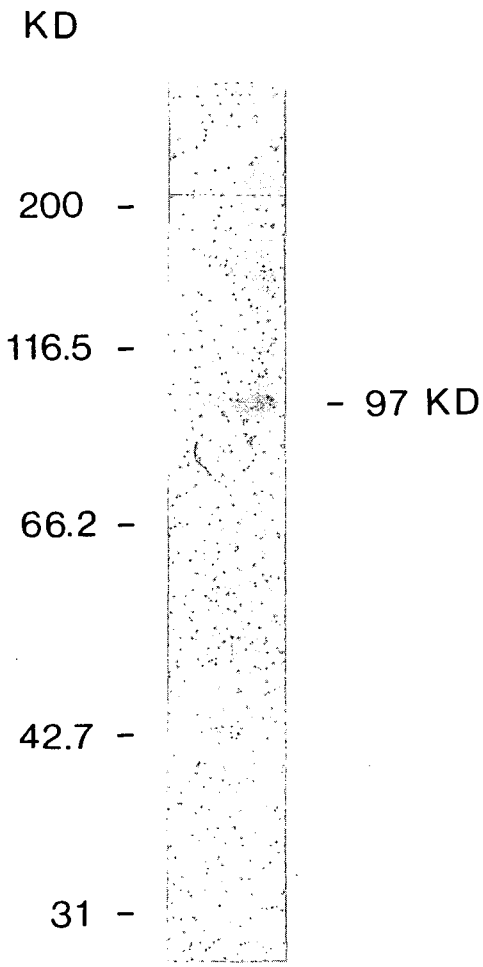

Fig. 2. Autoradiogram illustrating the ^{32}P labelling of the 97 KD insulin or/and IGF-I receptor subunit in cultured rabbit kidney proximal tubule cells.

depicts the time course for growth of renal proximal tubule cells in culture. Cell numbers increased logarithmically from days 5 to 13 followed by an apparent decline over the next 6 days. On day 9, insulin (216.47±28.20%) and IGF-I (202.46±18.36%) had a significant stimulatory effect on growth compared with control (100.00±15.84%) or IGF-II-treated cells (128.65±10.46%) ($p<0.05$). However, on other days, growth stimulatory effects of insulin or IGF-I were not significant as compared with control or IGF- II-treated cells. These observations are consistent with the presence of insulin and IGF-I receptors on the primary cultured cell membrane.

Identification of insulin and IGF receptor

WGA agarose chromatography was employed to examine whether insulin and IGF receptors are present in cultured primary proximal tubule cells. The interaction of insulin and IGF-I with their specific receptors results in the tyrosine phosphorylation of the β-subunit of the receptors, initiating a sequence of protein phosphorylation. Insulin was observed to stimulate the phosphorylation of a 97 KD protein (Fig. 2). This observation was consistent in the presence of an insulin receptor in the primary cultured cells (Czech, 1985). It is difficult to determine whether this band represents an insulin receptor and/or IGF-I receptor based upon this data. Conceivably, the β-subunit of the IGF-I receptor may be the phosphorylated protein in the WGA eluates from monolayers treated with higher insulin dosages, as well as with IGF-I. The band corresponds exactly to the position of the β-subunit of the insulin receptor in similar gels in which the receptor subunits are labelled either biosynthetically with amino acids or sugars, or by surface labelling.

Regulation of the expression of apical membrane proteins in primary cultured cells

Alkaline phosphatase exhibited markedly decreased activity from day 5 (Fig. 3A). Although the rate of decline diminished as cells because confluent, there was a consistent decline from day 5 through day 9 in culture. Fig. 3B illustrates the leucine aminopeptidase activities. The decrease in leucine aminopeptidase activity from day 5 to 9, was larger than from day 9 to 13. γ-glutamyl transpeptidase, an enzyme of critical importance in conserving and maintaining normal levels of glutathione within proximal tubule cells, was measured as a function of time in culture (Shmida et al, 1982). Cells exhibited a 45% reduction in γ-glutamyl transpepti-

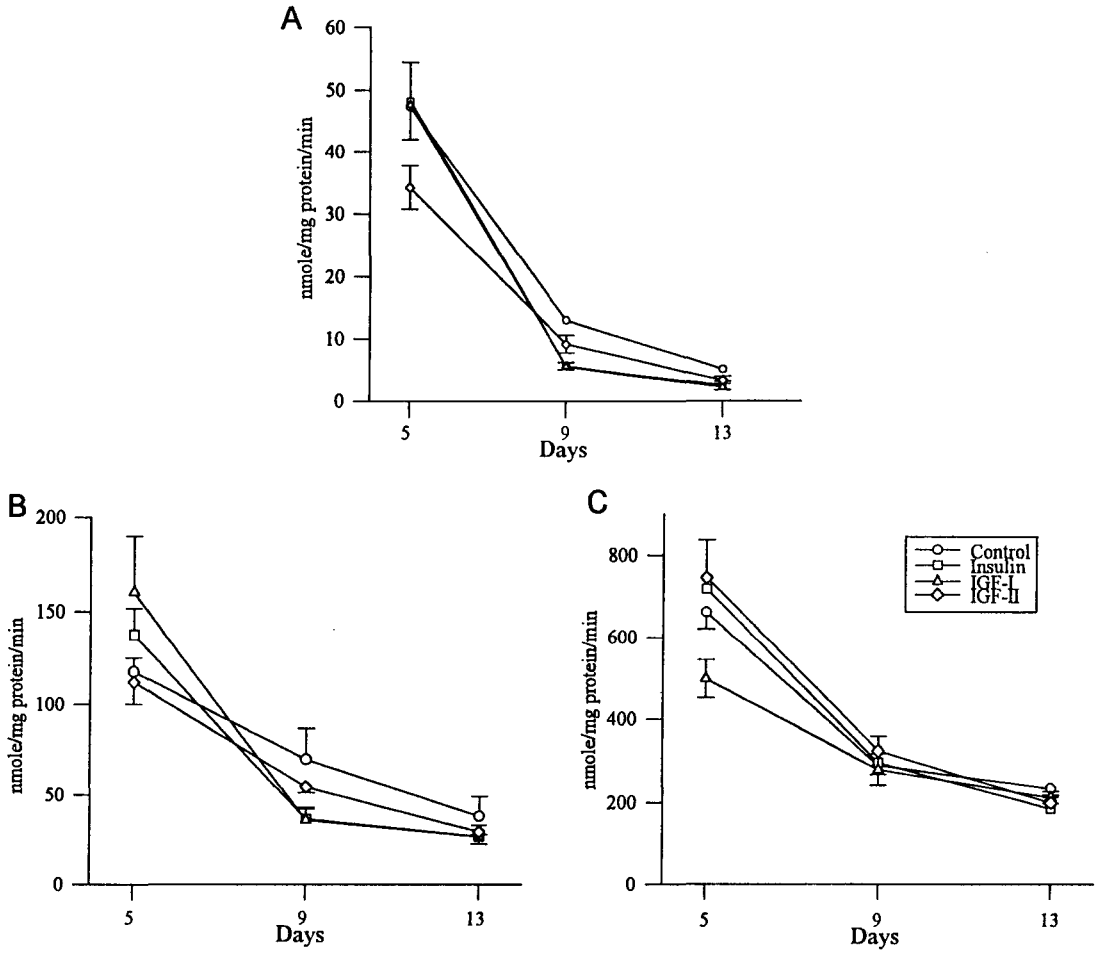

Fig. 3. Time course for the effect of insulin, IGF-I, and IGF-II on brush border marker enzymes activities over time. Primary proximal tubule cells were grown in DME/F12 supplemented with transferrin (5 μg/ml) and hydrocortisone only (5×10^{-8} M) (control) or in addition, with either insulin(5×10^{-10} M), IGF-I (5×10^{-10} M), or IGF-II (5×10^{-10} M). The medium was changed every 3 days. Alkaline phosphatase activity(A) was determined by measuring the hydrolysis of p-nitrophenyl phosphate, leucine aminopeptidase activity(B) was determined by measuring the hydrolysis of L-leucine-p-nitroanilide, γ-glutamyl transpeptidase activity(C) was measured by assaying the release of p-nitroanilide from L-γ-glutamyl-p-nitroanilide. See Materials and Methods for the details of these assays. Enzyme activity was determined on days 5, 9, and 13 in triplicate dishes. Values are the means±S.E of three independent determinations (n=9).

dase activity by day 9 and a further reduction of 71% by day 13 in culture (Fig. 3C).

Membrane transport studies

As in shown Fig. 4, α-MG uptake was significantly increased in proximal tubule cells incubated with insulin (26.20±0.25) or IGF-I (24.73±0.97) when compared with control (21.63±0.91) or IGF-II (20.84±0.96 pmole/mg protein/min) ($p<0.05$). ^{32}Pi uptake was significantly enhanced in proximal tubule cells incubated with insulin (186.70 ±3.11), IGF-I (194.73±1.16) when compared with

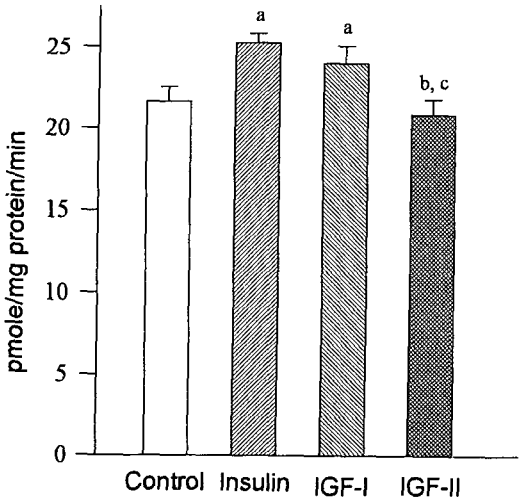

Fig. 4. Effect of insulin, IGF-I, and IGF-II on ^{14}C α-MG uptake. Primary proximal tubule cells were grown in DME/F12 supplemented with transferrin (5 μg/ml) and hydrocortisone only (5×10^{-8} M) (control) or in addition, with either insulin (5×10^{-10} M), IGF-I (5×10^{-10} M), or IGF-II (5×10^{-10} M). Intact monolayers were then utilized for uptake studies. Uptake were performed with 1mM α-MG and ^{14}C α-MG (0.5 μci/ml) at 37℃ for 30 mins. Uptake determinations were corrected for zero time uptake, and standardized with respect to protein as described in Material and Methods. Values are the means ±S.E. of three independent determinations (n=9). a, P<0.05 vs. control; b, P<0.05 vs. insulin; c, P<0.05 vs. IGF-I

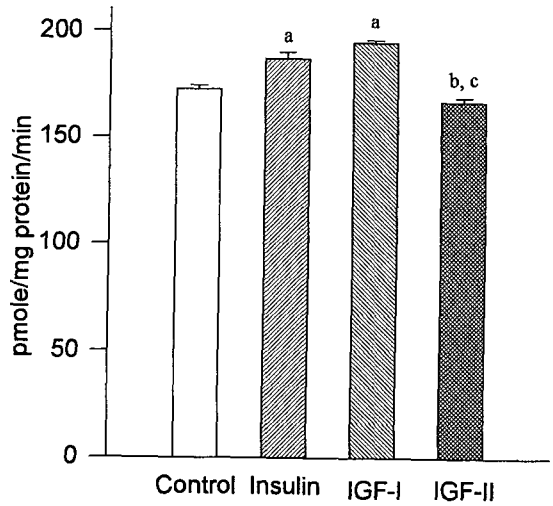

Fig. 5. Effect of insulin, IGF-I, and IGF-II on ^{32}Pi uptake. Primary rabbit kidney proximal tubule cell cultures were prepared as described in α-MG uptake study. Pi uptake studies were conducted with uptake buffer containing ^{32}Pi (0.5 μCi/ml) for 30 minutes at 37 ℃. Uptake determinations were corrected for zero time uptake, and standardized with respect to protein as described in Material and Methods. Results are means ± S.E. of three independent determinations (n=9). a, P<0.05 vs. control; b, P<0.05 vs. insulin; c, P<0.05 vs. IGF-I

control (172.65±1.65) or IGF-II-treated cells (166.75±2.09 pmole/mg protein/min) ($p < 0.05$) (Fig. 5). The sodium independent uptake of Pi, measured in the presence of choline chloride, was only 4~6% of that determined in sodium chloride-containing solution. It was not significantly modified by preincubation of cells with insulin or IGFs. The uptake of ^{22}Na into the primary proximal tubule cells were examined in ouabain-treated primary cell cultures. ^{22}Na uptake was significantly increased in proximal tubule cells incubated with insulin (51.26±3.29) or IGF-I (49.77±2.84), compared with control (37.32±3.80) or IGF-II-treated cells (37.88±1.59 pmole/mg protein/ min) ($p<0.05$) (Fig. 6). ^{86}Rb uptake was significantly increased in proximal tubule cells incubated with insulin (43.68±1.86), IGF-I (32.66±1.04), or IGF-II (35.06±0.62) when compared with control (26.61±1.11 pmole/mg protein/min) ($p<0.05$) (Fig. 7).

DISCUSSION

Kidney growth is regulated by various hormones and growth factors in this regard (IGFs, TGF, FGF and PDGF). Insulin and IGF-I are especially important (Fine & Norman, 1989). The effect upon DNA synthesis of a high concentration of insulin (0.2~1.0 μg/ml) could be achieved with a low concentration of IGF-I (10~20 ng/ml) (Zhang et al, 1991). Insulin stimulated primary proximal tubule cell growth at a concentration of 1×10^{-8} M and this

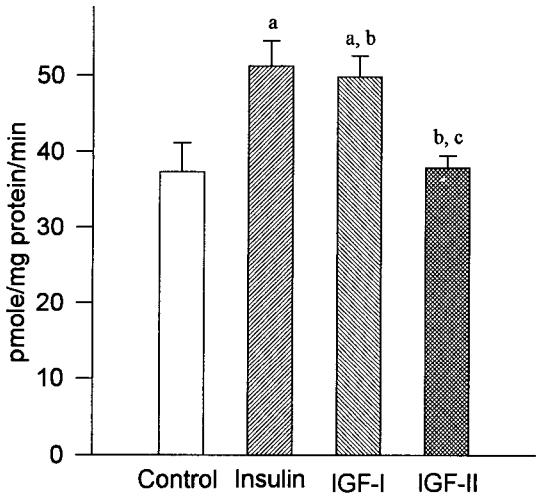

Fig. 6. Effect of insulin, IGF-I, and IGF-II on ^{22}Na uptake. Primary rabbit kidney proximal tubule cell cultures were prepared as described in α-MG uptake study. ^{22}Na uptake studies were conducted with uptake buffer containing ^{22}Na (0.25 μCi/ml) for 30 minutes at 37℃. Uptake determinations were corrected for zero time uptake, and standardized with respect to protein as described in Material and Methods. Results are means ± S.E. of three independent determinations (n=9). a, P<0.05 vs. control; b, P<0.05 vs. insulin; c, P<0.05 vs. IGF-I.

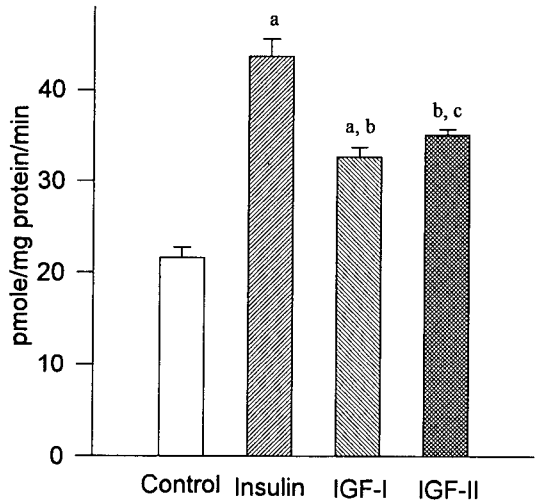

Fig. 7. Effect of insulin, IGF-I, and IGF-II on ^{86}Rb uptake. Primary rabbit kidney proximal tubule cell cultures were prepared as described in α-MG uptake study. ^{86}Rb uptake studies were conducted with uptake buffer containing ^{86}Rb (1 μCi/ml) for 30 minutes at 37℃. Uptake determinations were corrected for zero time uptake, and standardized with respect to protein as described in Material and Methods. Results are means ± S.E. of three independent determinations (n=9). a, P<0.05 vs. control; b, P<0.05 vs. insulin.

growth-stimulatory effect can be mediated by the IGF-I or IGF-II receptor. The growth stimulatory effect of insulin was dose-dependent and reached a peak at 1×10^{-5} M. Primary proximal tubule cell growth was also stimulated by 25 ng/ml IGF-I (3.3×10^{-9} M) and by 25 ng/ml IGF-II (2.5×10^{-9} M) (Wang & Taub, 1991). This growth stimulatory effect was significantly less than the effect which could be obtained with 5 μg/ml insulin (Wang & Taub, 1991). Our results show that insulin and IGF-I had a significant growth stimulatory effect at 5×10^{-10} M. IGF-II (5×10^{-10} M) did not stimulate significant cell growth. These observations are consistent with the presence of insulin and IGF-I receptors on the primary cultured proximal tubule cells. Previously, evidence has been presented for the existence of receptors for insulin, IGF-I, and IGF-II on the basolateral membrane of canine renal proximal tubules. Receptors for insulin were found to be at a seven-fold higher density in basolateral than in apical membranes. Receptors for IGF-I were found to be primarily localized in the basolateral membranes, whereas IGF-II receptors were found to be distributed more symmetrically (Hammerman, 1985). The present study demonstrates that insulin specifically stimulates the phosphorylation of a 97 KD protein. This protein has been tentatively identified as the β-subunit of the insulin receptor. Since the biological effects of insulin appear to be initiated by the interaction of the hormone with its receptors, the phosphorylation of one of the subunits of the insulin and/or IGF-I receptor may have

important physiological significance.

Renal hypertrophy in vivo is achieved by an increase in protein content per cell and an increase in cell size with minimal hyperplasia. Hypertrophied renal tubular cells remain quiescent and demonstrate an increase in transcellular transport rates. This situation was simulated in vitro by exposing a confluent, quiescent primary culture of rabbit proximal tubular cells to insulin (Fine et al, 1985). Insulin stimulates electrogenic sodium transport by rapidly activating amiloride-sensitive sodium channels in the apical cell membrane, in a manner similar to the action of vasopressin (Rodriguez-Commes, 1994). IGF-I stimulates vectorial Na^+ transport in a classical model of the mammalian distal nephron, the toad urinary bladder. Net mucosal to serosal Na^+ flux is stimulated by concentrations of IGF-I as low as 0.1 nM, and the response is maximal at 10 nM. Na^+ transport increases within minutes of the serosal addition of IGF-I, reaches a maximum in 2~3 hours, and is sustained for at least 5 hours. Neither the initial nor the sustained response to IGF-I is dependent on any new protein synthesis (Blazer-Yost & Cox, 1988). The cellular action of insulin is dependent, at least in part, on tyrosine kinase activation, probably in the proximal portion of the signal transduction pathway, and requires an increase in intracellular calcium to induce an apical membrane conductance for sodium (Rodriguez-Commes, 1994). The present study demonstrates that ^{22}Na uptake was significantly increased in proximal tubule cells incubated with insulin (5×10^{-10} M) or IGF-I (5×10^{-10} M) when compared with control or IGF-II-treated cells (5×10^{-10} M). Our findings are not consistent with IGF-II (10^{-6} M) causing stimulation of Na^+, H^+ exchange across the plasma membrane of the renal proximal tubule cells (Mellas et al, 1986). This activation has been postulated to signal the metabolic changes that result in cellular growth.

Previous studies have indicated that renal glucose production and utilization account for approximately 30% of glucose turnover in postabsorptive dogs. Physiological hyperinsulinemia suppresses renal glucose production and stimulates renal glucose uptake by approximately 75% (Cersosimo et al, 1994). In this study, α-MG uptake was significantly increased in proximal tubule cells incubated with insulin (5×10^{-10} M) or IGF-I (5×10^{-10} M) when compared with control or IGF-II-treated cells (5×10^{-10} M). The present data would suggest that insulin and IGF-I could play an important role in the control of the renal glucose transport in relation to growth. Quigley and Baum (1991) studied the issue of whether growth hormone or IGF-I affects proximal tubular Pi transport. Growth hormone had no effect on proximal tubular cell transport while IGF-I directly stimulated phosphate transport in rabbit proximal tubule. The stimulation of Pi transport by IGF-I is much greater when presented to the apical membrane than when presented to the basolateral membrane suggesting that apical receptors may play an important role in the regulation of phosphate transport in the proximal tubule. IGF-I (5×10^{-11} M) had a significantly stimulatory effect on phosphate transport. In this study, insulin significantly increased the Pi transport. In vivo, the most important regulatory factors influencing the transepithelial rate of Pi transport from the tubular lumen to blood, such as parathyroid hormone (PTH) or environmental Pi, affect the Vmax of the Pi transport system localized in the brush border membrane of the proximal tubule. Therefore, the present study suggests that IGF-I could be an important mediator of the effect of growth hormone on the renal handling of Pi, as observed in several physiological and pathological conditions. Since at steady state the tubular reabsorption of Pi is the most important regulator of the plasma level of Pi, the present data would also suggest that IGF-I plays a critical role in the control of the renal transport of Pi in relation to growth. IGF-I effect on Pi transport was mediated by the type I IGF receptor which is structurally similar to insulin receptors in

established opossum kidney (OK) cell line. Ligand binding to this class of receptor activates an intrinsic tyrosine kinase leading to the phosphorylation of tyrosine residues on both the receptor itself and other cellular proteins. Therefore protein tyrosine phosphorylation could be implicated in the regulation of renal cellular Pi transport by IGF-I (Caverzasio & Bonjour, 1992). Also in vitro experiments on renal epithelial cells indicate that IGF-I selectively enhances Pi transport across the plasma membrane via a cascade of events that requires the de novo synthesis of protein (Caverzasio & Bonjour, 1989). The effect of IGF-II is quite different from that of IGF-I.

Na^+ enters the cell at the apical(mucosal) surface via a variety of transport systems and is universally pumped out of the cell by the Na,K-ATPase on the basolateral membrane. Entry of nutrients via solute/Na symport can also occur at the basolateral membrane. The activity of the basolaterally-located Na, K-ATPase is increased in the proximal tubule of the remnant kidney. In vitro, proximal tubular hypertrophy is also associated with enhanced activity of the basolateral Na,K-ATPases. Na,K-ATPase normally operates below its Vmax with respect to intracellular Na concentration at a relatively constant level. Following an influx of Na^+, a parallel efflux of Na^+ is achieved by increasing the rate of transport of existing pumps. When Na^+ influx is sustained, the cell adapts by inserting additional transporters into the membrane, each of which presumably operates close to the original rate of transport. Cable analysis in microperfused rabbit proximal tubule also demonstrated a rise in basolateral membrane conductance with increased sodium transport, which was later found to occur in parallel with augmented basolateral potassium transference number(t_K) (Tsuchiya et al, 1992). Our results indicate that insulin and IGF-I cause a large increase in the Na-dependent transport systems of brush border membrane and that the resulting Na^+ influx increases the intracellular Na^+ concentration, thus activating the internal Na^+ transport sites of the Na,K-ATPase. Therefore, the Na^+ influx mediates the insulin- and IGF-I-induced stimulation of membrane Na,K-ATPase transport activity. Although IGF-II did not stimulate Na-dependent transport system of brush border membrane, Na,K-ATPase activity was increased. Therefore, further studies will be necessary to understand a role for IGF-II in renal transport functions. In conclusion, insulin (5×10^{-10} M) and IGF-I (5×10^{-10} M) exert stimulatory effects on growth and membrane transporter (glucose, Na, Pi, and Na-pump) activities in primary cultured rabbit kidney proximal tubule cells. IGF-II (5×10^{-10} M) had no effects on cell growth and membrane transporter(glucose, Na and Pi) activities.

REFERENCES

Blazer-Yost BL & Cox M (1988) Insulin-like growth factor I stimulates renal epithelial Na^+ transport. *Am J Physiol* **255,** C413-C417

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72,** 248-254

Brenner BM (1985) Nephron adaptation to renal injury or ablation. *Am J Physiol* **249,** F324-F337

Caverzasio J & Bonjour JP (1989) Insulin-like growth factor I stimulates Na-dependent Pi transport in cultured kidney cells. *Am J Physiol* **257,** F712-F717

Caverzasio J & Bonjour JP (1992) Tyrosine phosphorylation selectively regulates renal cellular phosphate transport. Evidence that it mediates the stimulatory effect of insulin-like growth factor-I. *Endo* **130,** 373- 380

Cersosimo E, Judd RL & Miles JM (1994) Insulin regulation of renal glucose metabolism in concious dogs. *J Clin Invest* **93(6),** 2584-2589

Chung SD, Alavi N, Livingston D, Hiller S & Taub M (1982) Characterization of primary rabbit kidney cultures that express proximal tubule functions in a hormonally defined medium. *J Cell Biol* **95,** 118-126

Cook WF & Pickering GW (1958) A rapid method for

seperating glomeruli from rabbit kidney. *Nature* **182,** 1103-1104

Cross M & Dexter TM (1991) Growth factors in development transformation and tumorigenesis. *Cell* **64,** 271-280

Czech MP (1985) The nature and regulation of the insulin receptor. Structure and function. *Annu Rev Physiol* **47,** 357-381

Fine LG, Badie-Dezfooly B, Lowe AG, Hamzeh A, Wells J & Salehmoghaddam S (1985) Stimulation of Na/H antiport is an early event in hypertrophy of renal proximal tubular cells. *Proc Natl Acad Sci USA* **82(6),** 1736-1740

Fine LG & Norman J (1989) Cellular events in renal hypertrophy. *Annu Rev Physiol* **51,** 19-32

Ham RG (1982) Importance of the basal nutrient medium in the design of hormonally defined media. *Cold Spring Harb Conf Cell Prolif* **9,** 39-60

Hammerman MR (1985) Interaction of insulin with the renal proximal tubular cell. *Am J Physiol* **249,** F1-F11

Kahn CR (1985) The molecular mechanism of insulin action. *Annu Rev Med* **36,** 429-451

Linhardt K & Walter K (1963) Phosphatases. In: Methods in Enzymatic Analysis. Bermeyer, ed. Academic press, New York, pp783-785

Mellas J, Gavin III JR & Hammerman MR (1986) Multiplication-stimulation activity-induced alkalization of canine renal proximal tubular cells. *J Biol Chem* **26,** 14437-14442

Mogensen CE (1987) Microalbuminuria as a predictor of clinical diabetic nephropathy. *Kidney Int* **33,** 855-867

Quigley R & Baum M (1991) Effects of growth hormone and insulin-like growth factor I on rabbit proximal convoluted tubule transport. *Am Soci Clin Invest* **88,** 368-374

Rechler MM & Nissley SD (1985) Receptors for insulin-like growth factors. In: *Receptors for polypeptide hormones.* B.I. Posner, ed. Dekker, Ny., 227-297

Rodriguez-Commes J, Isales C, Kalghati L, Gasalla-Herraiz J & Hayslett JP (1994) Mechanism of insulin-stimulated electrogenic sodium transport. *Kidney International* **46,** 666-674

Sakhrani LM, Behnam BD, Walter T, Nabil M, Andrew GL & Leon GF (1984) Transport and metabolism of glucose by renal proximal tubular cells in primary culture. *Am J Physiol* **246,** F757-F764

Sara VR & Hall K (1990) Insulin-like growth factors and their binding proteins. *Physiol Rev* **70,** 591-614

Shimada H, Endou H & Sakai F (1982) Distribution of gammaglutamyl transpeptidase and glutaminase isoenzymes in the rabbit single nephron. *Japan J Pharmacol* **32,** 121-129

Taub M (1990) The use of defined media in cell and tissue culture. *Toxico in vitro* **4,** 213

Tate SS & Meister A (1974) Interaction γ-glutamyl transpeptidase with amino acids, dipeptides, and derivatives and analogues of glutathione. *J Biol Chem* **249,** 7593-7602

Tsuchiya K, Wang W, Giebisch G & Welling PA (1992) ATP is a coupling modulator of parallel Na,K-ATPase-K-channel activity in the renal proximal tubule. *Proc Natl Acad Sci USA* **89,** 6418-6422

Wang Y & Taub M (1991) 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* **147,** 374-382

Zhang G, Ichimura T, Wallin A, Kan M & Stevens JL (1991) Regulation of rat proximal tubule epithelial cell growth by fibroblast growth factors insulin-like growth factor-I and transforming growth factor-β, and analysis of fibroblast growth factors in rat kidney. *J Cellular Physiol* **148,** 295-305