Role of insulin and IGF1 receptors in proliferation of cultured renal proximal tubule cells

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We have used a murine proximal tubule cell line (MCT cells) to determine the presence and binding characteristics of insulin and IGF1 receptors and to correlate these parameters with the concentration–response relationships for ligand-induced cellular proliferation. Separate insulin and IGF1 receptors were identified by equilibrium binding assays. Half-maximal displacement of either peptide occurred at 3–10 nM; crossover binding to the alternate receptor occurred with a 10– to 100-fold lower affinity. Peptide effects on cellular proliferation were determined by measuring [3H]thymidine incorporation. Both insulin and IGF1 stimulate thymidine incorporation in a dose-dependent manner with similar increases above the basal level. The estimated half-maximal stimulation (EC50) occurred at 4 nM for IGF1 and 8 nM for insulin. A comparison of the receptor binding affinities with the dose–response relationships for [3H]thymidine incorporation reveals that each growth factor appears to be exerting its effect via binding to its own receptor. Therefore, in this cell line, physiologic concentrations of either insulin or IGF1 can modulate cellular growth. To our knowledge this is the first demonstration of a mitogenic effect which may be modulated by ligand binding to the insulin receptor in proximal tubule epithelia.

Introduction

Insulin and insulin-like growth factor 1 (IGF1) are structurally related peptides whose functions are thought to have diverged during evolution. In general, insulin functions as an endocrine hormone which is critical in the control of carbohydrate, protein and lipid homeostasis. In contrast, IGF1 is classically considered a regulator of cellular growth and differentiation. Interestingly, however, in certain tissues the two peptides can also mediate similar cellular effects such as the stimulation of substrate and ion transport [1–3] and cellular proliferation [4–8].

Each peptide binds with high affinity to distinct receptors and it is this specific ligand-receptor interaction which initiates the physiological effects of the peptide. Overall the insulin and IGF1 receptors share remarkable homology both in primary structure and in organization. Not surprisingly, therefore, insulin and IGF1 exhibit crossover binding to the heterologous receptor, however, crossover binding occurs with a significant (10–100-fold) decrease in affinity [3,9–11]. The crossover binding phenomenon is often evoked to explain an apparent crossover in physiological effect. For example, insulin is a required component for growth of mammalian cells which are maintained in tissue culture using serum-free, defined media. Typically the growth-stimulatory effects of insulin are achieved at pharmacologic concentrations (1 μg/ml or greater) [12–18]. In contrast, the effects of IGF1 on cellular proliferation are often manifested at much lower (i.e., physiological) concentrations [2,9,11]. These findings suggest that the growth stimulatory effects of both insulin and IGF1 are manifested via binding to the IGF1 receptor. In contrast, several recent studies have suggested that in certain fibroblastic cell types both insulin and IGF1 can modulate cell growth via binding to their homologous receptors [5].

Insulin and IGF1 receptors co-exist on the same cells in many tissues. In the mammalian kidney, both
receptors have been demonstrated on glomerular mesangial cells [2] and proximal tubular epithelial cells [19,20]. In addition, in renal tissue, both insulin and IGF1 have been demonstrated to stimulate solute and fluid reabsorption [3,21-23] as well as promote growth in cell culture [24,25]. The co-existence of two receptors, each capable of binding either ligand, necessitates a careful correlation of binding affinities with physiological effect before a functional response can be ascribed to activation of either receptor.

In this study we used a proximal tubule cell line to test whether cellular proliferation is modulated by ligand binding to insulin and/or IGF1 receptors. The mouse cortical tubule (MCT) line was originally derived from microdissected proximal tubule segments from normal mice, immunoselected into a homogeneous cell population and stabilized in long-term culture by SV40 transformation [26]. This is a well characterized cell line which has proven to be a good model for polyacrylamide gel electrophoresis (PAGE) were purchased from Bio-Rad (Richmond CA). All other reagents were highest quality available and were obtained from commercial sources.

Cell Culture. The isolation and characterization of the MCT cell line were provided previously [26]. Multiple morphological and functional properties of this cell line are consistent with those of differentiated proximal tubule epithelial cells [26-28]. Briefly, the cells stain positive for cytokeratin and alkaline phosphatase and demonstrate apical microvilli and lateral tight junctions by electron microscopy. The cells respond to treatment with parathyroid hormone by an increase in cyclic AMP content [29]. In the studies described here, the culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's-F12 (HF12), supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine and 5 μg/ml human transferrin. The cells were passaged every 48-72 h and were carried in culture medium supplemented with 10% inactivated fetal calf serum. Cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

**Materials and Methods**

Media for cell culture were obtained from Gibco (Grand Island, NY). Recombinant human IGF1 (THR-59) was purchased from AMGen Biologicals (Thousand Oaks, CA). IGF1 stock solutions (μM) were prepared in 0.01 M HCl and stored at 5°C. IGF1 was iodinated to a specific activity of 1600-2300 Ci/mmol using a modified chloramine-T procedure [30]. Porcine insulin was kindly provided by Lilly Research (Eli Lilly, Indianapolis, IN). Insulin stock solutions (μM) were prepared in 0.05 M HCl and stored at 5°C. 125I-labeled porcine insulin, 2200 Ci/μmol, was purchased from New England Nuclear (Boston, MA). [3H]Thymidine, 5 Ci/μmol, was obtained from Amersham (Arlington Heights, IL). Disuccinimidyl suberate was obtained from Pierce Chemical (Rockford, IL). Disuccinimidyl suberate stock (5·10⁻⁵ M) was prepared in DMSO immediately before use. Electrophoresis grade reagents for polyacrylamide gel electrophoresis (PAGE) were purchased from Bio-Rad (Richmond CA). All other reagents were highest quality available and were obtained from commercial sources.

**Competitive binding studies.** In these experiments where competitive binding of insulin and IGF1 to MCT cells was determined, the cells were seeded onto 24 well tissue culture plates (density = 10⁵ cells/well). The cells were maintained in normal media for 24 h, then placed in serum-free media for 24-48 h prior to assay. Confluent cellular monolayers were washed and incubated at room temperature for 90 min in binding buffer (serum-free medium, 0.2% BSA, 25 mM Hepes, pH 7.4) containing (3-4)·10⁻¹⁰ M [125I]insulin or [125I]IGF1 and unlabeled competing peptides as indicated. At the end of the incubation period, the cells were washed three times with ice-cold phosphate-buffered saline and solubilized in 1 M NaOH, 0.1% Triton X-100. Aliquots of the solubilized cellular material were counted in a gamma scintillation counter. Nonspecific binding was defined as the number of counts bound in the presence of 10⁻⁷ M unlabeled peptide and this value was subtracted from all samples. In every experiment duplicate or triplicate assay wells were performed for each concentration. Nonspecific binding was less than 20% of total binding.

To further define the binding specificities of the receptor subunits, cells were incubated in binding buffer with (3-4)·10⁻¹⁰ M [125I]IGF1 in the presence or absence of unlabeled competing peptides. After a 90 min incubation period at room temperature, the cells were washed twice with cross-linking buffer (0.1 M Hepes, 0.12 M NaCl, 5 mM KCl, 1.2 mM MgSO₄, 8 mM glucose and 10 mg/ml BSA, pH 8.0). The bound peptide was covalently cross-linked to the receptor by a 15 min incubation with 0.1 M freshly prepared disuccinimidyl suberate in crosslinking buffer. The reaction was terminated by the addition of 0.1 M Tris (pH 8.8). The cells were washed and solubilized in SDS-PAGE buffer (3% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.05 M Tris, pH 6.8). The solubilized samples were stored at -20°C.

**PAGE.** SDS-PAGE was performed on 15 × 13 cm slab gels with a linear 5–15% acrylamide gradient and a 4% stacking gel. The aliquots applied to the gel were matched for cell number. The preparations were subjected to electrophoresis under constant current conditions (30 mA/gel for 20 min followed by 25 mA/gel...
for 4 h). The gels were then fixed, dried and exposed to Kodak XAR-5 film in cassettes containing X-Omatic fine intensifying screens (Eastman Kodak, Rochester, NY).

[^3]H/Thymidine incorporation studies. Cells in culture were released by trypsin-EDTA, washed twice in serum-free medium and adjusted to a final concentration of 1 × 10⁶ cells/ml. 10,000 cells were subcultured in flat-bottom 96-microtiter wells, each containing 200 µl serum-free medium. After 48–72 h of quiescence the media were removed and replaced with fresh serum-free medium without or with the addition of various concentrations of insulin or IGFI as noted in the text. All growth studies were performed on rested cells at approx. 70% subconfluence. Cells were allowed to grow for an additional 24 h. During the last 6 h of culture, the cells were pulsed with [^3]H]thymidine (1 µCi/well). The media were removed and the cells were released with trypsin-EDTA for subsequent lysis and collection with a cell harvester (Brandel, Gaithersburg, MD) onto glass-microfiber filter paper (934-AH, Whatman, U.K.). The incorporated radioactivity in cellular nuclei was assayed by counting filters in scintillation cocktail.

[^3]H]Thymidine incorporation into cell DNA was taken as an index of cell proliferation and was expressed in counts per min per well. Each experimental condition was tested in four to six replicate wells and the mean was taken to represent an individual experiment. In parallel experiments, measurements of cell number were performed in order to verify that the observed increase in thymidine incorporation was associated with an increase in cell number.

Statistics. The data are presented as means ± S.E. with n indicating the number of different experiments. Comparisons were performed using Student’s t-test for paired or unpaired values as appropriate; values of P < 0.05 were considered significant. Binding studies were analyzed graphically.

Results

Competitive binding studies

Equilibrium binding assays were performed to determine the presence and specificity of insulin and IGFI receptors on MCT cells. Fig. 1 illustrates the specificity of human recombinant IGFI binding: half maximal displacement of [¹²⁵]IGFI occurs at an unlabeled IGFI concentration between 3 and 10 nM. Insulin was an effective competitive ligand only at approx. 100-fold greater concentrations. Fig. 2 illustrates the specificity of binding of porcine insulin: half maximal displacement of [¹²⁵]insulin occurs at an unlabeled insulin concentration between 3 and 10 nM. IGFI also displaced insulin but only at approx. 10–30-fold greater concentrations. Thus, the affinity of insulin for the insulin receptor is very similar to that of IGFI for the IGFI receptor; crossovers binding to each receptor does occur but only with a 10- to 100-fold lower affinity.

The relative number of insulin and IGFI receptors appears to differ in MCT cells. Using our standard incubation conditions with approximately equal concentrations of peptides which had been iodinated to a
Fig. 3. [\(^{125}\)I]IGF1 binding to mouse proximal tubule cell receptor subunits in the presence of various concentrations of unlabeled IGF1 and insulin. To show that receptor specificity was manifested at the level of the \(\alpha\) subunit of the IGF1 receptor, selected samples from the competitive binding curves were treated with the crosslinking reagent disuccinimidyl suberate to covalently couple bound ligand to the receptor. The crosslinked samples were solubilized in a reducing buffer, separated by one-dimensional PAGE and visualized by autoradiography. Unlabeled competing ligands (final concentrations indicated) were present in the incubation buffer before crosslinking. The arrow indicates the position of the \(\alpha\) subunit of the receptor.

Positions of the molecular mass markers (in kDa) are indicated.

similar specific activity, 3–6% of the [\(^{125}\)I]IGF1 was specifically bound by the MCT cells; in contrast, only 0.1–0.4% of the [\(^{125}\)I]insulin was specifically bound in this cell line.

To show that the binding specificity was manifested at the level of the \(\alpha\)-subunits of the MCT receptors, in some competitive binding experiments the bound IGF1 was crosslinked to the receptor with disuccinimidyl suberate. Fig. 3 illustrates [\(^{125}\)I]IGF1 binding to MCT cells in the presence of various concentrations of unlabeled IGF1 or insulin. Maximal [\(^{125}\)I]I incorporation into proteins having an apparent molecular mass of 130 kDa is observed in samples incubated with no competing ligand or with 1 nM unlabeled IGF1. As anticipated from the competitive binding curves, 100 nM unlabeled IGF1 completely inhibited the binding of [\(^{125}\)I]IGF1, whereas 10 nM IGF1 partially inhibited binding. 100 nM unlabeled insulin only slightly inhibited the binding of [\(^{125}\)I]IGF1. The presence of low concentrations of insulin (1 and 10 nM) did not inhibit and may stimulate [\(^{125}\)I]IGF1 incorporation. The basis for this apparent increase in IGF1 binding in the presence of physiological concentrations of insulin is unknown. The diffuse high molecular mass (> 200 kDa) bound in these studies likely results from the crosslinking of \(\alpha/\beta\) and/or \(\alpha/\alpha\) receptor subunits by the divalent crosslinking reagent, disuccinimidyl suberate [2]. Due to the extremely low number of insulin receptors, we were unable to incorporate sufficient [\(^{125}\)I]insulin to analyze the [\(^{125}\)I]insulin binding curves by PAGE.

\[^{3}\text{H}]\text{Thymidine incorporation studies}\]

Peptide effects on cellular proliferation were determined by measuring [\(^{3}\text{H}\)]thymidine incorporation in the presence of various concentrations of insulin and IGF1 (Fig. 4). Both insulin and IGF1 stimulated thymidine incorporation in a dose-dependent manner with similar maximal increases above the basal level. The estimated half-maximal stimulation (EC\(_{50}\)) is 4 nM for IGF1 and 8 nM for insulin; these concentrations fall within the physiologic range of either peptide.

A maximal effect of IGF1 on cellular proliferation appears to be manifested within a narrow concentration range. Maximal [\(^{3}\text{H}\)]thymidine incorporation was achieved at 10 nM and further increases in peptide concentration result in a submaximal proliferative response (Fig. 4).

To confirm the ability of insulin to stimulate cell proliferation, parallel subconfluent cultures in 12-well plastic plates were incubated in the presence and absence of 32 nM insulin. At the end of the 48 h incubation period the control cultures contained 6.75 ± 0.36 \(\times\) 10\(^5\) cells/well while the insulin treated cultures contained 8.95 ± 0.61 \(\times\) 10\(^5\) cells/well (\(n = 5\), \(P < 0.05\)).

Fig. 4. Comparison of tritiated thymidine incorporation in response to insulin and IGF1 in proximal tubule cells. [\(^{3}\text{H}\)]Thymidine incorporation in response to various concentrations of insulin and IGF1 is expressed relative to control cultures which were incubated in serum-free media during the experimental period. The data shown represent seven separate experiments. The standard errors of the mean are indicated by vertical bars. * indicates concentrations where the responses to insulin and IGF1 are statistically different (\(P < 0.02\)).

- - - - O, insulin; •——•, IGF1.
We have also examined the proliferative response to incubation with combinations of insulin, IGF1 and epidermal growth factor (EGF) (Fig. 5). Incubation with submaximal doses of IGF1 and insulin (3.2 nM) resulted in a significantly higher stimulation of thymidine incorporation compared to that elicited by either hormone alone. In contrast, the combined treatment with maximal doses of IGF1 and insulin produced a proliferative stimulation that was not different from the response to either hormone alone. When maximal doses of either IGF1 or insulin are combined with epidermal growth factor (EGF, 10 ng/ml), the stimulation in thymidine incorporation is significantly higher than that exhibited by treatment with EGF alone.

**Discussion**

In the kidney insulin is thought to modulate metabolic and transport functions while IGF1 is considered to be a regulator of growth and differentiation [21,31,34]. Many of the studies which have given rise to this general concept have used intact organs or membranes isolated from specific areas of the kidney. While such studies have been extremely valuable in establishing the functions of various nephron segments, it is difficult to precisely determine the role of growth factors and metabolic modulators in these experimental systems. A continuous cell line derived from a defined area of the kidney tubule provides the cellular homogeneity and stability necessary to assess the contribution of various factors to the growth of a specific cell type. In this study we used the MCT cell line as a model for proximal tubule cell growth. Previous investigations have demonstrated that this cell line retains the characteristics of differentiated proximal tubule cells and provides a suitable model to investigate proliferation of renal epithelia [26–29].

While the physiological role of insulin in renal growth remains unclear, it is known that kidney cells in culture require insulin in the supporting medium. Hormone-supplemented, serum-free medium has been described for maintenance of the Madin-Darby canine kidney (MDCK) cell line [12], the LLC-PK1 epithelial cell line from pig kidney [15], and for primary cultures of canine renal epithelia [13], baby mouse kidney cells [14] and rabbit kidney epithelial cells [16,17]. IGF1 was not a component of the hormone supplements but insulin was present in micromolar concentrations. The assumption, explicit or implicit, is that at these supra-physiological concentrations insulin modulates growth and differentiation via crossover binding to IGF1 receptors. However, this assumption may not be valid for all cell types.

Physiological concentrations of either insulin or IGF1 promote cell growth and proliferation via binding to their homologous receptors in a rat osteosarcoma cell line, UMR-106-01 [8] and in Swiss 3T3 fibroblasts [6] as well as in normal skin fibroblasts in primary [4] and continuous [7] culture. In a careful study, Furlanetto et al. [5] used specific anti-receptor antibodies to show that insulin, working via an insulin receptor, was not mitogenic in two lines of human embryonic lung fibroblasts but, under identical conditions, was mitogenic in a human dermal fibroblast cell line, a human embryonic skin fibroblast line and an osteogenic sarcoma cell line. Thus, it appears that, although activation of IGF1 receptors is uniformly capable of mediating a mitogenic response, the ability of activated insulin receptors to stimulate proliferation is cell-specific.

Both insulin and IGF1 receptors have previously been demonstrated on proximal tubular epithelia [19,20,32]. In agreement with these studies, we have found that mouse proximal tubule cells in culture contain two separate receptors, each with similar displacement curves (Figs. 1 and 2). The affinity of insulin for the insulin receptor is very similar to that of IGF1 for the IGF1 receptor; crossover binding does occur but only with 10–100-fold lower affinity. The displacement curves for insulin and IGF1 binding to their specific receptors are very similar to those reported in other tissues and cell lines [2,3,11,33].
hybrid receptors have been assembled in vitro [35] and have been identified in NIH3T3 and HepG2 cells [36] and in transfected rodent cells which overexpress human insulin and IGF1 receptors [37]. Our binding curves are consistent with the existence of separate insulin and IGF1 receptor populations, however, we cannot rule out the existence of a small number of insulin/IGF1 hybrid receptors. The molecular weight of the α subunit of MCT cell IGF1 receptor is comparable to other IGF1 receptors [38] and the ligand binding specificity is manifested at the level of the α subunit (Fig. 3).

In our studies the amount of IGF1 specifically bound by the proximal tubule cells was at least an order of magnitude greater than that of insulin indicating a relatively higher number of IGF1 receptors. This is similar to isolated rat renal tubules [33] and cultured rat renal mesangial cells where specific binding of [125I]IGF1 was >200-fold higher than insulin binding [2]. In contrast, the specific binding of insulin was approx. 4-fold higher than that of IGF1 in proximal tubule basolateral membranes prepared from canine kidneys [32]. It is unknown whether the differences in magnitude of receptor expression in the proximal tubule represents natural differences in species, heterogeneity in cell type found in intact kidney or changes in receptor expression during the process of cellular immortalization.

Both insulin and IGF1 stimulate [3H]thymidine incorporation in proximal tubule cells with an EC50 between 3 and 10 nM; a maximal dose of IGF1 stimulated thymidine incorporation 2.2-fold, while a maximal concentration of insulin elicited a 2-fold stimulation. A comparison of the receptor-ligand binding affinities (Figs. 1 and 2) with the dose–response relationships for [3H]thymidine incorporation (Fig. 4) reveals that each growth factor appears to stimulate growth via binding to its own receptor. Therefore, in this proximal tubule cell line, physiologic concentrations of either insulin or IGF1 can modulate cellular growth. It is possible that the slightly higher potency and magnitude of stimulation by IGF1 is due to the higher density of the IGF1 receptor compared with that of the insulin receptor.

To our knowledge this is the first demonstration of a mitogenic effect which may be modulated by ligand binding to the insulin receptor in proximal tubule epithelia. Very little, in general, is known regarding growth effects of insulin on epithelial cells. In contrast, IGF1 has been implicated as a major growth factor in renal tissue. Renal hypertrophy is a manifestation of acromegaly [39] which is characterized by an increase in circulating IGF1 levels. In addition, in response to certain stimuli, such as the loss of renal mass, the cortical and medullary collecting duct cells of the mammalian kidney are known to synthesize IGF1 [34,40–42].

The demonstration of specific receptors for IGF1 in the proximal tubule support the concept of a paracrine ‘loop’ for IGF1 [34].

Interestingly, maximal stimulation of [3H]thymidine incorporation occurs at insulin concentrations measured at and above 32 nM, whereas IGF1 is only maximally effective over a very narrow concentration range. A similar sharp decrease in the magnitude of IGF1 stimulation of [3H]thymidine incorporation at peptide levels above 10 nM has been demonstrated in cultured rat renal mesangial cells [2]. This relatively narrow effective concentration range may be physiologically relevant. However, a complete understanding of this phenomenon is limited by imprecise measurements of effective in vivo concentrations of IGF1.

In vivo, insulin levels in the circulation vary widely and rapidly according to the metabolic requirements of the organism. Despite this fluctuation, the effective circulating concentration of insulin is known to be much lower than the total concentration of IGF1. IGF1, unlike insulin, is bound by several high affinity, soluble binding proteins which may modulate peptide–receptor interactions and, therefore, the effective circulating concentration is difficult to ascertain [11,31]. Also, in contrast to insulin, IGF1 is a paracrine or autocrine factor in the kidney [34].

Although the growth stimulatory responses to both insulin and IGF1 appear to be initiated by ligand binding to distinct receptors, the cascade of events that culminate in cell proliferation may overlap at a point subsequent to ligand-receptor binding. The stimulatory response to a combination of maximal concentrations of both insulin and IGF1 is not statistically greater than the response to a maximal stimulation of either peptide alone (Fig. 5). This was not due to the inability of the epithelium to show a further increase in thymidine incorporation: a maximal concentration of EGF in combination with a maximal concentration of insulin stimulates MCT cells to a greater degree than either peptide alone.

A similar overlap in the intracellular pathways stimulated by insulin and IGF1 has been observed in the toad urinary bladder, a model of the mammalian distal nephron [3]. In this high resistance epithelium insulin or IGF1 binding to distinct receptors stimulates transepithelial Na+ flux. The natriferic pathways activated by insulin and IGF1 appear to converge subsequent to ligand-receptor binding but prior to the final transport step(s). The generality of this post-receptor convergence in intracellular pathways remains to be tested.

The role of IGF1 as a renal growth factor has been delineated by various studies; the role of insulin in renal growth remains more elusive. The current data suggest that insulin may be an important factor in renal cell proliferation. This peptide may play a role in repair and recovery after insult or may be important as
a maintenance factor during normal epithelial turnover and regeneration; however, the exact function of insulin-stimulated proliferation remains to be defined.

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