Binding and Degradation of ^{125}I -Insulin by Renal Glomeruli and Tubules Isolated from Rats

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Summary. Isolated rat renal glomeruli and tubules were shown to exhibit specific binding of ^{125}I -insulin and enzymatic degradation of the hormone. Binding to both renal fractions reached a plateau by 1 h at 22 °C and increased linearly with increasing protein concentrations. Binding was inhibited in both preparations by insulin and its analogues in the order of relative potency: insulin > despentapeptide insulin > proinsulin, but insulin was ten times more potent in inhibiting ^{125}I -insulin binding to glomeruli than that to tubules, indicating a different affinity of receptors for the hormone in the two renal fractions (about 17 versus 210 μg unlabelled insulin/l inhibiting 50% of the ^{125}I -insulin binding to glomeruli and tubules, respectively). Bound ^{125}I -insulin dissociated at a faster rate from tubules than from glomeruli; this release was accelerated by unlabelled insulin in both renal fractions, but to a greater extent in glomeruli than in tubules. Two-thirds of the total bound material released from glomeruli was found to be intact insulin as measured by trichloroacetic acid precipitation, whereas only one-third of the material released from tubules was intact. No direct relationship between binding and degradation of ^{125}I -insulin in these renal fractions could be demonstrated, however, because of the release of proteolytic enzymes into the incubation medium resulting in almost all degradation being extracellular. Although differing in their affinity for ^{125}I -insulin the high affinity glomerular insulin receptor and the lower affinity tubular insulin receptor have characteristics similar to those of insulin receptors in insulin responsive tissues.

Key words: Insulin receptor, insulin binding, insulin degradation, kidney, renal glomeruli, renal tubules, insulin analogues.

Although insulin binding and degradation have been studied in vitro in a number of systems ranging from intact cells to isolated plasma membranes and solubilized cell fractions [1], relatively little is known about these processes in isolated renal preparations. Renal cell membranes possess both binding and degradative capacity towards insulin [2, 3], and preliminary evidence indicates that these activities are present in both glomerular and tubular fractions [4–8]. The kidney is a major site of microangiopathic complications in diabetes mellitus [9], specifically those which affect glomerular histology and function, and the role of insulin therapy in preventing or ameliorating such complications is unsettled and controversial [10–12]. The activities of several enzymes involved in the biosynthesis of renal basement membranes are increased in experimental diabetes and are restored to normal levels by careful insulin treatment [13–15], possibly indicating a direct effect of insulin on the metabolism of this extracellular matrix. Renal electrolyte balance has also been shown to be modulated by insulin levels [16, 17] suggesting an effect of the hormone on tubular transport functions.

The localization and properties of receptor and degradative sites for insulin in the renal cortex are therefore of particular interest and have been investigated using purified preparations of rat renal glomeruli and tubules isolated concurrently from kidneys without collagenase digestion.

Materials and Methods

Isolation of Renal Fractions

Isolated rat renal glomeruli and tubules were prepared from 300–400 g male Wistar rats by the following modifications of the procedure using magnetic iron oxide described previously [18–20]. The renal cortices of three to five rats were pooled for each experi-

ment and disrupted first by five up and down strokes of a loose fitting tapered Teflon pestle in a smooth glass tube and then by ten additional strokes with a tighter fitting pestle. The cortical material initially retained on the 210 μm nylon sieve was rehomogenized with the tight fitting pestle to increase the yield of renal fractions. After passage of the 210 μm nylon sieve filtrate through a 153 μm sieve to remove remaining undisrupted large aggregates of cortical material, the iron-containing glomerular and iron-free tubular fractions were caught on a 44 μm sieve and transferred to a Petri dish. The magnetic glomerular fraction remaining in the dish after repeated washing and decantation over a bar magnet was used in the experiments. The decanted material containing tubules and some glomeruli was concentrated using a 44 μm sieve and again transferred to a Petri dish and decanted over a bar magnet. This decanted material consisted almost entirely of tubules with a small number of glomeruli which could be removed by inserting a magnetic stirring bar into the dish and removing the iron-containing particles. The isolation procedure was monitored using a binocular dissecting microscope at 10–40 $\times$ magnification to follow the purifications. The final preparations were < 2% cross-contaminated with each other. All isolation steps and subsequent incubations with the renal fractions were carried out in Krebs-Ringer bicarbonate buffer (pH 7.4) which was supplemented with 1.5% bovine serum albumin (Fraction V, Sigma) for the binding and dissociation experiments.

Binding Experiments

Glomeruli and tubules were incubated in medium containing 1 μg ^{125}I -insulin/l with or without the addition of unlabelled insulin. All experiments were performed at 22 °C; tubes to measure the non-specific binding of ^{125}I -insulin to the renal fractions (defined as the binding measured in the presence of unlabelled insulin 50 mg/l) were included in each experiment. The degradation of ^{125}I -insulin as determined by the trichloroacetic acid non-precipitable counts in the supernatant fractions of the incubations was also followed in each experiment. Although degradation of ^{125}I -insulin associated with the renal pellets was not followed in these experiments, in additional experiments it was found to parallel the ratio of degraded/intact ^{125}I -insulin seen in the medium.

Separate experiments were performed to investigate the possible binding of fragments of ^{125}I -insulin produced by incubation with the renal fractions, or by digestion of ^{125}I -insulin with papain or *Streptococcus aureus* V8 protease. Although glomeruli and tubules could take up such fragments from the medium, no specific binding of these fragments was observed. In four experiments, 0.7%–2.3% of the total counts of ^{125}I -insulin degradation products in the medium were taken up by tubules and glomeruli. The extent of binding of these labelled degradation products did not exceed the levels of non-specific binding observed in parallel incubations with equal levels of intact ^{125}I -insulin in the presence of 50 mg unlabelled insulin/l.

In order to follow the time course of ^{125}I -insulin binding and degradation, glomeruli and tubules were suspended in 10–12 ml of incubation medium (glomerular protein 0.5–0.6 mg/ml; tubular protein 0.3–0.7 mg/ml) and incubated at 22 °C with shaking. Triplicate 0.25 ml aliquots of the incubation mixtures were removed at several time points and layered onto 0.5 ml of a 1:2 mixture of dinonylphthalate:dibutylphthalate oil in 1.5 ml microcentrifuge tubes. Centrifugation for 3 min in a Beckman microcentrifuge rapidly sedimented the renal fractions through the oil layer, and 0.125 ml aliquots of the upper supernatant solutions were pipetted into 0.125 ml of cold 20% trichloroacetic acid (TCA). The resulting protein precipitates were removed by centrifugation and the supernatants were counted to determine insulin degradation. The remaining supernatant and oil layers were then carefully aspirated from the renal pellets. The tips of the microcentrifuge tubes con-

taining the pellets were cut off to minimize extraneous binding to the tube walls and counted for radioactivity. After counting, the pellets were solubilized in 0.5 ml of NaOH 1 mol/l and protein was determined by the method of Lowry et al. [21]. With good agitation before removal of each aliquot, the variation in sampling these rapidly-settling renal fractions was less than 5%.

The binding and degradation of ^{125}I -insulin as a fraction of protein concentration and in the presence of varying concentrations of unlabelled insulin and analogues were measured by incubating glomeruli (0.03–1.3 mg protein/ml) and tubules (0.1–2.1 mg protein/ml) in 1.5 ml microcentrifuge tubes at 22 °C for 75–90 min in a total volume of 1.06 ml containing ^{125}I -insulin (1 μg /l) and unlabelled insulin or analogues, 0–50 mg/l. Triplicate 0.25 ml aliquots of the incubation mixtures were then removed and pellets and supernatant fractions assayed for ^{125}I -insulin binding and degradation as described above.

Dissociation Experiments

In order to determine the time course of dissociation of bound ^{125}I -insulin into a large volume of incubation medium, glomeruli (2.9 mg protein/ml) and tubules (4.8 mg protein/ml) obtained from ten rat kidneys were incubated at 22 °C for 75 min in 7 ml of incubation medium containing ^{125}I -insulin (1 μg /l) in the presence and absence of unlabelled insulin (50 mg/l) to achieve a plateau in binding. Triplicate 0.1 ml aliquots were then removed to measure binding. Specific ^{125}I -insulin binding was 8.96% and 3.20% of the total counts to tubules and glomeruli respectively. Non-specific binding was 40% of the total bound counts in each fraction. The incubation mixtures were then centrifuged, the supernatant medium removed, and the pellets resuspended in an equivalent volume of ice-cold medium. The carry-over of the initial labelling medium in this step was 0.36% for tubules and 0.11% for glomeruli. Triplicate 0.1 ml aliquots were again immediately removed to determine zero time binding, and multiple 0.1 ml aliquots were quickly dispensed into conical centrifuge tubes containing 7.5 ml of medium with or without unlabelled insulin 1 mg/l. Duplicate tubes were centrifuged at several time points and the supernatant medium immediately aspirated off the pellets which were then counted for radioactivity. The ^{125}I -insulin binding measurements at the plateau and at the zero time immediately before dilution were identical within 5%.

In order to determine the nature of the bound material released during the dissociation, glomeruli (2.8 mg protein/ml) and tubules (4.1 mg protein/ml) were allowed to achieve a plateau in binding with tracer amounts of ^{125}I -insulin as described above. Total ^{125}I -insulin binding to tubules and glomeruli was 7.80% and 4.07%, and specific binding was 5.09% and 2.26% of the total counts, respectively. Multiple 0.1 ml aliquots of the incubation mixtures were then dispensed into 1.5 ml microcentrifuge tubes and the supernatant medium removed by careful aspiration after centrifugation. The carry-over of the initial labelling medium in this step was 1.35% for tubules and 0.66% for glomeruli. Dissociation was initiated by the rapid addition to the pellets of 1 ml of incubation buffer with or without unlabelled insulin 1 mg/l. The pellets were resuspended in the buffer and triplicate tubes were removed at different times and rapidly centrifuged after prior addition of 0.1 ml of a 1:2 mixture of dinonyl:dibutylphthalate oil to allow complete separation of the pellet and supernatant fractions. Remaining bound radioactivity in the pellet and intact and degraded ^{125}I -insulin in the supernatant fractions were then determined as described above.

Insulin and Analogues

^{125}I -insulin (specific activity 200–220 $\mu\text{Ci}/\mu\text{g}$) was prepared by iodination and Sephadex G-50 chromatography as described previously [22]. Porcine monocomponent insulin and glucagon and por-

cine proinsulin were gifts from J. Schlichtkrull (Novo Research Institute, Copenhagen, Denmark) and G. Jouve (Novo Paris, France). Despentapeptide insulin (a derivative with the last five carboxy-terminal B chain residues missing), Al-Boc-Gly-insulin (a derivative with a blocked amino-terminal glycine in the A chain), and a cross-linked A1B1-insulin derivative with a 20 atom bridge referred to as (Z-lys) 2 (AD) [23] were gifts of D. Brandenburg (Deutsches Wollforschungsinstitut, Aachen, FGR).

Results

The binding of ^{125}I -insulin to both glomerular and tubular fractions (Fig. 1) proceeded rapidly at 22 °C and reached a plateau between 40 and 90 min. Although the percentage binding per mg of protein/ml varied from experiment to experiment and sometimes differed between glomeruli and tubules, the time course to the plateau was essentially constant. Non-specific binding was maximal at 10 min and remained constant thereafter.

Both non-specific and total binding of ^{125}I -insulin to isolated renal glomeruli and tubules were essentially linear over the range of concentrations examined (Fig. 2). All experiments were performed at protein concentrations above 0.2 mg/ml. Tubules had a greater binding capacity than glomeruli at higher protein concentrations.

The degradation of ^{125}I -insulin by glomerular and tubular preparations was linear over a 90 min incubation period with degradation products appearing as early as 10 min after the start of incubation (not shown). This degradative activity was predominantly due to release of proteolytic enzymes into the medium from the glomerular and tubular fractions, since medium first exposed to tissue in the absence of ^{125}I -insulin for periods of 75–90 min, and then separated and incubated without tissue for an additional 75–90 min with ^{125}I -insulin, had 66%–100% of the degradative activity seen in incubation mixtures of renal fractions and medium incubated with tracer for identical times under standard assay conditions. Preincubation of the renal fractions with medium, followed by centrifugation and replacement of the old medium with fresh incubation medium and ^{125}I -insulin, had only a slight effect in reducing extracellular degradation, as proteolytic activity was shown to be released into the medium throughout the course of incubation.

Degradation of ^{125}I -insulin by proteolytic activity from both isolated glomeruli and tubules increased progressively as a function of protein concentration of the renal fractions (Fig. 3). Tubular degradation was markedly higher than glomerular degradation at all protein concentrations examined.

The degradation of ^{125}I -insulin increased progressively with increasing substrate concentrations in in-

Fig. 1. Time course of ^{125}I -insulin binding. Renal glomeruli and tubules were incubated at 22 °C with ^{125}I -insulin (1 $\mu\text{g}/\text{l}$) in the presence (non-specific binding) and absence (total binding) of unlabelled insulin (50 mg/l); binding was measured at 10, 20, 30, 40, 60 and 90 min. Data have been corrected for degradation of ^{125}I -insulin measured at each time point. $\circ$ — $\circ$ = total binding glomeruli; $\bullet$ — $\bullet$ = total binding tubules; $\triangle$ — $\triangle$ = non-specific binding glomeruli; $\blacktriangle$ — $\blacktriangle$ = non-specific binding tubules

Fig. 2. Binding of ^{125}I -insulin as a function of protein concentration. Renal glomeruli and tubules at different protein concentrations were incubated for 90 min at 22 °C with ^{125}I -insulin (1 $\mu\text{g}/\text{l}$) in the presence (non-specific binding) and absence (total binding) of unlabelled insulin (50 mg/l). Binding was determined at the completion of the incubation period. Binding has been corrected for degradation of ^{125}I -insulin determined at each time point (glomerular degradation: 2.3%–12.5% of total radioactivity; tubular degradation: 5.6%–53.2% of total radioactivity). $\bullet$ — $\bullet$ = total binding tubules; $\circ$ — $\circ$ = total binding glomeruli; $\blacktriangle$ — $\blacktriangle$ = non-specific binding tubules; $\triangle$ — $\triangle$ = non-specific binding glomeruli

cubations of both glomerular and tubular preparations (Fig. 4) and the degradative systems were not saturated at levels of hormone as high as 170 nmol/l (approximately 1 mg/l). At all substrate concentrations proteolytic activity derived from tubules was greater than that derived from glomeruli.

Fig. 3. Degradation of ^{125}I -insulin as a function of protein concentration. Renal glomeruli and tubules at different protein concentrations were incubated at 22°C for 90 min with ^{125}I -insulin ($1\text{ }\mu\text{g/l}$) and degradation determined at the completion of the incubation by TCA precipitation of the supernatant solutions as described in Methods. $\circ\text{---}\circ$ = tubules; $\bullet\text{---}\bullet$ = glomeruli

Fig. 4. Degradation of ^{125}I -insulin as a function of hormone concentration. Renal glomeruli (0.6 mg protein/ml) and tubules (1.3 mg protein/ml) were incubated at 22°C for 75 min with ^{125}I -insulin ($1\text{ }\mu\text{g/l}$) and varying concentrations of unlabelled insulin. Degradation was then measured in the medium supernatant fractions as described in Methods. $\circ\text{---}\circ$ = tubules; $\bullet\text{---}\bullet$ = glomeruli

When the binding of ^{125}I -insulin to glomeruli and tubules was examined in the presence of varying concentrations of unlabelled insulin and analogues, ranging from $5\text{--}50,000\text{ }\mu\text{g/l}$ (approximately $1\text{--}10,000\text{ nmol/l}$), a continuous inhibition of binding was seen over the entire range of insulin concentrations ex-

Fig. 5. Inhibition of ^{125}I -insulin binding to glomeruli by unlabelled insulin ($\bullet\text{---}\bullet$), proinsulin ($\circ\text{---}\circ$), despentapeptide insulin ($\square\text{---}\square$), A1-Boc-Gly-insulin ($\blacktriangle\text{---}\blacktriangle$), A1-B1 cross-linked insulin ($\triangle\text{---}\triangle$), and glucagon ($\blacksquare$). Glomeruli were incubated for 75 min at 22°C with ^{125}I -insulin ($1\text{ }\mu\text{g/l}$) in the absence (initial binding) or presence of unlabelled hormones at the indicated concentrations ($5\text{--}50,000\text{ }\mu\text{g/l}$). Binding is expressed as percentage of initial binding of ^{125}I -insulin. The data have been corrected for non-specific binding and degradation at each point. Each point for insulin is the mean $\pm$ SEM of two to four experiments using renal fractions from four animals per experiment. The points for the insulin analogues and glucagon are the mean of results in two separate experiments

Fig. 6. Inhibition of ^{125}I -insulin binding to tubules by unlabelled insulin ($\bullet\text{---}\bullet$), proinsulin ($\circ\text{---}\circ$), despentapeptide insulin ($\square\text{---}\square$), A1-Boc-Gly-insulin ($\blacktriangle\text{---}\blacktriangle$), A1-B1 cross-linked insulin ($\triangle\text{---}\triangle$), and glucagon ($\blacksquare$). Experimental conditions and expression of results are as in Fig. 5

amined (Figs. 5 and 6). In both glomeruli (Fig. 5) and tubules (Fig. 6), the insulin analogues were considerably less potent than insulin itself in inhibiting ^{125}I -insulin binding, being effective only at concentrations $> 10\text{ nmol/l}$, whereas significant inhibition was seen for insulin at 1 and 2 nmol/l in glomeruli and tubules,

Table 1. Hormone concentrations at which half-maximal inhibition of ^{125}I -insulin binding is observed

Hormone	Concentration (mol/l)	
	Glomeruli	Tubules
Insulin	2.8×10^{-9}	3.5×10^{-8}
Proinsulin	1.4×10^{-7}	2.3×10^{-7}
Despentapeptide insulin	3.5×10^{-8}	4.5×10^{-8}
A1-Boc-Gly-insulin	4.0×10^{-8}	7.6×10^{-8}
A1-B1 cross-linked insulin [23]	1.2×10^{-6}	1.4×10^{-6}

Data are taken from points at which 50% inhibition of ^{125}I -insulin binding in the presence of tracer alone (initial binding) was observed in Figures 5 and 6.

The inhibition curves for insulin were significantly different between glomeruli and tubules at concentrations of 5 ($p < 0.01$), 10 ($p < 0.05$), 20 ($p < 0.01$), 70 ($p < 0.05$), 250 ($p < 0.01$) and 5000 ($p < 0.001$) $\mu\text{g/l}$ insulin using Student's *t*-test. Those for proinsulin, despentapeptide insulin and A1-B1 cross-linked insulin were not significantly different at any concentration tested. The inhibition curves for A1-Boc-Gly insulin were significantly different at concentrations of the analogue of 500 $\mu\text{g/l}$ ($p < 0.05$) and 1000 $\mu\text{g/l}$ ($p < 0.005$).

respectively. Glucagon was not an effective inhibitor of ^{125}I -insulin binding even at concentrations of 2.7 $\mu\text{mol/l}$.

The concentrations of hormone necessary to achieve 50% inhibition of ^{125}I -insulin binding are summarized in Table 1. Insulin was ten times more potent in glomeruli than in tubules in inhibiting ^{125}I -insulin binding. The insulin analogues studied as inhibitors of ^{125}I -insulin binding ranged from equipotent in glomeruli and tubules (e.g., the A1-B1 cross-linked derivative) to almost twice as potent in glomeruli as in tubules (e.g., proinsulin and A1-Boc-Gly-insulin). While the insulin analogues were all less potent than insulin in inhibiting the binding of ^{125}I -insulin to both glomeruli and tubules, their potency in relation to insulin was greater in tubules than in glomeruli. Thus, the concentration of despentapeptide insulin required to achieve half-maximal inhibition of ^{125}I -insulin binding in glomeruli was ten times higher than the equivalent insulin concentration, but in tubules it was < 1.5 times higher.

When the binding of ^{125}I -insulin to glomeruli and tubules was examined in the presence of bacitracin (0.8 mg/ml) to inhibit insulin degradation, concentrations of unlabelled insulin from 5–50,000 $\mu\text{g/l}$ (approximately 1–10,000 nmol/l) produced a continuous inhibition of binding (Fig. 7). This was similar to that observed in experiments where insulin degradation was not inhibited (Figs. 5 and 6) except that the concentration of hormone at which half maximal inhibition of ^{125}I -insulin binding was observed was lowered, being 1.4 and 5.4 nmol/l for glomeruli and tubules, respectively. A greater inhibition of ^{125}I -insulin binding

Fig. 7. Inhibition of ^{125}I -insulin binding to glomeruli (●—●) and tubules (○—○) by unlabelled insulin in the presence of bacitracin (0.8 mg/ml) in the medium to inhibit insulin degradation. Glomeruli and tubules were incubated for 75 min at 22°C with ^{125}I -insulin (1 $\mu\text{g/l}$) in the absence (initial binding) or presence of unlabelled insulin at the indicated concentrations (5–50,000 $\mu\text{g/l}$). Binding is expressed as a percentage of initial binding of ^{125}I -insulin. The data have been corrected for non-specific binding at each point. Degradation of ^{125}I -insulin determined at the completion of the incubations by TCA precipitation of the supernatant solutions was $< 3.5\%$ of the total counts at all insulin concentrations used, which was identical to the degradation seen in incubations of ^{125}I -insulin in medium without tissue.

in glomeruli than in tubules was seen at all concentrations of unlabelled insulin tested.

When tubules and glomeruli which had achieved a plateau in binding at tracer levels of ^{125}I -insulin were diluted into a large volume of medium in the absence (dilution alone) or presence of unlabelled insulin 1 mg/l (170 nmol/l) (dilution + insulin), there was a rapid dissociation of bound radioactivity from the renal fractions which was accelerated in the presence of insulin in the dilution medium (Fig. 8). The more rapid dissociation observed in the presence of insulin was pronounced in the glomerular fraction and less marked in the case of the tubular preparation (Fig. 8). In both renal fractions non-specific binding dissociated very rapidly in the first 2.5 min of dilution and only very slowly thereafter, with no difference between the rates observed in the absence and presence of insulin in the dilution medium.

The nature of the ^{125}I -labelled material dissociating from glomerular and tubular binding sites was examined in an experiment where the volume of dilution medium was kept small enough to permit analysis of the dissociation products by TCA precipitation. The results of such an experiment with glomeruli indicated that of the total counts dissociated in 2 h (approximately 80% of total bound) two-thirds were precipitable by TCA while the remainder were non-TCA precipitable (Fig. 9a). A similar analysis of the incubation medium in which the initial plateau binding

Fig. 8. Time course of dissociation of bound ^{125}I -insulin. Renal glomeruli and tubules which had achieved a plateau in binding with ^{125}I -insulin (1 $\mu\text{g}/\text{l}$) for 75 min at 22°C were resuspended in a volume of ice-cold medium equivalent to the initial incubation volume (7 ml); aliquots were promptly diluted into 75 volumes of medium at 22°C with unlabelled insulin 1 mg/l (170 nmol/l) (dilution + insulin) or without unlabelled insulin (dilution), and the time course of dissociation followed as described in Methods. Data are corrected for non-specific binding, and are expressed as the percentage of ^{125}I -insulin binding measured at the plateau before dilution (100%). ●—● = dilution; ○—○ = dilution + insulin; ▲—▲ = dilution, non-specific binding; △—△ = dilution + insulin, non-specific binding

Fig. 9. Time course of dissociation of intact and degraded products of ^{125}I -insulin binding. Renal glomeruli and tubules which had achieved a plateau in binding with ^{125}I -insulin (1 $\mu\text{g}/\text{l}$) for 75 min at 22°C were distributed in small aliquots into 1.5 ml microcentrifuge tubes and diluted with 1 ml of medium containing unlabelled insulin 1 mg/l (170 nmol/l) (dilution + insulin) or with insulin-free medium (dilution alone). The time course of appearance of dissociation products in the medium and of their separation into intact and degraded fractions by TCA precipitation was performed as described in Methods. Data are *not* corrected for non-specific binding since at the level of unlabelled insulin at which such binding is determined (50 mg/l) ^{125}I -insulin degradation is almost completely inhibited. ■—■ = total ^{125}I -insulin released; ○—○ = intact ^{125}I -insulin released; ●—● = ^{125}I -insulin remaining bound; △—△ = degraded ^{125}I -insulin released

was carried out revealed that 88% of the total radioactivity was TCA-precipitable.

In contrast, examination of the dissociation products from tubular binding (Fig. 9b) revealed that of the bound radioactivity released (almost 80% of the total), less than one-third was TCA-precipitable. Analysis of the initial tubular binding medium showed that almost two-thirds (63%) of the ^{125}I counts were TCA-precipitable. With both glomerular and tubular preparations, the increased radioactivity dissociated into the medium in the presence of unlabelled insulin 1 mg/l was mainly intact (not shown).

Discussion

This study demonstrates that ^{125}I -insulin binding and degradative activities, previously reported in renal cell membrane preparations [2, 3] and in tubules obtained by collagenase digestion [8], are located in both the glomerular and tubular fractions of rat renal nephrons [4–8]. The procedures employed here allow concurrent isolation of morphologically intact glomeruli and tubules [18–20] from the same kidneys without the necessity for enzyme digestion and the coincident risk of proteolytic alteration of receptor sites. Our results indicate that such preparations can yield kinetic binding and degradation data comparable to those obtained with isolated cells or cell membrane preparations [1, 24, 25].

Degradation of ^{125}I -insulin was appreciable in both glomerular and tubular preparations, but was more extensive in incubations with tubules, in accordance with the role of this renal fraction in recovering and degrading small peptides and proteins [26, 27]. Insulin degradation increased progressively with time, protein concentration and substrate concentration up to 170 nmol insulin/l, in agreement with the properties and K_m estimated for the renal degradative system from cell membrane preparations [2, 3]. In our experiments, however, in contrast to those of Kurokawa et al. [5, 7, 8], much if not all of the ^{125}I -insulin degradative activity was associated with release of proteolytic activity from the renal fractions into the medium during the course of the incubations. The observed extent of degradation varied from experiment to experiment. This may be the result of cellular damage occurring during the isolation and/or incubation of the renal fractions. The degradative activity of the isolated renal fractions could be markedly increased (e.g. from 13.5% to 48.5% in glomeruli and from 7.2% to 49.4% in tubules in a typical experiment) by cellular disruption with a glass homogenizer or Polytron, supporting the intracellular source of the degradative activity. Our results are consistent with reports showing

that in work on ^{125}I -insulin binding and degradation there is commonly leakage of degradative enzymes into the incubation medium, due to cell damage during isolation and/or incubation of cells from several different tissues, including isolated hepatocytes and adipocytes [28–35]. Therefore, it was not possible for us to examine directly the possible relationship between binding and degradation of ^{125}I -insulin in glomeruli and tubules in these studies.

Both glomerular and tubular preparations exhibited a specificity for binding of ^{125}I -insulin, since insulin analogues with lower biological potency than insulin itself were less potent than the native hormone in competing with ^{125}I -insulin for the binding sites; glucagon was ineffective as an inhibitor even at very high concentrations. However, the apparent affinity of insulin for its receptor(s) in isolated glomeruli was about ten times greater than the affinity for the tubular receptor(s).

The inclusion of bacitracin in the incubation medium resulted in steeper displacement curves and higher affinities of both glomeruli and tubules for ^{125}I -insulin, but the glomerular receptors maintained their higher affinity for the hormone compared with those of tubules. In these experiments bacitracin effectively inhibited all ^{125}I -insulin degradation as monitored by sampling the extracellular medium. However, the integrity of intracellular insulin was not monitored in these experiments.

The difference in apparent affinity of the renal receptor(s) was maintained even after correction for non-specific binding and elimination of extracellular degradation, and is remarkable in view of the cellular diversity of the glomerulus which would be expected to dilute high affinity sites if receptor sites of differing affinity were present in the three cell types. Although the affinity of the glomerular and tubular receptors for insulin differed markedly, the affinity of the receptors for the insulin analogues differed appreciably between glomeruli and tubules only at high concentrations of peptide. The relative affinities (compared with insulin) of the analogues for the receptor in tubules were thus significantly greater than the relative affinities of the analogues for the glomerular receptors (Table 2). These differences in absolute and relative affinities are also related to the shape of the ^{125}I -insulin binding competition curves (Figs. 5 and 6), which have an appreciably steeper slope for despentapeptide insulin and A1-Boc-Gly-insulin in the region of 50% inhibition in both the glomerular and tubular preparations than for insulin, proinsulin and the A1-B1 cross-linked derivative. This difference may be due to receptor heterogeneity or varying ability in inducing negative cooperativity which has been reported for some insulin analogues [36]. Taken together,

Table 2. Relative potencies of insulin, glucagon and insulin analogs in inhibiting ^{125}I -insulin binding and degradation by glomeruli and tubules

Hormone	Glomeruli		Tubules	
	Binding	Degradation	Binding	Degradation
Insulin	100	100	100	100
Proinsulin	2.0	18.2	15.2	26.5
Despentapeptide insulin	8.0	149	77.8	236
A1-Boc-Gly-insulin	7.0	81.5	46.1	75.4
A1-B1 cross-linked insulin	0.23	561 ^a	0.25	427 ^a
Glucagon ^a	0.01	3.1	0.02	3.2

Values in table are calculated from the peptide concentrations at which half-maximal inhibition of ^{125}I -insulin binding was observed (Figs. 5 and 6 and Table 1), and at which half-maximal inhibition of ^{125}I -insulin degradation was observed in the same experiments (data not shown)

$$\text{Relative potency} = \frac{\text{Molar concentration of insulin for 50\% inhibition}}{\text{Molar concentration of analogue for 50\% inhibition}} \times 100$$

^a Values are relative potencies calculated from insulin concentrations at which inhibition of ^{125}I -insulin binding or degradation equivalent to that observed at 1 mg/l (1.64×10^{-7} mol/l) A1-B1 cross-linked insulin and 10 mg/l (2.70×10^{-6} mol/l) glucagon were observed

the data (Figs. 5 and 6, Table 1) suggest that the glomerular receptors have a greater overall affinity for insulin than the tubular receptors, a conclusion which is also suggested by the faster apparent rates of dissociation of ^{125}I -radioactivity from tubules than from glomeruli (Fig. 8). Negative cooperativity of the type described by DeMeys et al. [36, 37] was demonstrated in both glomerular and tubular preparations by dilution experiments in the presence and absence of unlabelled insulin 1 mg/l (170 nmol/l), a concentration which was reported to produce the maximum increase in dissociation rate of bound ^{125}I -insulin in human cultured lymphocytes [37]. An increase in the dissociation rate of bound ^{125}I -insulin was seen in both glomerular and tubular fractions, but was more marked in the glomeruli, although the effect was less pronounced than that observed in cultured lymphocytes [37].

Two-thirds of the total bound material released into the medium from glomeruli was found to be intact insulin as measured by TCA precipitation, compared with only one-third of that released from tubules. Appreciable amounts of receptor-bound ^{125}I -insulin have also been shown previously to be released in a degraded form in studies with isolated rat hepatocytes [31, 38] and adipocytes [30, 39, 40]. Our

results are in agreement with the known degradative capacity of proximal tubules for proteins [26, 27], suggesting that one function of the insulin binding site(s) in tubules may be to mediate the degradation of the hormone. Insulin analogues, however, show a pronounced discrimination in their ability to inhibit binding versus degradation of ^{125}I -insulin in both glomeruli and tubules (Table 2). For example, the A1-B1 cross-linked derivative of insulin, the poorest inhibitor of ^{125}I -insulin binding tested, was nevertheless the most potent inhibitor of ^{125}I -insulin degradation in both glomeruli and tubules, indicating that these activities are not carried out by the same system.

Whether the observed differences in apparent affinity of the glomerular and tubular receptors are due to the presence of different receptors [24] in these two renal fractions, or to differences in cooperative interactions [36, 37], or differences in interactions between insulin-receptor complexes and various types of effectors [41], will require further experimentation with insulin and its analogues. Speculation regarding the function of insulin receptors in renal glomeruli and tubules and their possible involvement in the pathology of disease states will await further characterization of the receptors.

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