Binding and Degradation of Insulin by Isolated Renal Brush Border Membranes

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SUMMARY

Filtered proteins including insulin are absorbed in the proximal tubule by means of pinocytosis. The first step in this process is binding of the protein to brush border membrane. As it is not known whether absorption exhibits specificity, we set out to determine whether specific binding sites for insulin are present in brush border membranes. Rabbit-isolated brush border membranes were incubated with ^{125}I-insulin and varying concentrations of cold insulin or other peptide hormones. Binding and degradation of ^{125}I-insulin occurred in a time- and temperature-dependent manner. Native insulin competitively inhibited ^{125}I-insulin binding, but calcitonin, arginine vasopressin, glucagon, and growth hormone (10^{-6} M) were relatively ineffective. Nonspecific binding averaged one-third of the total radioactivity bound. Scatchard analysis of binding data revealed two classes of insulin receptors: high affinity, low capacity receptors and low affinity, high capacity receptors. Gel filtration analysis of ^{125}I-insulin exposed to brush border membrane revealed the formation of low-molecular-weight products similar to that produced by intact kidneys. The degrading process exhibited some specificity, for cold insulin (10^{-6} M) was more effective than calcitonin, vasopressin, glucagon, or growth hormone in inhibiting degradation (32% versus <13% inhibition; $P < 0.01$). Whether this reflects inhibition of insulin specific binding before exposure to degradation or inhibition of specific enzymes is unclear. In summary, it appears that renal brush border membranes have a major insulin-specific receptor component that could potentially mediate tubular insulin absorption. In addition, there is a smaller nonspecific component that may also have the potential to mediate insulin absorption. Finally, it appears that brush border membranes have the ability to degrade insulin to low-molecular-weight products by a process that exhibits some specificity for insulin. DIABETES *31*:618–623, July 1982.

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The kidney is a major site for the removal of insulin from the systemic circulation; this is accomplished by glomerular filtration and to a lesser extent by extraction from the postglomerular circulation.[1] Following filtration, insulin, like other low-molecular-weight proteins, is absorbed in the proximal tubule by means of pinocytosis and is then degraded intracellularly.[2,3] There has been much debate about the specificity of the absorption process.[3,4] In particular, studies by Just and Habermann[3,5] have suggested that polypeptides bind to the renal brush border membrane by means of a nonspecific charge-related process and that binding is the event that initiates pinocytosis. Previous studies by Blanchard et al.[6] and by Duckworth[7] have revealed the presence of insulin-specific receptors on renal tubular plasma membrane preparations, but as these preparations were relatively impure, precise localization of the receptors to either the brush border or basolateral membrane is not possible.

In view of the potentially important role of ligand binding in initiating the pinocytotic process, we set out to determine whether insulin-specific binding sites are present in renal brush border membranes. The results of this study indicate that there are indeed insulin-specific receptors on brush border membranes. Furthermore, evidence is provided that these membranes degrade insulin and that this process is in part insulin specific.

MATERIALS AND METHODS

Materials. ^{125}I-insulin with less than 1 (0.6) iodine atom/molecule (specific activity ~210 μCi/μg) was prepared by a modification of the chloramine T method.[8] ^{125}I-insulin was separated from unreacted iodide by filtration on a cellulose acetate column. If required, before use it was further purified by filtration on a Sephadex G-50 column (0.9 × 60 cm) in phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin (BSA). This rendered the final product

at least 95% trichloroacetic acid (TCA) precipitable and 86% immunoprecipitable. $^{125}I^-$ was obtained from Cambridge Nuclear (Billerica, Maine); bovine serum albumin, Fraction V from Miles Laboratory, Inc. (Elkhart, Indiana); porcine insulin, proinsulin, and glucagon were gifts from Eli Lilly and Co. (Indianapolis, Indiana) and rat growth hormone from the National Institute of Arthritis, Metabolism and Digestive Diseases, reference preparation B-6. Synthetic salmon calcitonin was obtained from Armour Pharmaceutical Co. (Kankakee, Illinois) and arginine vasopressin and Triton X-100 from Sigma Corporation (St. Louis, Missouri).

Renal cortical brush border membranes were prepared from female New Zealand white rabbits by a modification of the method of divalent cation precipitation with differential centrifugation.[9] The final yield of brush border membranes as judged by alkaline phosphatase activity was 8%. Alkaline phosphatase, Na-K-ATPase, and acid phosphatase activity were enriched 8-, 0.5-, and 0.6-fold, respectively, compared with crude homogenate.

Binding studies. A typical assay consisted of incubating in triplicate ^{125}I-insulin (1.7×10^{-10} M) with membranes (~60 μg protein/tube) and unlabeled hormones as indicated, in 100 μl Krebs-Ringers phosphate (KRP) containing 0.5% BSA (pH 7.4). Following incubation at times and temperature to be specified, membrane-bound and free insulin were separated by filtering and washing under reduced pressure on BSA-coated millipore cellulose acetate filters (EAWP, 0.2 μm) as described by Cuatrecasas.[10] The wash consisted of 6 ml cold KRP with 0.1% BSA. Binding was corrected for degradation, as measured by tricholoroacetic acid (TCA) precipitation of the filtrate, and for nonspecific binding, which was taken to be the radioactivity bound in the presence of 10^{-5} M unlabeled insulin. To distinguish between uptake due to binding versus uptake due to transport into membrane vesicles, the form assumed by the renal brush border membranes after isolation, the uptake of ^{125}I-insulin was measured in the presence and absence of 0.025% Triton X-100.[11]

Degradation studies. A standard degradation study involved triplicate incubations of ^{125}I-insulin and brush border membrane in 100 μl buffer as described above. At the end of each incubation period, 200 μl cold KRP containing 0.5% BSA was added to the reaction mixture, which was then centrifuged in the cold at 17,000 *g* for 5 min. Duplicate 100-μl aliquots of the supernate were each added to 0.5 ml of cold KRP containing 0.5% BSA to which 12% TCA was added. The precipitate was collected by centrifugation at 3000 rpm. Immunoprecipitable ^{125}I-insulin was measured in samples obtained before and after 60 min incubation by a modified double antibody method[12] by the addition of an excess guinea pig anti-insulin antibody with precipitation of the antibody-antigen complex 48 h later. Although TCA precipitation overestimates immunoprecipitable radioactivity, a significant correlation between the two methods ($y = 0.819x + 23.84$; $r = 0.98$) was observed. Thus, the appearance of TCA soluble material was taken as a measure of degradation.

The nature of the labeled material present after 60-min incubation with membranes at 37°C was assessed chromatographically on a Sephadex G-50-fine column (0.9 × 60 cm) by elution with 1 M acetic acid containing 0.1% BSA. The column was standardized with blue dextran (void volume), ^{125}I-insulin, $^{125}I^-$, and monoiodotyrosine. Sixty 1-ml fractions were collected and recovery of added radioactivity was greater than 92%.

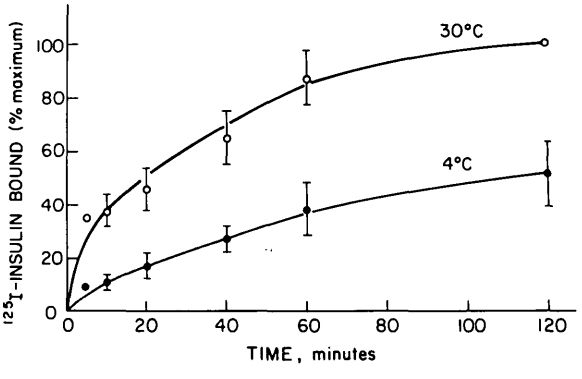
$^{125}I^-$ was counted in a Beckman 4000 gamma counter. Protein was determined by the method of Lowry et al.[13] Alkaline phosphatase and NA-K-ATPase was assayed as previously described.[14] Phosphate produced from ATP was measured by the method of Ames[15] and acid phosphatase as described by Bergmeyer.[16]

The results were expressed as the mean ± SEM, and unless otherwise stated are representative of four separate membrane preparations. The data were statistically analyzed with Student's *t* test, but when multiple groups were compared a one-way analysis of variance was used. If the F-value was significant, the data were further analyzed by means of Dunnett's test.[17] A P value of <0.05 was taken as significant.

RESULTS

Binding studies. The specific binding of ^{125}I-insulin to the brush border membrane exhibited both time and temperature dependency (Figure 1). At 30°C, there was initial rapid binding, and after 60 min near maximum binding was achieved. In seven separate experiments, specific binding at 60 min averaged 5 ± 0.5%/100 μg protein, which at the insulin concentration studied (1.7×10^{-10} M) is equivalent to 50 ± 5 pg/mg protein. In these seven experiments, specific binding represented 67.7 ± 3.8% of the total binding and nonspecific binding accounted for the rest. At 4°C, specific binding was significantly depressed. Binding also exhibited dependency on membrane concentration; over the range of membrane protein concentration 22–130 μg, increasing protein concentrations were associated with proportional increases in total ^{125}I-insulin binding ($y = 0.48x + 0.674$, $r = 0.84$; $P < 0.01$). Theoretically, binding to the brush border membrane preparation could predominantly reflect binding to contaminating basolateral membranes. To exclude this possibility, ^{125}I-insulin binding studies were performed with four kidney homogenates and their corresponding brush border membrane preparations (enriched <0.3-fold in the basolateral membrane marker, NA-K-ATPase). Incubations were performed at 30°C for 60 min in the presence of 10 mM N-ethylmaleimide (NEM), the latter to inhibit degradation.[7] Residual degrading activity

FIGURE 1. Time course of ^{125}I-insulin binding to renal brush border membranes incubated at 30°C (○) or 4°C (●). Data are corrected for nonspecific binding and degradation and are expressed as percentage of the value obtained after 120 min of incubation. Results are the mean ± SEM of four separate membrane preparations.



over the period of study averaged 3.4 ± 0.5% and 3.4 ± 0.4% for the brush border membranes and homogenates, respectively. Specific binding of ^{125}I-insulin, corrected for degradation, was significantly greater with the brush border membranes than with the homogenates; brush border membrane binding exceeded binding to homogenate by 70 ± 15% ($P < 0.01$). This suggests that a significant portion of the binding to the membrane preparation represents true brush border membrane binding. By contrast, binding to the homogenate may represent binding to several subcellular fractions.

Binding to brush border membranes also exhibited a significant nonspecific component, 32 ± 4% of the total radioactivity bound after 60 min at 30°C. This component was temperature sensitive, for at 4°C it accounted for 8 ± 3.2% of the total radioactivity bound. Incubation of membranes (N = 4) at 30°C for varying time periods revealed that between 20 and 60 min, nonspecific binding increased in parallel to specific binding and accounted for 38 ± 5.3%, 37 ± 4.4%, and 36 ± 5.2% of the total radioactivity bound at 20, 40, and 60 min, respectively. After 120 min nonspecific binding continued to rise but as specific binding increased little (Figure 1), nonspecific binding now constituted a greater proportion, 48.3 ± 5.3% of the total radioactivity bound. There was a significant correlation between membrane protein concentration and nonspecific binding ($r = 0.84$; $P < 0.01$).

Incubation of membranes with ^{125}I-insulin and increasing concentrations of native insulin at 30°C resulted in competitive inhibition of ^{125}I-insulin binding (Figure 2). The Scatchard plot of the binding data (Figure 3) was consistent with either two classes of receptors (high affinity, low capacity and low affinity, high capacity receptors) or the presence of a single class of receptors with a negative cooperative type of hormone receptor interaction.[18] Affinity constants of 2×10^{10} and 8.7×10^{8} M^{-1} and binding capacities of 0.5 and 15.5 pM/mg protein were obtained for the high and low affinity sites, respectively.[19,20] These calculations assume the presence of steady-state binding. When membranes were incubated with increasing concentrations of proinsulin, it was apparent that proinsulin was less effective than insulin in inhibiting ^{125}I-insulin binding (Figure 4). The inhibitory effect of various non-insulin peptides on ^{125}I-insulin binding was examined and it was observed that calcitonin, arginine vasopressin, rat growth hormone, and glucagon in concentrations of 10^{-6} M were relatively ineffective when compared with native insulin ($P < 0.01$; Table 1). Binding was not significantly altered by the presence of 0.025% Triton X-100, a detergent that disrupts brush border membranes vesicles.[11] The latter finding suggests that uptake of radioactivity does indeed represent binding and not transport with intravesicular accumulation, for accumulation would be expected to fall in "leaky" vesicles.[21]

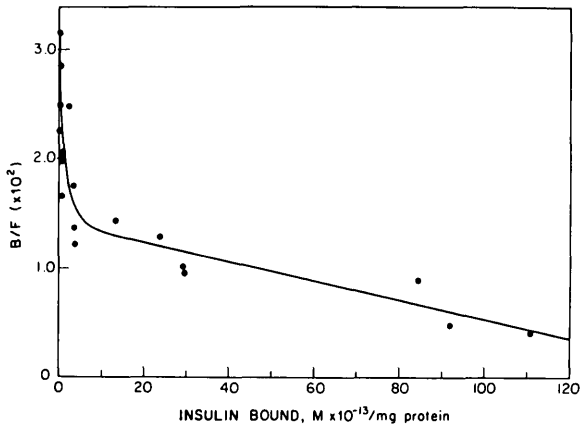


FIGURE 3. Scatchard analysis of data presented in Figure 2.

Degradation studies. ^{125}I-insulin exposed to brush border membrane was degraded in a time- and temperature-dependent manner (Figure 5). Degradation at 37°C was significantly greater than at 30°C, while lowering the temperature to 4°C was associated with little degradation. At 30°C there was a significant correlation between degradation and time

FIGURE 2. Competitive inhibition of ^{125}I-insulin binding to brush border membrane by native insulin. Four membrane preparations were incubated with ^{125}I-insulin (1.7×10^{-10} M) and varying concentrations of native insulin for 60 min at 30°C. The ^{125}I-insulin bound in the presence of native insulin is expressed as a percentage of that bound in the absence of native insulin. Data are corrected for nonspecific binding and degradation.

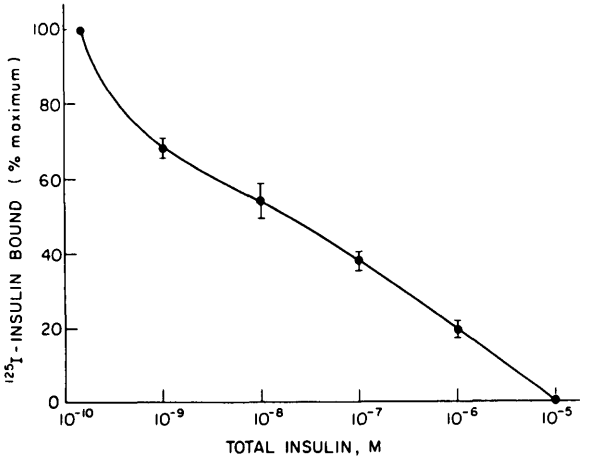


FIGURE 4. Comparison of the effects of varying concentrations of native insulin and of proinsulin on ^{125}I-insulin (1.7×10^{-10} M) binding to brush border membrane. Results are the mean of two separate membrane preparations.

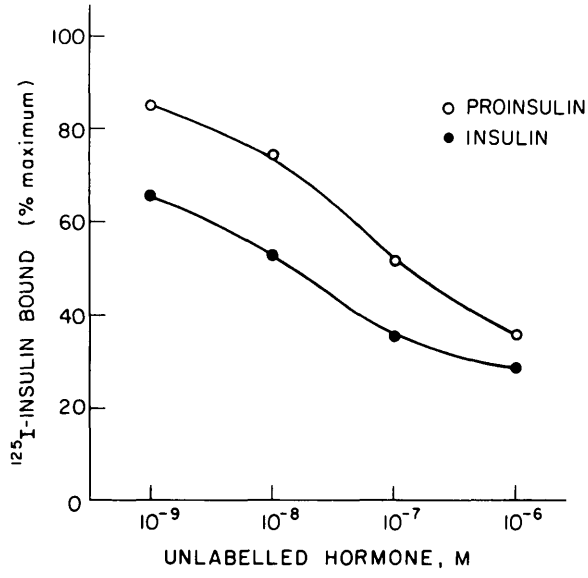


TABLE 1
Effect of various peptide hormones on renal brush border membrane binding and degradation of ^{125}I-insulin

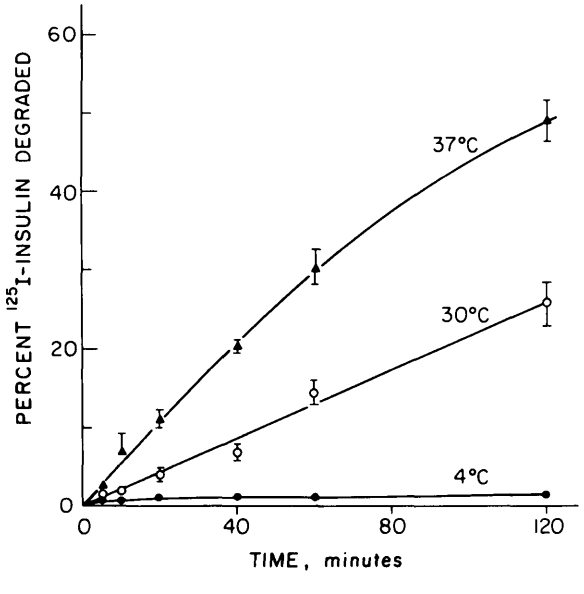
Hormone (10^{-6} M)	Total binding (percent control)	Degradation (percent control)
None (control)	100	100
Pork insulin	52.1 ± 1.6*	67.5 ± 1.9*
Salmon calcitonin	92.8 ± 2.7	86.6 ± 2.9
Arginine vasopressin	89.4 ± 5.8	88.2 ± 2.3
Rat growth hormone	90.1 ± 2.4	91.0 ± 3.9
Pork glucagon	97.4 ± 7.7	90.1 ± 3.2

Four separate brush border membrane preparations were incubated in triplicate at 30°C for 60 min with ^{125}I-insulin (1.7×10^{-10} M) and the various hormones listed. Values are mean ± SEM. Binding is uncorrected for nonspecific binding but is corrected for degradation.
* $P < 0.01$ when compared with the other hormones.

of incubation (Figure 5; $y = 0.219x - 0.297$; $r = 0.95$) and between degradation and membrane protein concentration (Figure 6; $y = 0.174x - 0.252$; $r = 0.95$). At 37°C in the presence of insulin concentrations of 1.7×10^{-10} M, the degrading activity averaged 10 ± 0.5 pg/mg protein/min when measured over a 60-min time period (Figure 5). The presence of native insulin in high concentration, 10^{-6} M, inhibited ^{125}I-insulin degradation (Table 1). Indeed native insulin, 10^{-6} M, produced significantly greater inhibition than the same molar concentrations of calcitonin, arginine vasopressin, rat growth hormone, or glucagon ($P < 0.001$). Degradation of ^{125}I-insulin exposed to brush border membranes for 60 min at 30°C was not significantly altered by the presence of 0.025% Triton X-100 (13.3 ± 1.0% versus 13.5 ± 1.0%).

The products of degradation formed after incubating ^{125}I-insulin with brush border membranes for 60 min at 37°C were studied by gel filtration. As depicted in Figure 7, three main peaks were seen after exposure; peak 1 is the void volume, peak 2 is the insulin peak, and peak 4 corresponds to

FIGURE 5. Time course of ^{125}I-insulin degradation by renal brush border membrane preparations incubated at 37°C, 30°C, and 4°C. Results are the mean ± SEM of four separate membrane preparations.



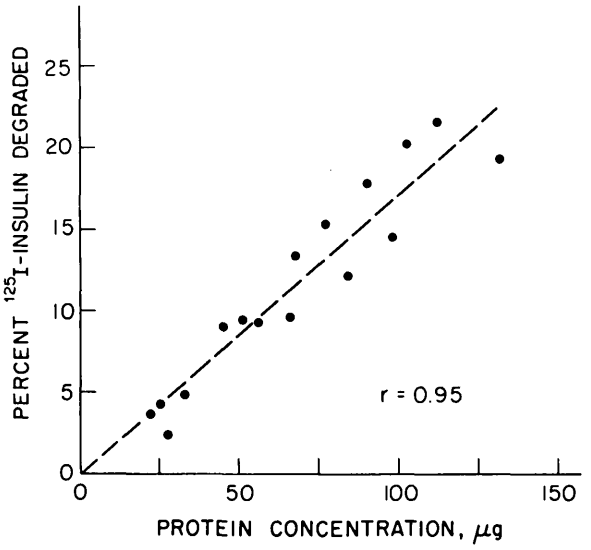


FIGURE 6. Relationship between membrane protein concentration and ^{125}I-insulin (1.7×10^{-10} M) degradation. Each point represents the results of individual experiments in triplicate obtained with membranes incubated for 60 min at 30°C.

the elution volume of monoiodotyrosine. There was a negligible increase in material in the ^{125}I$^-$ elution volume (peak 3). Table 2 shows the percentage of labeled material eluting in each peak and the results of immunoprecipitation of the incubate before and after exposure to the brush border membranes. After 60-min incubation, there was a decrease in radioactivity eluting in the insulin peak and a major increase in lower-molecular-weight material eluting in peak 4. There was a small increase in peak 1 material and slight increase in peak 3 material. On the assumption that all the immunoprecipitable radioactivity resides in the insulin peak, we have expressed the immunoprecipitable radioactivity of the incubate as a percentage of the radioactivity eluting in this peak (Table 2). The percentage immunoreactivity was essentially unaltered by exposure to the brush border membranes, being 93.0 ± 2.0% before and 92.8 ± 2.1% after exposure.

FIGURE 7. Gel filtration pattern of ^{125}I-insulin before and after incubation with renal brush border membranes for 60 min at 37°C.

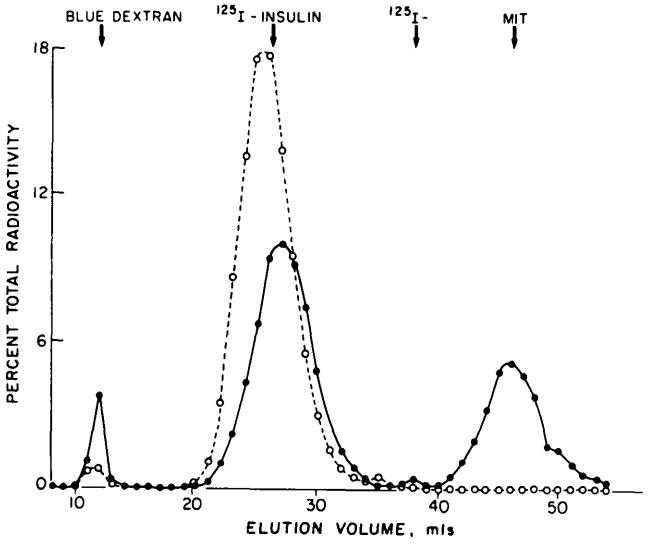


TABLE 2
Analysis of gel filtration profile and immunoprecipitability of ^{125}I-insulin before and after incubation with renal brush border membranes for 60 min at 37°C (N = 4)

Period	Gel filtration radioactive pattern (%)				Immuno-precipitation (%)	Percent immuno-precipitation (percent peak 2 radioactivity)
	Peak 1	Peak 2	Peak 3	Peak 4		
Before	1.6 ± 0.4	97 ± 0.9	1.4 ± 0.5	0	90.1 ± 2.0	93.0 ± 2.0
After	6.1 ± 0.9	59.3 ± 4.4	2.7 ± 1.2	32.1 ± 3.5	51.4 ± 2.7	92.8 ± 2.1

DISCUSSION

The present study reveals the presence of insulin-specific receptors in renal brush border membranes and the ability of these membranes to degrade insulin. Both binding and degradation are time- and temperature-dependent processes. Competitive binding studies indicate that an insulin-specific binding process is indeed present, with little inhibition of binding by calcitonin, arginine vasopressin, rat growth hormone, or glucagon. Proinsulin, which has less biologic activity than insulin, was less effective than native insulin in displacing ^{125}I-insulin. Scatchard analysis of the ^{125}I-insulin binding data obtained in the presence of varying concentrations of native insulin is consistent with either the presence of two classes of receptors, namely, high affinity, low capacity receptors and low affinity, high capacity receptors, or of a single class of receptors exhibiting negative cooperativity. The calculated high affinity constant (2×10^{10} M^{-1}) is higher than that reported with a crude renal plasma membrane preparation (1.3×10^{9} M^{-1}).[6] Review of studies with other tissues[20] reveals binding sites with high affinity constants of the order 10^{8}–10^{10} M^{-1}. Binding to the brush border membranes also exhibited a significant nonspecific component that is time and temperature dependent and cannot be saturated at high insulin concentrations. At 30°C, this nonspecific component accounts for approximately one-third of total radioactivity bound. Studies to further characterize this nonspecific process and to determine the nature of the material bound are required.

The main product of the brush border membrane degradation was a low-molecular-weight form that eluted with monoiodotyrosine. Small amounts of material eluting in the void volume were also formed. The exact nature of this high-molecular-weight material is not known, but production of this material also occurs in the isolated kidney[22] and in other tissues.[23,24] A negligible increase in material eluting in the ^{125}I$^-$ peak was observed; this differs from the functioning isolated kidney and from kidney homogenates where large amounts of ^{125}I$^-$ appear consequent to deiodination of ^{125}I-tyrosine.[4,22] To assess whether exposure to brush border membranes results in a partial modification of the insulin molecule with loss of immunoreactivity but without a change in its gel filtration characteristics, the material eluting in the insulin peak was evaluated for immunoreactivity. This was achieved by assuming that all the immunoreactive material resides in the insulin peak, and then expressing the percentage of immunoprecipitable radioactivity in the sample as a percentage of the radioactivity eluting in the insulin peak. Based on this calculation, the percent immunoreactivity of the insulin peak material was essentially unchanged after exposure to the brush border membrane. This is unlike the situation observed after exposure of insulin to the contraluminal aspect of tubular cells in the isolated perfused rat kidney[22] or exposure to isolated liver plasma membranes.[23] Under both these circumstances, nonimmunoreactive material eluting in the insulin peak and lower-molecular-weight material are produced. However, the present findings are consistent with the fate of insulin removed from the circulation of the isolated perfused rat kidney by means of glomerular filtration;[22] apparently the filtered insulin exposed to the brush border membrane and absorbed in the proximal tubule undergoes complete degradation. It is likely that the insulin-degrading activity of the brush border membrane contributes to the complete degradation of filtered insulin.

Brush border membrane insulin-degrading activity appears to consist of a specific and a nonspecific component. Specificity is suggested by the finding that an excess of native insulin 10^{-6} M inhibited insulin degradation by 32%, whereas several other unrelated peptides were significantly less effective, inhibiting degradation by less than 13%. It is not apparent whether this difference reflects inhibition of specific receptors or enzymes. The presence of nonspecific degradation is suggested by the residual degrading activity in the presence of an excess of native insulin. It is noteworthy that brush border membranes degrade other peptide hormones, such as calcitonin[25] and especially small linear peptides, e.g., angiotensin and bradykinin.[26]

The exact relationship between binding and degradation of insulin by plasma membranes from other tissues is unclear.[27] Although earlier studies suggest that binding and degradation may be dissociated,[28] recent studies indicate an essential role of the insulin receptor in insulin degradation:[29,30] binding to the receptor resulting in exposure to insulin-degrading enzymes. Evidence supporting a relationship between binding and degradation in the kidney has been provided in a study with isolated renal tubules,[31] where pretreatment with trypsin abolished both binding and degradation of insulin. In this latter preparation, insulin is exposed to the contraluminal membranes and to a lesser extent the brush border membranes.

The binding of the insulin to plasma membranes in general may serve important physiologic functions, namely, delivery or exposure of insulin to degrading enzymes and initiation of hormone action. It is unclear whether the binding of insulin to its specific receptor in the brush border membrane is the sole initiator of pinocytosis of the hormone, or whether nonspecific binding also has this effect. If both processes have this effect, then uptake would consist of both a specific and a nonspecific component. The exact nature of this latter component bears further study, but it is perhaps relevant that Just and Habermann[3] made observations suggesting that nonhormone peptides bind to the brush border membranes in a nonspecific charge-related manner and

that this binding may promote pinocytosis. The relationship of brush border membrane insulin-specific binding to the action of the hormone is of particular interest. Other peptide hormones, such as parathyroid hormone,[32] initiate their actions by binding to specific receptors on the contraluminal plasma membranes, a process likely to be true for insulin.[22,33] Whether binding to brush border membrane insulin-specific receptors plays any role in the action of insulin on the kidney is an important question that requires further study.

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