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**Research Article**

To investigate a possible action of insulin on the glomerulus, the binding of 125I-insulin to the isolated glomeruli prepared from rat kidney was examined. When incubated at 22 degrees C, 125I-insulin binding proceeded with time and reached a steady state at 45 min at which time nonspecific binding was less than 25% of total binding. A small fraction of 125I-insulin was degraded during incubation. This binding was specific to insulin in that it was inhibited by unlabeled porcine and beef insulins and to a lesser extent by porcine proinsulin and desalanine-desasparagine insulin, but not by glucagon, parathyroid hormone, vasopressin, calcitonin, and angiotensin II. Increasing concentrations of nonlabeled insulin displaced 125I-insulin binding in a dose-dependent fashion. Scatchard plot of the data was curvilinear consistent with either two classes of receptors with different affinities or a single class of receptors that demonstrate negative cooperativity. The addition of excess nonlabeled insulin to the glomeruli preincubated with 125I-insulin resulted in a rapid dissociation of approximately or equal to 70% of bound 125I-insulin. Insulin decreased the increments in glomerular cyclic AMP levels by epinephrine and by prostaglandin E2, but not those by histamine. These data showed the presence of specific insulin receptors in the glomeruli, and that insulin action may be, at least in part, through modulation of glomerular cyclic AMP concentrations. Such action of insulin [...]

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# Binding of $^{125}\text{I}$ -Insulin to the Isolated Glomeruli of Rat Kidney

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**ABSTRACT** To investigate a possible action of insulin on the glomerulus, the binding  $^{125}\text{I}$ -insulin to the isolated glomeruli prepared from rat kidney was examined. When incubated at  $22^\circ\text{C}$ ,  $^{125}\text{I}$ -insulin binding proceeded with time and reached a steady state at 45 min at which time nonspecific binding was  $<25\%$  of total binding. A small fraction of  $^{125}\text{I}$ -insulin was degraded during incubation. This binding was specific to insulin in that it was inhibited by unlabeled porcine and beef insulins and to a lesser extent by porcine proinsulin and desalanine-desasparagine insulin, but not by glucagon, parathyroid hormone, vasopressin, calcitonin, and angiotensin II. Increasing concentrations of nonlabeled insulin displaced  $^{125}\text{I}$ -insulin binding in a dose-dependent fashion. Scatchard plot of the data was curvilinear consistent with either two classes of receptors with different affinities or a single class of receptors that demonstrate negative cooperativity. The addition of excess nonlabeled insulin to the glomeruli preincubated with  $^{125}\text{I}$ -insulin resulted in a rapid dissociation of  $\cong 70\%$  of bound  $^{125}\text{I}$ -insulin. Insulin decreased the increments in glomerular cyclic AMP levels by epinephrine and by prostaglandin  $\text{E}_2$ , but not those by histamine. These data showed the presence of specific insulin receptors in the glomeruli, and that insulin action may be, at least in part, through modulation of glomerular cyclic AMP concentrations. Such action of insulin may underlie the alteration in glo-

merular ultrafiltration and the development of glomerular lesions in diabetes mellitus, a disease in which insulin deficiency or the tissue resistance to insulin exists.

## INTRODUCTION

Several lines of evidence suggest that insulin may have biological actions on glomerular function and metabolism. First, distinct glomerular lesions develop in patients with diabetes mellitus (1, 2). Second, the glomerular filtration rate (GFR)<sup>1</sup> becomes higher than normal early in the course of juvenile onset diabetes mellitus while renal plasma flow remains normal, resulting in an elevation of the filtration fraction; filtration fractions averaging 28–30% have been reported (3–5). Because diabetes mellitus is a condition in which deficiency of insulin or tissue resistance to insulin exists, insulin may play some role in the regulation of glomerular function and metabolism. It has been well established that most, but maybe not all, of insulin actions are initiated by the binding of insulin to its specific receptors on plasma membrane of target cells (6, 7). It is possible, therefore, that insulin receptors will exist in the glomerulus if insulin is to have direct actions on glomerular cells. Based on this assumption we carried out a study to examine whether insulin receptors are present in the glomerulus and, if so, to characterize these receptors.

## METHODS

Wistar male rats, weighing 200–250 g, were used. Rats were sacrificed by decapitation and kidneys were quickly removed and placed in an ice-cold isotonic saline. Isolated glomeruli were prepared using the methods of Sraer et al. (8) and Nørgaard

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<sup>1</sup>Abbreviations used in this paper: GFR, glomerular filtration rate; KRT, Krebs-Ringer-tris-HCl buffer.

(9) with minor modifications. The cortical tissue was passed through a metal sieve (150 mesh, 105  $\mu\text{m}$ ) into a Petri dish placed on ice with the aid of a spatula. The material from the undersurface of the sieve was collected with a clean spatula and the cortical homogenate was suspended in cold calcium- and glucose-free Hanks' solution (only calcium- and glucose-free Hanks' solution was used in this study and will be referred to as Hanks' solution in this paper). The cortical tissue suspension was passed through a 25-gauge needle five times and the suspension was centrifuged at 1,000 g for 10 min at 4°C. The pellet was resuspended in 27% Ficoll (Sigma Chemical Co., St. Louis, Mo.) in Hanks' solution kept at 4°C. 3 ml each of the Ficoll solution containing cortical homogenate from one kidney was transferred to a centrifuge tube, and layers of Ficoll in the Hanks' solution in decreasing concentrations were then carefully placed over this suspension (2 ml, 23%; 2 ml, 20%; and 3 ml, 11%) to give a total gradient volume of 10 ml. After centrifugation at 1,150 g for 10 min at 4°C, glomeruli appeared at the 11/20% interphase and occasionally also at the 20/23% interphase with virtually no contamination with other renal cortical cells. These two layers were collected with the aid of a Pasteur pipette, diluted with the cold Hanks' solution, and centrifuged at 3,000 rpm for 10 min at 4°C. The pellet was resuspended and centrifuged again. This procedure was repeated two more times. The final pellet was suspended in Krebs-Ringer-tris-HCl (KRT) buffer, pH 7.4 (114 mM NaCl, 5 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 1.0 mM  $\text{CaCl}_2$ , 17 mM  $\text{NaHCO}_3$ , and 16 mM tris-HCl), to give a protein concentration of 1.5–2.5 mg/ml. Initially, kidneys were perfused with ice-cold isotonic saline *in situ* through a polyethylene (PE-100) catheter inserted in the abdominal aorta to wash out blood from glomerular capillaries. However, it was found that no detectable binding of insulin was observed to the blood cells obtained from peripheral circulation at comparable hemoglobin concentrations contained in the glomerular preparations. Thus, we did not routinely perfuse kidney *in situ* to prepare isolated glomeruli.

The isolated glomeruli (150–250  $\mu\text{g}$  protein) were incubated in a flat-bottom 17  $\times$  52 mm glass scintillation vial containing 200  $\mu\text{l}$  KRT buffer, pH 7.4, containing 3% bovine serum albumin (Fraction V; radioimmunoassay grade, Sigma Chemical Co.), 1 mg/ml glucose, 0.5 nM monoiodinated- $^{125}\text{I}$ -insulin ( $^{125}\text{I}$ -porcine monocomponent insulin, a gift from Eli Lilly & Co., Indianapolis, Ind.;  $\approx 500$ –600 Ci/mmol sp act; kindly prepared by Dr. S. Kaplan, University of California, Los Angeles, School of Medicine, Los Angeles, Calif. using the method of Freychet et al. [10] with a modification of Olefsky and Reaven [11]) with or without varying concentrations of nonlabeled porcine insulin, other insulins and peptide hormones, or inhibitors of insulin degradation (Results). In most experiments the isolated glomeruli were incubated at 22°C for 45 min unless otherwise specified in Results. At the end of the incubation, 3 ml of ice-cold KRT buffer was added to the incubation vial, and the contents of the vial were transferred to a plastic conical centrifuge tube and centrifuged at 3,000 rpm for 3 min at 4°C. The supernate was transferred to a test tube; another 3 ml of ice-cold KRT buffer was carefully layered onto the pellet and centrifuged again. The resultant supernate was aspirated by suction and the pellet was counted for radioactivity. The initial supernate was divided into aliquots; to each aliquot an equal volume of 10% trichloroacetic acid (TCA) was added, mixed, and centrifuged at 3,000 rpm for 10 min at 4°C. The radioactivities of the initial supernate and the TCA-treated supernate were counted separately for each incubation. Additionally, an aliquot of glomerular suspension was added to the incubation mixture containing 0.5 nM  $^{125}\text{I}$ -insulin and 3 ml of ice-cold KRT buffer, immediately mixed, centrifuged, and the

pellet was rinsed with another 3 ml portion of ice-cold KRT buffer as described above for the separation of glomeruli from the incubation medium; the radioactivity "trapped" in the pellet was <0.3% of total radioactivity added. This trapped  $^{125}\text{I}$ -insulin was determined in each experiment, and the value was subtracted from the radioactivity in the pellet.

In some experiments, the isolated glomeruli were incubated with 10  $\mu\text{g}/\text{ml}$  trypsin (bovine pancreas, type XI, Sigma Chemical Co.) at 37°C for 15 min. Then, soybean trypsin inhibitor (type I-S, Sigma Chemical Co.) (50  $\mu\text{g}/\text{ml}$ ) was added to the incubation medium and the incubation was continued at 37°C for another 10 min. At the end of this 25 min preincubation, the incubation vials were placed on ice; 0.5 nM  $^{125}\text{I}$ -insulin was added to the incubation mixture, and the glomeruli were incubated at 22°C for 45 min, and the insulin binding was determined. Appropriate control incubations for this experiment were run in parallel as indicated in Results.

To determine changes in cyclic nucleotide levels in the glomeruli, the isolated glomeruli were first incubated at 37°C for 10 min in 200  $\mu\text{l}$  KRT buffer, pH 7.4, containing 3% bovine serum albumin and 0.2 mM methyl-isobutylxanthine. Then, the test hormone or appropriate vehicle in a volume of 10  $\mu\text{l}$  was added and the incubation was continued for an additional 3 min. The incubation was then terminated by the addition of 0.5 ml 10% TCA. After removing the denatured protein by centrifugation, the TCA in the supernate was extracted three times in a glass tube with ether saturated with water. Residual ether was removed by placing the tube in a boiling water bath for 3 min. Aliquots of the extracts were assayed after acetylation for cyclic AMP and cyclic GMP by the radioimmunoassay using the specific antibodies to cyclic nucleotides prepared in our laboratory as described by Steiner et al. [12]. Since 25-fold greater concentration of cyclic GMP did not interfere with the binding of cyclic AMP to anticyclic AMP antibody and since 50-fold greater concentration of cyclic AMP did not interfere with the binding of cyclic GMP to the anticyclic GMP antibody, we did not separate cyclic AMP and cyclic GMP in the glomerular extracts before the assays in this study. We measured the cyclic nucleotides in both intraglomerular space and the incubation medium. In separate studies we found that cyclic AMP in the incubation medium under these incubation conditions represents <10% of total cyclic AMP (in the glomeruli plus in the incubation medium), and that any increment in cyclic AMP in response to the agonists was limited to the intraglomerular space.

Glomerular protein concentrations were measured using the Protein Assay Kit supplied by Bio-Rad Laboratories, Richmond, Calif. All experiments were repeated at least twice. Data were expressed as mean  $\pm$  SD, and analyzed by Student's *t* test.

### Calculations and Analyses of Data

The radioactivity in the pellet after subtracting the trapped count is referred to as "total bound" insulin. "Nonspecific" binding represents radioactivity in the pellet obtained in the presence of a large amount of nonlabeled porcine insulin, 1.7  $\mu\text{M}$  or greater. "Specifically bound" insulin is derived from total bound minus nonspecific and will represent "insulin bound" in this report unless otherwise indicated. The fraction in the supernate precipitable by 5% TCA (final concentration) was referred to as "intact" hormone, and that not precipitable as "degraded" hormone (13, 14). In separate studies, it was found that the fraction not precipitated by TCA did not bind to the glomeruli. The preparations of  $^{125}\text{I}$ -insulin used were 94–97% precipitable by TCA, and the incubations of  $^{125}\text{I}$ -insulin at 22°C for 2 h without glomeruli did not increase the fraction of the radioactivity not precipitable by TCA. The

TABLE I  
Percentage of Intact Insulin Assessed by TCA Precipitation and by the Binding to the Excess Anti-Insulin Antibody

	TCA precipitation	Binding to anti-insulin antibody
Intact <sup>125</sup> I-insulin, %		
Before incubation	95.0±0.8	92.3±3.5
After 45 min incubation at 22°C without glomeruli	94.2±0.5	90.0±4.1
After 45 min incubation at 22°C with glomeruli	89.3±0.5	84.8±2.2
Insulin bound, %	1.93±0.15	1.99±0.16
Intact insulin bound, %	2.06±0.16	2.17±0.17

Values are the mean±SD of quadruplicate incubations. <sup>125</sup>I-Insulin, 0.5 nM, was incubated in KRT buffer with or without isolated glomeruli (210 μg protein) at 22°C for 45 min.

TCA-nonprecipitable fraction of <sup>125</sup>I-insulin present before the incubation was subtracted to calculate total <sup>125</sup>I-insulin added and changes in intact and degraded insulin. Percentage of insulin bound represents a fraction of total insulin that is specifically bound to the glomeruli (15). Because the TCA precipitation method may underestimate the degradation of insulin (13, 14), we compared the fraction of insulin degraded by TCA precipitation with excess anti-insulin antibody. As shown in Table I, there was no significant difference in the fraction of intact <sup>125</sup>I-insulin preparation assessed by TCA and antibody precipitation methods. After incubation with isolated glomeruli, 89.3±0.5% of <sup>125</sup>I-insulin remained intact judged by TCA method, whereas 84.8±2.2% was intact by the antibody precipitation method. These discrepant results are in agreement with previous observations that TCA precipitation may underestimate true degradation of insulin when a significant fraction of the hormone is degraded (13, 14). Since the total bound fraction was a minute fraction of the total <sup>125</sup>I-insulin present in the incubation medium, and since the degradation of <sup>125</sup>I-insulin by the glomeruli was small under the incubation conditions employed (<10% in most of the experiments), the insulin binding was expressed as percentage of total <sup>125</sup>I-insulin. In addition to the several assumptions made for the treatment of data of this kind as described (15), it is assumed that all bound hormone is intact for the calculation of the fractional insulin binding. Although a minor fraction of "bound" insulin may be located intracellularly and may not be intact (Results), this assumption is a useful simplification for the analyses of data.

## RESULTS

*Time-course of insulin binding to the glomerulus and the effects of glomerular concentrations.* As shown in Fig. 1A, there was an initial rapid binding of <sup>125</sup>I-insulin to the glomeruli that slowed after 30 min of incubation. The nonspecific binding increased in a linear fashion; thus, the specific binding of insulin, i.e., the difference between the total insulin binding minus the nonspecific binding, reached an apparent steady state at 45 min of incubation. A small fraction of radioactivity in the incubation medium became nonprecipitable by TCA, providing a rough estimate of insulin degradation (Fig. 1B). Therefore, most of the experiments were carried out at 22°C for 45 min at

which time insulin degradation was <10% by TCA precipitation, and the results were expressed as percentage of total insulin bound to the glomeruli unless otherwise indicated.

As shown in Fig. 2, there was a linear relation between glomerular protein concentration and the percentage of insulin bound. Thus, the glomerular protein concentrations of 150–250 μg/incubation were used throughout the study.

*Specificity of insulin binding to the glomeruli.* When the glomeruli were incubated with 0.5 nM <sup>125</sup>I-insulin with various peptide hormones, which may act on the glomeruli, none of the peptide hormones tested but insulin had any significant effect on the binding of <sup>125</sup>I-insulin (Table II). To further define the specificity of insulin binding to the glomerulus, the displacement of <sup>125</sup>I-insulin by nonlabeled insulins were examined. As shown in Table II, the displacement of <sup>125</sup>I-insulin

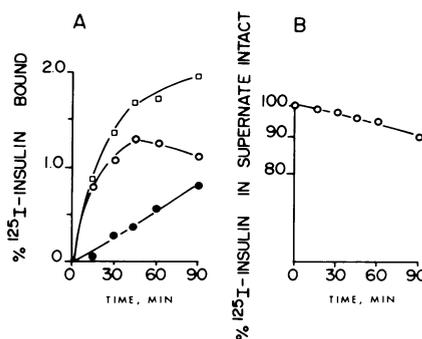


FIGURE 1 Time-course of insulin binding and degradation by the isolated glomeruli at 22°C. (A) Time-course of <sup>125</sup>I-insulin binding to the glomeruli at 22°C with 0.5 nM <sup>125</sup>I-insulin with (nonspecific binding, ●) or without (total binding, □) 1.7 μM nonlabeled insulin. The specific binding (○) is the difference between the total binding and the nonspecific binding. (B) <sup>125</sup>I-insulin degradation assessed by TCA precipitation method. Glomerular protein concentration was 147 μg. Each point is the mean of duplicate incubations.

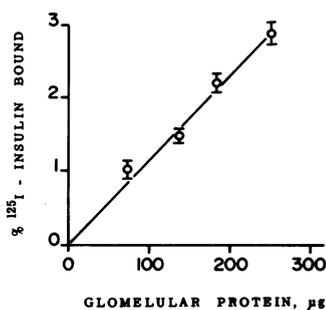


FIGURE 2 Relation between glomerular protein concentrations and the percentage of insulin bound to the glomeruli. Each point is the mean of triplicate incubations with vertical bars representing SD.

by porcine proinsulin and desalanine-desasparagine porcine insulin was less than that by porcine insulin at an equimolar concentration, whereas the displacement by beef insulin was comparable to that by porcine insulin.

TABLE II  
Effects of Various Insulins and other Hormones on  $^{125}\text{I}$ -Insulin Binding to Isolated Glomeruli

Hormone added	Concentration	$^{125}\text{I}$ -insulin binding % of control
1 None		100.0±5.1
Porcine insulin	0.002 $\mu\text{M}$	78.2±4.6
	0.17 $\mu\text{M}$	30.4±2.4
Beef insulin	0.002 $\mu\text{M}$	78.4±5.3
	0.17 $\mu\text{M}$	29.4±3.6
Porcine proinsulin	0.002 $\mu\text{M}$	91.3±5.3
	0.17 $\mu\text{M}$	52.7±4.0
Desalanine-desasparagine porcine insulin	0.002 $\mu\text{M}$	97.5±5.4
	0.17 $\mu\text{M}$	78.8±4.1
2 None		100.0±6.5
Glucagon	14 $\mu\text{M}$	98.4±7.3
Parathyroid extract	5 U/ml	110.6±5.1
Arginine vasopressin	0.1 U/ml	91.9±3.6
Salmon calcitonin	0.2 U/ml	99.6±4.2
Angiotensin II	50 $\mu\text{M}$	97.7±5.1
Porcine insulin	1.7 $\mu\text{M}$	27.5±2.9

Isolated glomeruli were incubated at 22°C for 45 min with 0.5 nM  $^{125}\text{I}$ -porcine insulin with or without various test hormones indicated, and the binding of  $^{125}\text{I}$ -insulin to glomeruli was examined. The mean value of the binding in the absence of nonlabeled test hormone was taken as 100%. Values are the mean±SD of triplicate incubations. Insulins, glucagon, and parathyroid extract were obtained from Eli Lilly & Co., arginine vasopressin from Dr. Richard Weitzman, Harbor General Hospital, Los Angeles, Calif., salmon calcitonin from Armour Pharmaceutical Co., Scottsdale, Ariz., and angiotensin II from CIBA-Geigy Corp., Summit, N. J.

Effects of increasing concentrations of nonlabeled insulin on the  $^{125}\text{I}$ -insulin binding. When the glomeruli were incubated with 0.5 nM  $^{125}\text{I}$ -insulin together with increasing concentrations of nonlabeled porcine insulin at 22°C for 45 min, the competitive displacement of  $^{125}\text{I}$ -insulin binding to the glomeruli was observed as shown in Fig. 3. The Scatchard plot of the data based on the assumption that the binding was in a steady state was curvilinear (Fig. 4). A high affinity association constant of  $1.8 \times 10^8$  liter/mol was obtained. The curvilinear pattern of the plot appears to be consistent with either the presence of two classes of insulin binding sites with different affinities or the presence of a single class of receptors with a negative cooperative type of hormone receptor interaction (15, 16).

Dissociation of  $^{125}\text{I}$ -insulin binding by excess nonlabeled insulin. The addition of excess nonlabeled porcine insulin (1.7  $\mu\text{M}$ ) to the incubation medium after 45 min incubation of the glomeruli with 0.5 nM  $^{125}\text{I}$ -insulin resulted in a rapid dissociation of bound radioactivity as shown in Fig. 5. This dissociation was not complete leaving  $\approx 30\%$  of bound radioactivity remaining with the glomeruli.

Effects of inhibitors on insulin binding. The addition of *N*-ethylmaleimide (0.5 mM) completely inhibited the degradation of insulin judged by TCA precipitation but without effect on the insulin binding to the glomeruli (data not shown). The pretreatment of the glomeruli with trypsin resulted in a complete inhibition of both binding and degradation of insulin by the glomeruli as shown in Table III. The pretreatment of glomeruli by trypsin-trypsin inhibitor complex or trypsin inhibitor alone was without effect on both insulin binding and degradation.

Effects of insulin on glomerular cyclic nucleotide levels. It has been demonstrated that various hormonal

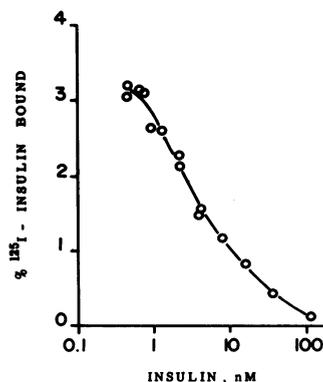


FIGURE 3 Competitive displacement of  $^{125}\text{I}$ -insulin binding to the glomeruli by nonlabeled insulin. The isolated glomeruli (170  $\mu\text{g}$  protein) were incubated with 0.5 nM  $^{125}\text{I}$ -insulin with varying concentrations of nonlabeled porcine insulin at 22°C for 45 min. Each point represents a single incubation.

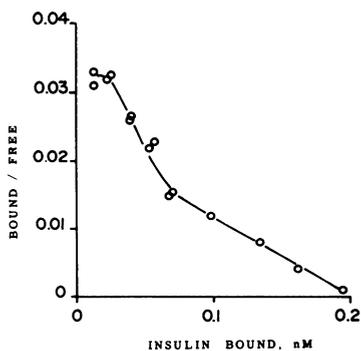


FIGURE 4 Scatchard analysis of the insulin binding. Data were calculated from Fig. 3.

agents increase glomerular cyclic AMP levels by stimulating adenylate cyclase activity, and that insulin may modulate cyclic nucleotide levels in various tissues by interacting with other peptide and amine hormones (17–22). It is possible, therefore, that insulin may affect glomerular cyclic nucleotide levels. As shown in Table IV, insulin itself had little effect on cyclic AMP levels in the glomeruli. However, insulin attenuated the rises in cyclic AMP levels induced by epinephrine and prostaglandin  $E_2$ , whereas it was without effect on the rises in cyclic AMP induced by histamine. Insulin had no effect on cyclic GMP concentrations in the glomeruli with or without these agonists (data not shown).

## DISCUSSION

These results demonstrate the presence of insulin binding and degradation in the isolated glomeruli of rat kidney. This binding was specific for insulin and dependent on the incubation period and the concentra-

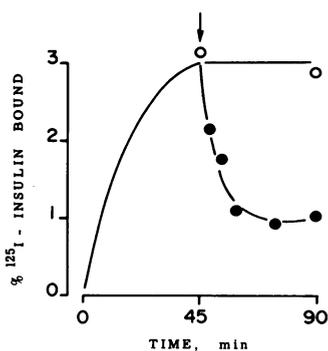


FIGURE 5 Dissociation of  $^{125}\text{I}$ -insulin bound to the glomeruli by the addition of an excess nonlabeled insulin. Isolated glomeruli (280  $\mu\text{g}$ ) were incubated at  $22^\circ\text{C}$  for 90 min with 0.5 nM  $^{125}\text{I}$ -insulin with (●) or without (○) subsequent addition of 3.3  $\mu\text{M}$  nonlabeled insulin at 45 min as indicated by an arrow. Upon the addition of nonlabeled insulin,  $\cong 70\%$  of specifically bound  $^{125}\text{I}$ -insulin was rapidly dissociated from the glomeruli. Each point represents the mean of duplicate incubations.

TABLE III  
Effects of Trypsin Pretreatment on the Binding and Degradation of  $^{125}\text{I}$ -Insulin by the Isolated Glomeruli

Additions in the preincubation		$^{125}\text{I}$ -insulin bound	Degradation
1st (15 min)	2nd (10 min)		
		%	%
None	None	$1.95 \pm 0.08$	$6.8 \pm 0.6$
trypsin	inhibitor	$0.20 \pm 0.40$	$0.2 \pm 0.4$
None	inhibitor	$2.09 \pm 0.14$	$6.9 \pm 0.6$
None	trypsin + inhibitor	$2.15 \pm 0.20$	$6.7 \pm 0.4$

Isolated glomeruli were incubated at  $37^\circ\text{C}$  for 15 min with or without trypsin, 10  $\mu\text{g}/\text{ml}$ , followed by 10 min incubation at  $37^\circ\text{C}$  with or without trypsin inhibitor, 50  $\mu\text{g}/\text{ml}$  (preincubation). After cooling the incubation mixture, isolated glomeruli were incubated at  $22^\circ\text{C}$  for 45 min with 0.5 nM  $^{125}\text{I}$ -insulin, and the insulin binding and degradation (by TCA precipitation) were determined. Values are the mean  $\pm$  SD of quadruplicate incubations. See text for details.

tion of the glomeruli. When incubated at  $22^\circ\text{C}$ , insulin binding to the glomeruli reached a steady state at 45 min, at which time nonspecific binding was  $<25\%$  of total insulin binding. The results of competitive binding studies indicate the specificity of the glomerular insulin receptors. Thus, unlabeled porcine and beef insulin and, to a lesser extent the biologically less active insulins

TABLE IV  
Effects of Insulin, Epinephrine, Prostaglandin  $E_2$ , and Histamine on Cyclic AMP Levels in the Isolated Glomeruli

Experiment	Additions	Cyclic AMP
		pmol/mg protein
1	None	$14.1 \pm 1.6$
	insulin, 3.4 $\mu\text{M}$	$11.8 \pm 1.8$
	epinephrine, 1.0 $\mu\text{M}$	$27.4 \pm 0.6$
	epinephrine + insulin	$20.9 \pm 1.9^*$
2	None	$25.2 \pm 2.8$
	insulin, 2.0 $\mu\text{M}$	$23.8 \pm 1.1$
	prostaglandin $E_2$ , 1.0 $\mu\text{M}$	$48.3 \pm 3.4$
	prostaglandin $E_2$ + insulin	$36.5 \pm 2.5^*$
	histamine, 4.4 $\mu\text{M}$	$39.7 \pm 2.4$
	histamine + insulin	$38.3 \pm 0.7$
3	None	$14.5 \pm 1.8$
	insulin, 2.0 $\mu\text{M}$	$15.0 \pm 1.0$
	histamine, 1.0 $\mu\text{M}$	$27.5 \pm 2.8$
	histamine + insulin	$27.0 \pm 2.2$

Values are the mean  $\pm$  SD of quadruplicate incubations. See text for details.

\* Significantly ( $P < 0.01$ ) different from values with each hormone alone.

such as porcine proinsulin and desalanine-desasparagine porcine insulin (23), were able to displace  $^{125}\text{I}$ -insulin, whereas no displacement was observed with glucagon, parathyroid hormone, vasopressin, calcitonin, and angiotensin II.

Increasing concentrations of unlabeled insulin resulted in a dose-dependent displacement of  $^{125}\text{I}$ -insulin binding to the glomeruli. The Scatchard plot of the data was curvilinear and consistent with either two classes of binding sites of high affinity low capacity and low affinity high capacity or a negative cooperative type of a single class of receptors (15, 16). The calculated association constant for high affinity sites was somewhat lower than those reported in other cell systems by some investigators, but similar to those by others (15). Relatively high concentrations of  $^{125}\text{I}$ -insulin (0.5 nM) were used in this study because of a small fraction of insulin binding and of relatively low specific activities of  $^{125}\text{I}$ -insulin compared with those used in other studies. Therefore, it is possible that the highest affinity binding sites were not detected and instead the "intermediate" affinity sites were obtained (15). In addition, the binding studies were done after 45 min, which is slightly short of equilibrium which tends to underestimate the true affinity. Because the glomerulus consists of a heterogeneous cell population, these analyses of the data may be of limited value. However, these data provide a basis to compare the properties of insulin binding to the isolated glomeruli with those observed in other tissues.

The addition of excess nonlabeled porcine insulin to the glomeruli that were in equilibrium with  $^{125}\text{I}$ -insulin resulted in a rapid dissociation of  $^{125}\text{I}$ -insulin from the glomeruli, suggesting that the majority ( $\approx 70\%$ ) of  $^{125}\text{I}$ -insulin was bound in a reversible fashion on the surface of glomerular cells, probably to its receptors in the plasma membranes. The fraction of bound radioactivity not dissociated by the subsequent addition of excess nonlabeled insulin may represent  $^{125}\text{I}$ -insulin bound irreversibly to the plasma membranes or located intracellularly, either as an intact hormone or as degradation products. Several recent studies demonstrated that insulin may enter the cells as an intact molecule, and such a process may be operative in glomerular cells (24, 25).

The inhibition of insulin degradation by *N*-ethylmaleimide without any significant effect on insulin binding may suggest a possible involvement of insulin-glutathione-transhydryase in the insulin degradation which is preceded or independent of insulin binding to its receptor sites. It has been demonstrated in liver and fat cell membranes that the trypsin pretreatment can inhibit insulin binding to the receptors. That the pretreatment of glomeruli with trypsin abolished the binding and degradation of insulin may suggest that

the insulin-receptor binding may be linked to insulin degradation as suggested in the liver (26).

The glomerulus is composed of three major cell types: epithelium, endothelium, and mesangium. Preliminary autoradiographic studies showed the insulin binding to the surface of the epithelium and the endothelium (unpublished observation). The presence of insulin receptors in the vascular endothelium has been demonstrated by Bar et al. (27). These data suggest a possible role of insulin to regulate metabolism and function of glomerular epithelium and endothelium through the binding to the receptors on the plasma membranes, and cyclic AMP may be involved as a mechanism. In diabetes mellitus, a condition in which deficiency of insulin or tissue resistance to insulin exists, the presence of abnormalities in the glomerular basement membrane collagen biosynthesis has been demonstrated in both man and in experimental animals. Thus, the glomerular basement membrane collagen in diabetes is characterized by an increased hydroxylysine content with increased glucosyltransferase and lysyl-hydroxylase activities (28, 29). These abnormalities in glomerular basement membrane collagen biosynthesis can be prevented by insulin treatment in experimental diabetic animals, even in the absence of complete correction of hyperglycemia, suggesting the role of insulin on the regulation of basement membrane collagen biosynthesis (30). On the other hand, studies by Wahl and his associates (31) and by Beisswenger (32) failed to show abnormal glomerular basement membrane metabolism and composition in diabetic animals. Nonetheless, if the glomerular epithelium is the primary cell type responsible for the production of basement membrane collagen (33), the possible presence of insulin binding to the epithelium may be associated with a role of insulin on the epithelial cell function and collagen biosynthesis.

Early in the course of diabetes mellitus in man a greater than normal GFR with normal renal blood flow and, thus, an elevated filtration fraction have been reported (3-5). Although the exact mechanisms responsible for the elevated GFR and filtration fraction have not been elucidated, elevated plasma levels of growth hormone and glucose have been proposed as possible mechanisms (5). That the growth hormone tends to increase both GFR and renal blood flow and that hyperglycemia by glucose infusion in normal and diabetic patients increased GFR only minimally, but not to the extent seen in untreated early diabetic patients, suggest roles played by elevated plasma growth hormone levels and hyperglycemia are of minor significance (34). Insulin administration to these diabetic patients with high GFR and high filtration fraction results in a fall in GFR and filtration fraction toward normal within 1-2 wk, an observation demonstrating the reversible

nature of the abnormal glomerular functions in these patients (5). What cell types are responsible for the elevated GFR and filtration fraction and how such changes are induced in diabetes are not known.

It has been demonstrated that various hormonal agents such as parathyroid hormone, vasopressin, prostaglandins, catecholamines, and histamine stimulate glomerular adenylate cyclase, probably through the binding to their specific receptors, and increase glomerular cyclic AMP levels (17–22). Baylis et al. (35–37), using micropuncture techniques, demonstrated that these hormonal agonists may affect GFR accompanied by changes in the glomerular capillary ultrafiltration coefficient and suggested that their effects on the glomerular capillary ultrafiltration coefficient may be mediated by cyclic AMP. The determinants of glomerular ultrafiltration other than ultrafiltration coefficient may also change with these hormonal agents and therefore the fall in ultrafiltration coefficient may not necessarily be accompanied by a reduction in GFR. As shown in this study, insulin does attenuate the epinephrine and prostaglandin E<sub>2</sub>-induced rises in cyclic AMP levels in the glomeruli, although insulin per se was without effect. This observation is of important in two respects; on the one hand, the data demonstrate a possible biological significance of insulin receptors in the glomeruli, and on the other, the data imply a possible role of insulin in the regulation of GFR. It is of note that both clearance and micropuncture studies failed to show the effect of both exogenous and endogenous hyperinsulinemia on GFR in normal humans and in dogs (38, 39). The circulating and local (intraglomerular) concentrations of catecholamines and prostaglandins may be different in normal and disease states such as diabetes mellitus. Thus, it is possible that insulin may affect GFR by modulating the effects of catecholamines and prostaglandins on the determinants of glomerular ultrafiltration, and as such insulin effects on GFR may become apparent only in certain conditions such as diabetes mellitus. Considering the complex nature of factors governing glomerular ultrafiltration, further studies seem warranted to investigate in detail the effects of insulin on the determinants of GFR.

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