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The Renal Metabolism of Insulin: Urinary Insulin Excretion in Patients With Mutant Insulin Syndrome (Insulin Wakayama)

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Many studies have shown that the kidney plays an important role in the metabolism of many proteins and small peptides. To understand insulin handling in the kidney, we examined urinary insulin excretion under several conditions in patients with mutant insulin syndrome (MIS; insulin Wakayama). Urinary excretion of insulin was studied using high-performance liquid chromatography analysis in patients with MIS. In these patients, most of the insulin extracted from a 24-hour urine collection and from urine collected after stimulation of insulin secretion by glucose or glucagon was normal insulin, whereas 90% of serum insulin is structurally abnormal (Leu-A3 insulin). On the other hand, arginine, which is known as an inhibitor of renal tubular reabsorption, increased urinary excretion of Leu-A3 insulin. The ratio of Leu-A3 and normal insulin in urine after arginine was similar to that in serum. A large amount of Leu-A3 insulin is excreted in urine when reabsorption of insulin at renal tubules is inhibited by arginine. These data indicate that normal and Leu-A3 insulin are filtered through the glomerulus with relatively little restriction. Using the fact that basal urine has a high concentration of normal insulin and an extremely low concentration of Leu-A3 insulin, which has less receptor-binding affinity, we speculated some possibilities. One possibility is that both forms of insulin are reabsorbed by the tubular cells, but with different efficiencies. Leu-A3 insulin absorption in more complete, and this suggests differences in the uptake pathways that may account for the differences in response to arginine infusions. Another possibility is that only normal insulin is secreted from tubules into urine which is mediated by receptors. Our results provide new insight into renal metabolism of insulin and showed that MIS is a useful model for studying it.

INSULIN, synthesized in the β cells of the pancreas, is secreted into the portal vein, then the liver removes approximately 50%.[1] The rest of the secreted insulin goes into the peripheral circulation and affects muscle and fatty tissues. It is then removed from circulation by the kidney.[1] Only 1% or less of excreted insulin can be found in urine.[2-4] Many studies have shown that the kidney plays an important role in the metabolism of many proteins and small peptides.[5] The origin and role of insulin in urine are not completely clear. The renal clearance of insulin has 2 different routes; one is luminal and the other is peritubular.[6] The major route is glomerular filtration. After filtration, insulin is reabsorbed by proximal tubular cells, and $<$1% appears in the urine.[7,8]

We have reported 2 cases of familial hyperinsulinemia associated with mutant insulin syndrome (MIS; insulin Wakayama).[9,10] This abnormal insulin comes from the abnormal allele of the insulin gene, showing a thymine-for-guanine substitution at nucleotide position 1298 from the putative cap site, resulting in a leucine-for-valine substitution at position 3 of the insulin A (Leu-A3 insulin). Because patients with this syndrome are heterozygous and have both normal and mutated alleles, 2 different insulins are secreted into the circulation. Leu-A3 insulin has $<$0.5% of the receptor binding and biologic activity of normal insulin.[10] To understand insulin handling in the kidney, it is important to see urinary insulin excretion under several conditions, as in patients with MIS, in which 2 different insulins exist in circulation. The high-performance liquid chromatography (HPLC) method was used to separate and identify

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the mutant human insulin.[11,12] We tried to separate and quantitate normal and Leu-A3 insulin in the serum and the urine with HPLC. Next we studied how levels of urinary insulin were altered by stimulation of insulin secretion with glucose and suppression of the functions of reabsorption by arginine injection in normal subjects and in patients with MIS who have 2 different insulins in serum. Clarification of insulin handling under different conditions makes renal insulin handling and the importance of urinary insulin clear.

SUBJECTS AND METHODS

Methods of Extraction, Identification, and Quantification of Normal or Leu-A3 Insulin From Serum and Urine Samples

The method of sample preparation for HPLC is described elsewhere.[9,12] Insulin was extracted from 10 mL of serum using Sep Pak C18 column (Waters Millipore, Bedford, MA) and lyophilized. The urine samples were treated the same as the serum samples after precipitation with 50% trichloroacetic acid and resolved in 20 mL of phosphate-buffered saline (PBS, pH 7.4). The lyophilized samples were resolved in PBS (pH 7.4) at a concentration of 1 μU/μL, and 100 μL of the sample was analyzed using HPLC.

We used the Tri-Roter-IV as a pump, the UVDEC-100- IV as a detection module, and the DS-L300 (Japan Spectrophonic, Japan) for data processing. The HPLC column was C-18 ion-pairing column, 25 $\times$ 0.46 cm (Beckman, Fullerton, CA). The mobile phase was 29.7% (vol/vol) acetonitrile (ACN) aqueous solution (pH 3.0) containing 0.02 mol/L triethylamine, 0.05 mol/L $NaClO_4$, and 0.1 mol/L phosphate. The samples were eluted with a flow rate of 1 mL/min at 21°C, and 0.5 mL of fractions was collected, lyophilized, and dissolved in phosphate-buffered saline (PBS). The levels of insulin-like immunoreactivity (IRI) and C-peptide (CPR) were measured by radioimmunoassay (Fadesef insulin kit RIA and CPR kit RIA, Shionogi, Japan). RIA dilution curves of the abnormal insulin were parallel with that of human insulin standard.[9] We conclude that our RIA method has the same sensitivity and specificity with these 2 insulins. The elution pattern of HPLC was made with absorbance levels at 280 nm in high-dose samples and the levels of IRI in each tube of the low-dose samples. Synthetic human and Leu-A3 insulins (Peptide Institute, Japan) were used as standards. The quantitation of each insulins was calculated from the area under the curve (AUC) of the elution pattern.

To examine the recovery rate of these insulins using this method, 2 mU each of synthetic human and Leu-A3 insulins were added to 10 mL of serum or 100 mL of urine. Samples were incubated at 37°C for 24 hours, and insulin was extracted from each sample as described above. The IRI levels and elution patterns of these samples were examined.

Urinary Insulin Levels and Elution Pattern in Normal Subjects and Patients With MIS

Normal subjects (n = 3, age 36 to 41 years) with no family history of diabetes or liver or renal disorders and patients with MIS (n = 3, age 45 to 48 years) with both normal and mutant alleles by genomic DNA sequencing and 2 different insulins in the serum by HPLC were examined. Serum creatinine levels were 0.67 $\pm$ 0.29 mg/dL (normal subjects) and 0.73 $\pm$ 0.12 mg/dL (MIS patients; normal range, 0.4 to 1.5 mg/dL). Ten milliliters of postprandial serum and 200 mL of urine from 24-hour collection were obtained from the subjects, and insulin was extracted. Elution patterns of extracted insulins were analyzed by HPLC.

Serum and Urinary Insulin During 75 g Oral Glucose Tolerance Test, 1 mg Glucagon Test, and 4 g Arginine Injection Test

Seventy-five gram oral glucose tolerance test (OGTT), 1 mg glucagon injection test, and 4 g arginine injection test were performed in the same subjects. After overnight fasting, 75 g of glucose was administrated orally, and blood samples were collected 0, 30, 60, 90, and 120 minutes after loading. Each subject urinated completely 30 minutes before the examination, and urine samples between −30 and 0 minutes and between 0 and 120 minutes after load were collected. After overnight fasting, 1 mg of glucagon (Novo Nordisk, Denmark) or 40 mL of 10% arginine (Hoechst, Japan) was injected intravenously. Blood samples were collected 0, 5, and 30 minutes after injection, and urine samples were collected between −30 and 0 minutes and between 0 and 30 minutes.

Serum and urinary insulin levels before and after loading were measured by radioimmunoassay. Insulin extracted from urine samples using the methods described above were analyzed by HPLC. All results are expressed as means $\pm$ SEM.

RESULTS

Recovery Rate of Insulin From Serum or Urine and Elution Patterns of Insulins

The recovery rates of human insulin and Leu-A3 insulin were 84.4% $\pm$ 1.6% and 82.4% $\pm$ 3.7% from serum and 58.0% $\pm$ 3.8% and 57.1% $\pm$ 6.1% from urine (n = 3). There was no difference in recovery rates between the 2 insulins.

Each synthetic human, porcine, and Leu-A3 insulin had a single peak and good separation under these conditions (data not shown). The elution patterns of normal and Leu-A3 insulin in the serum and urine were same as those of standard insulins. These data suggest that insulin should have the same immunoreactivity and hydrophobicity in urine as in serum.

Insulin in Serum and Urine in Patients With MIS

The elution patterns of insulin extracted from serum and urine from normal subjects showed that the single peak coincided with that of standard human insulin. Serum insulin from patients showed 2 peaks, the normal insulin peak and the Leu-A3 insulin peak, and the ratio of the 2 insulins was 1:9 (Fig 1). Insulin extracted from urine was eluted on the same position of normal insulin and not on that of Leu-A3 insulin (Fig 1).

Serum and Urinary Insulin Response and Their Elution Patterns During 75 g OGTT and 1 mg Glucagon Injection Test in Patients With MIS

After oral glucose loading, insulin levels in the serum and the urinary excretion rate of insulin were elevated (Table 1). The elution pattern of serum insulin after OGTT was the same as that of the serum at fasting (data not shown). The elution pattern of insulin extracted from urine had only 1 peak coinciding with that of normal insulin. The peak of the Leu-A3 insulin was very small (Fig 2). After the other insulin stimulation test, such as glucagon 1 mg intravenously, the elution patterns of serum and urinary insulin were the same as in the oral glucose tolerance test (OGTT; data not shown).

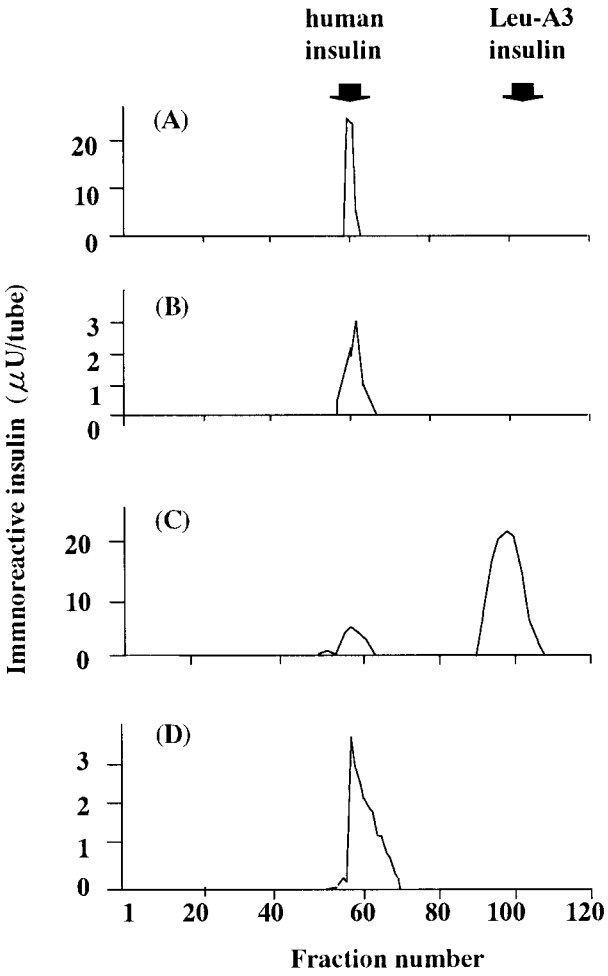

Fig 1. HPLC elution patterns of insulin extracted from (A) serum and (B) urine of a normal subject and (C) serum and (D) urine of a patient with MIS.

Urinary Insulin Response and Elution Patterns After 4 g Arginine Injection Test

The insulin levels in serum and urinary excretion rate of insulin were increased after arginine injection (Table 1). Elution of insulin in serum appeared in the same pattern as that during fasting and after glucose loading. However, insulin extracted from urine was eluted in the positions of both normal and Leu-A3 insulin (Fig 3). The ratio of normal insulin and Leu-A3 insulin in urine was 15:85, which was the same as that in the serum.

DISCUSSION

The kidney plays an important role in the metabolism of insulin. Approximately half of the secreted insulin is removed by the kidney. Insulin is metabolized in the kidney by 2 routes; one is glomerular, and the other is peritubular.[2] The major route is glomerular. Less than 20% to 40% of insulin is removed by the peritubular route.[2,7] The rate of glomerular filtration is affected by its size, charge, and hydrophobicity and by glomerular permeability and effective renal plasma flow.

Insulin as it exists in serum is not bound to plasma proteins in its monometric form. Therefore, insulin is filtered almost freely in subjects with normal renal function.[2] In humans, glomerular filtration accounts for 60% to 80% of renal clearance of insulin.[2] After filtration, insulin is degraded and reabsorbed in proximal tubular cells. Small linear proteins such as glucagon are degraded at the renal brush border membrane of the proximal tubular cells.[13,14] However, insulin, which contains disulfide bonds, prevents its hydrolysis at the tubular brush border membrane.[15] For the most part, insulin is removed by the reabsorption process. This process may be mediated by the insulin receptor, possibly by endocytosis.[6] Recently, it has been suggested that the endocytic membrane receptor megalin binds insulin and epidermal growth factor and is responsible for the reabsorption of small peptides.[7] In cultured proximal-like opossum cells, insulin is internalized by receptor-mediated endocytosis for degradation. These cells release internalized insulin into the cultured medium by retroendocytosis.[16,17] However, whether the insulin retroendocytosis actually occurs in vivo is unknown. It is believed that very little insulin escapes from reabsorption to appear in urine.

We have reported the usefulness of HPLC in separating and quantitating human and porcine insulin.[18] These 2 insulins are different in only 1 amino acid and resemble each other in hydrophobicities. We can separate and quantitate these 2 and Leu-A3 insulin with HPLC. Using our extraction methods, normal and Leu-A3 insulins showed the same recovery rate from serum or urine, despite differences in amino acid– and receptor–binding activities. Therefore, our methods of extraction and separation are reliable for separation and quantitation of these insulins.

To elucidate the insulin metabolism in the kidney, we examined urinary insulin excretion under several conditions in patients with MIS using the HPLC method. Patients with MIS have 2 different insulins in their serum; one is normal and the

Table 1. Insulin and C-Peptide Levels in Serum and Urine After Oral Glucose Loading and Arginine Injection

	Insulin		C-Peptide	
	Normal	MIS	Normal	MIS
Serum at fasting	8.95 ± 1.24 μU/mL	70.3 ± 7.33 μU/mL	2.68 ± 0.43 ng/mL	1.71 ± 0.22 ng/mL
Urine at fasting	2.86 ± 0.63 μU/min	0.48 ± 0.03 μU/min	5.56 ± 1.07 ng/min	5.02 ± 1.26 ng/min
Urine after OGTT	7.06 ± 2.46 μU/min	0.85 ± 0.23 μU/min	4.30 ± 0.99 ng/min	5.42 ± 2.51 ng/min
Urine after arginine injection	0.34 ± 0.55 μU/min	5.94 ± 0.23 μU/min	1.27 ± 0.51 ng/min	3.07 ± 0.43 ng/min

NOTE. Data represent means ± SEM.

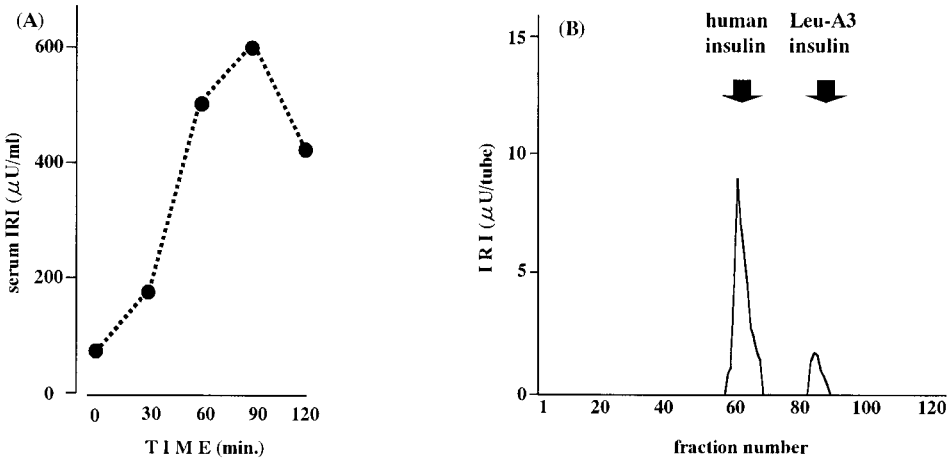

Fig 2. (A) Serum levels of insulin before and after oral glucose loading in a patient with MIS. (B) Elution patterns of insulin extracted from urine after oral glucose loading in a patient with MIS. IRI, immunoreactive insulin.

other is abnormal.[9] These 2 insulins are different in only 1 amino acid and therefore resemble each other except for receptor-binding activity.[10] Therefore, we can examine the differences in metabolism of the 2 different insulins in the same subjects and under the same conditions. In normal subjects, intact insulin can be found in urine. In patients with MIS, most of the insulin extracted from 24-hour urine collections was normal, whereas 90% of serum insulin was Leu-A3 insulin. After insulin concentration was increased by glucose and glucagon stimuli, Leu-A3 insulin was not seen in urine, which is the same pattern as in normal subjects.

These data suggest that the amount of Leu-A3 insulin excreted in urine is extremely low compared with that of normal insulin. To elucidate the low clearance of abnormal insulin, we used an inhibitor of renal tubular functions. Arginine, one of the basic amino acids, is an inhibitor of renal tubular reabsorption.[19-21] After arginine injection, large amounts of Leu-A3 insulin and a small amount of normal insulin were found in the urine. The ratio of Leu-A3 and normal insulin in urine after arginine injection was similar to that of serum. These data indicate that normal and Leu-A3 insulin are filtered through the glomerulus with relatively little restriction. Because basal urine has a high ratio of normal insulin and extremely low ratio of Leu-A3 insulin, we considered the some possibilities. One possibility is that both forms of insulin are reabsorbed by the tubular cells, but with different efficiencies. Leu-A3 insulin absorption is more complete and this suggests differences in the uptake pathways and possibly accounting for the differences in response to arginine infusions. The relative increase in urinary excretion of Leu-A3 insulin after arginine infusion suggests that this reabsorption pathway is more sensitive to arginine than the normal insulin reabsorption pathway. The other possibility is that both insulins are filtered from glomeruli and are reabsorbed completely at tubules in a process not mediated by receptors, possibly by endocytosis. Only secretion of normal insulin into urine is receptor mediated after glomeular filtration and tubular reabsorption. The insulin receptor groups in nephron, proximal, distal, and/or convoluted tubules may be responsible for transporting normal insulin into urine because the insulin receptor can be found in almost all nephron seg-

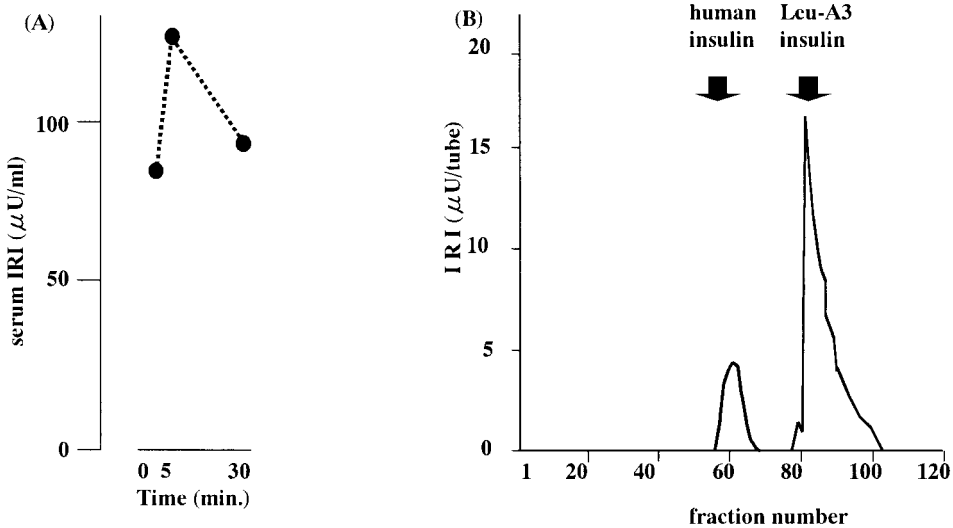

Fig 3. (A) Serum levels of insulin before and after arginine injection in a patient with MIS (B) HPLC elution patterns of insulin extracted from urine after arginine injection in a patient with MIS. IRI, immunoreactive insulin.

ments, especially in the proximal and distal convoluted tubules.[22] The presence of insulin in urine may reflect transcytosis of capillary insulin across the tubule cell to the lumen of the tubule. This hypothesis also explains the difference in urinary insulin clearance between 2 insulins in patients with MIS.[23] An alternate but unlikely explanation would invoke the presence of a tubular secretary pathway for Leu-A3 insulin that is activated by arginine.

In summary, glomerular filtration of insulin occurs in a nonspecific manner, but tubular reabsorption and a tubular secretory pathway may involve some receptor-mediated process affected by amino acids. In addition, examination of patients with MIS is useful for studying insulin metabolism in vivo.

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